EMERGING INFECTIOUS DISEASES®

Coronaviruses and Influenza Viruses

April 2014

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EMERGING INFECTIOUS DISEASES

April 2014

On the Cover

John Frederick Lewis (1804–1876)

On the Banks of the Nile, Upper Egypt (detail)

Oil on panel, 1876
Yale Center for British Art, Paul Mellon Collection

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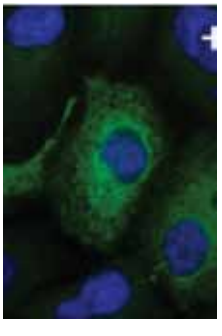

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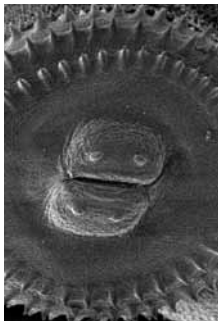

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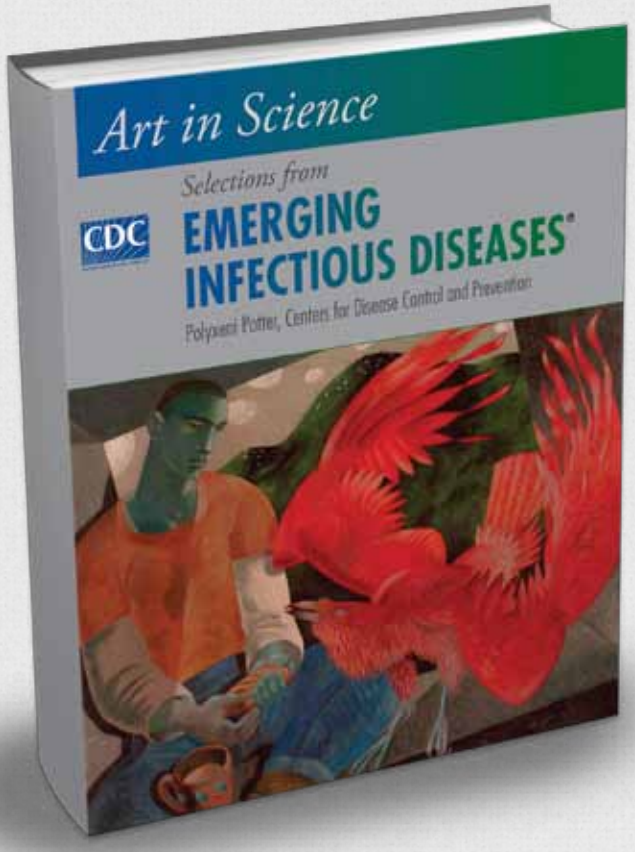

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This collection of 92 excerpts and covers from **Emerging Infectious Diseases** will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.

Distribution of Pandemic Influenza Vaccine and Reporting of Doses Administered, New York, New York, USA

Roopa Kalyanaraman Marcello,[1] Vikki Papadouka, Mark Misener, Edward Wake, Rebecca Mandell,[2] and Jane R. Zucker

In 2009, the New York City Department of Health and Mental Hygiene delivered influenza A(H1N1)pdm09 (pH1N1) vaccine to health care providers, who were required to report all administered doses to the Citywide Immunization Registry. Using data from this registry and a provider survey, we estimated the number of all pH1N1 vaccine doses administered. Of 2.8 million doses distributed during October 1, 2009–March 4, 2010, a total of 988,298 doses were administered and reported; another 172,289 doses were administered but not reported, for a total of 1,160,587 doses administered during this period. Reported doses represented an estimated 80%–85% of actual doses administered. Reporting by a wide range of provider types was feasible during a pandemic. Pediatric-care providers had the highest reporting rate (93%). Other private-care providers who routinely did not report vaccinations indicated that they had few, if any, problems, thereby suggesting that mandatory reporting of all vaccines would be feasible.

In April 2009, a novel swine-origin influenza A (H1N1) virus (now called influenza A(H1N1)pdm09 [pH1N1]) was detected in the United States (*1*). During the next 2 months, more than 1 in 10 New York City (NYC) residents reported influenza-like illness; cases occurred primarily among children and young adults (*2*). By June 2009, the World Health Organization had declared an influenza A(H1N1) pandemic (*3*). In July 2009, in anticipation of limited vaccine supply, the Advisory Committee on Immunization Practices and the Centers for Disease Control and Prevention prioritized groups for receipt of monovalent pH1N1 vaccine (*4*,*5*).

Building on previous pandemic influenza preparedness planning, in the summer of 2009, the NYC Department of Health and Mental Hygiene (DOHMH) began planning for pH1N1 vaccine allocation and distribution. To efficiently provide limited doses to a diverse population of providers in a large urban setting, DOHMH developed an allocation plan that included hospitals, private care providers (including adult, pediatric, and obstetric practices), and other outpatient facilities (including federally qualified health centers, pharmacies, DOHMH walk-in immunization clinics, and NYC agencies with a medical unit). In addition, DOHMH conducted a large-scale school-located vaccination program that offered pH1N1 vaccine to virtually all of the 1.4 million NYC schoolchildren in kindergarten through grade 12 (*6*). DOHMH also conducted 58 point-of-dispensing mass vaccination clinics over 5 weekends (*7*).

In NYC, all vaccine doses administered to persons <19 years of age must be reported to the Citywide Immunization Registry (CIR), DOHMH Immunization Information System; this requirement includes influenza vaccine (*8*). Vaccine doses administered to patients ≥19 years of age can be reported to the CIR with the patient's consent. Electronic files containing birth certificates are entered into the CIR on a weekly basis to establish a population base and to facilitate reporting among pediatric-care providers. Since 2008, the CIR has been one of the Immunization Information System sentinel sites in the United States and has met data-quality and population-capture requirements, including those of receiving complete and timely data from at least 85% of providers and participation of at least 85% of children <19 years of age (*9*).

Author affiliations: New York City Department of Health and Mental Hygiene, Queens, New York, USA (R.K. Marcello, V. Papadouka, M. Misener, E. Wake, R. Mandell, J.R. Zucker); and Centers for Disease Control and Prevention, Atlanta, Georgia, USA (E. Wake, J.R. Zucker)

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[1]Current affiliation: The Corkery Group, New York, New York, USA.
[2]Current affiliation: University of Michigan, Ann Arbor, Michigan, USA.

On October 28, 2009, because of the pH1N1 pandemic, various provisions of New York State public health law were suspended, including the requirement to obtain consent to report vaccines given to adults. This change authorized the NYC Health Commissioner to issue a Declaration of a Public Health Emergency and to modify the NYC Health Code to require reporting to the CIR of all pH1N1 influenza vaccinations administered, including those administered to persons ≥19 years of age. This change was made to increase provider accountability, track vaccine uptake, and assist with estimating vaccine coverage.

Distribution of pH1N1 Vaccine and Reporting of Doses Administered

Vaccine Ordering and CIR Registration

All medical providers in NYC were invited to order pH1N1 influenza vaccine. Providers who ordered vaccine were required to register with the CIR if they had not previously done so and to electronically sign a memorandum of agreement. Receipt of this memorandum, a faxed vaccine order form, and fulfillment of the order were recorded in the CIR. To rapidly train a large number of providers who served adult patients but had no CIR reporting experience, DOHMH provided instruction by email and through a series of webinars.

Vaccine shipments began in early October 2009. Initial doses were distributed primarily to pediatric-care providers, obstetricians, hospitals (for patients and health care workers), federally qualified health centers, and uniformed personnel (e.g., in fire and police departments). Distribution of doses to adult-care providers and DOHMH walk-in clinics began in the middle of October; most providers received doses at the end of the month. Pharmacies and residential facilities (e.g., nursing homes) began to receive vaccine in mid-December when demand was low. A portion of each weekly shipment was set aside for use in school vaccination programs and weekend point-of-dispensing clinics. Because of the limited supply, providers initially did not receive the full quantity of doses they had ordered; additional vaccine was sent as it became available.

Because vaccine supply was constantly changing and to address the many questions received from the medical community, DOHMH had to maintain continuous 2-way communication with providers throughout the entire period of vaccine allocation and distribution. To that end, the NYC Bureau of Immunization provided weekly email updates to all participating providers and posted these updates on the agency's public website. DOHMH also held weekly conference calls with private providers, federally qualified health centers, and hospitals to provide updates and answer questions. To ensure that providers were able to contact the Bureau of Immunization with questions or concerns and to check on the status of a vaccine order, a dedicated email account was established so that inquiries could be answered within 48 hours. This email account supplemented the existing Bureau of Immunization hotline, which regularly provides information about vaccines to providers and the public; the hotline was staffed with extra personnel to handle the increased volume of calls.

Data Collection and Reporting

Providers could report administration of pH1N1 vaccine doses in 1 of 2 ways: 1) by using the online CIR application, or 2) by sending electronic files, which conformed to a prespecified file format, directly to the CIR. Providers of child and adolescent care were expected to report administration of pH1N1 vaccine doses in the same way that they reported administration of other childhood vaccines. In addition, DOHMH designed a scannable paper reporting form for the school-based vaccination program and point-of-dispensing clinics; the completed forms were scanned, and the data were then automatically uploaded into the CIR.

From October 1, 2009, through March 4, 2010, a total of 988,298 doses of pH1N1 influenza vaccine were reported to the CIR as having been administered. These doses represented 35.5% of the 2,781,700 doses delivered to providers. Reporting to the CIR varied by provider type (Table 1). Those provider types that previously had been required to report to the CIR reported a larger proportion of the pH1N1 vaccine doses received; 53%, 46%, and 43% of total doses received by the Health and Hospitals Corporation (the NYC public hospital system), private pediatric-care providers, and federally qualified health centers, respectively, were reported. Colleges, private hospitals, and adult-care providers reported a smaller percentage of doses received: 29%, 28%, and 20%, respectively. The lowest proportion of doses reported was by residential facilities (4%) and pharmacies (0%). These provider types were the last to receive vaccine in December, when demand was very low citywide.

Provider Survey

Because a large number of pH1N1 vaccine doses were distributed but not reported to the CIR, the Bureau of Immunization conducted a survey to estimate the number of doses actually administered by providers. The survey, which consisted of 19 questions, sought to identify how many doses providers administered and how many viable doses and expired/spoiled doses were still in stock. Providers were told the number of doses distributed to them and the number reported to the CIR. They were then asked if this was the actual number of doses given and, if not, how many more were given but not reported to the CIR. The survey also included questions about the quality of communications received from DOHMH and about providers' experiences using the CIR.

Table 1. Doses of pH1N1 vaccine distributed and reported to the Citywide Immunization Registry, New York, New York, USA, October 1, 2009–March 4, 2010*

Facility type	No. providers who received pH1N1 vaccine	No. doses received	No. doses administered reported to CIR (% of all vaccine received)
Private care provider			
Adult care	1,471	425,500	84,877 (19.9)
Pediatric care	1,176	635,300	294,324 (46.3)
Private hospital	258	528,300	149,963 (28.4)
Residential facility	129	35,900	3,928 (10.9)
Federally qualified health center	126	132,200	57,345 (43.4)
Health and hospitals corporation†	65	194,300	103,176 (53.1)
Pharmacy	29	139,600	19,679 (14.1)
College	21	18,600	5,459 (29.3)
Uniformed personnel	10	16,000	11,423 (71.4)
DOHMH‡	1	656,000	258,124 (39.3)
Total	3,286	2,781,700	988,298 (35.5)

*pH1N1, influenza A(H1N1)pdm09 virus; CIR, Citywide Immunization Registry; DOHMH, Department of Health and Mental Hygiene.
†New York City public hospital system.
‡Includes all DOHMH-conducted vaccination activities, including walk-in immunization clinics, school vaccination programs, and point-of-dispensing sites.

All facilities and providers that had ordered pH1N1 vaccine from DOHMH by March 4, 2010, were eligible for inclusion in the survey. DOHMH clinics, point-of-dispensing clinics, and the school vaccination program were excluded because DOHMH conducted these operations. The complete list of potential participants was stratified into 9 groups according to facility type: private adult-care providers, private pediatric-care providers, private hospitals, colleges and universities, federally qualified health centers, the Health and Hospitals Corporation, pharmacies, residential and long-term care facilities, and uniformed personnel. A 12% computerized random sample by facility type resulted in a final pool of 395 potential participants, which included at least 1 provider from each of the 9 facility types.

DOHMH used its in-house call center to conduct the survey from March 15 through April 7, 2010. Call center staff entered responses from providers into the online survey tool Survey Monkey (www.surveymonkey.com). The survey was pre-populated with CIR data about the number of doses ordered and administered by the facility. The Bureau of Immunization trained the call center staff and provided background information on pH1N1 vaccination, DOHMH vaccine distribution methods, and CIR reporting methods. Up to 4 calls were made to each of the selected sites during March and April 2010. After 4 unanswered calls or refusal to participate, facilities were considered nonresponders. The survey took ≈5 minutes to complete.

Survey Results

Of the 395 facilities sampled, 228 completed the survey (58% response rate) (Table 2). Nearly all providers acknowledged that they did not report a portion of administered doses to the CIR; across all provider groups, 9,297 (8%) of the 116,600 doses received by facilities that responded to the survey were reported as administered but not reported to the CIR. Reporting was achieved across provider types: 79% of private pediatric-care providers, 49% of adult-care providers, and 44% of residential facilities surveyed reported at least some of the administered doses to the CIR.

For most providers, a large proportion of vaccine remained in storage in their facilities as either viable or expired/spoiled. Among all providers surveyed, nearly 27,812 doses (24% of doses received) remained at the facilities. In addition, 40,069 doses (34% of doses received) were unaccounted for, meaning that they were not reported to the CIR or through the survey as administered and not reported as remaining in storage or expired/spoiled.

If the number of doses the surveyed providers reported that they administered is considered to be a reasonable estimate of the true number of doses administered, then 81% of all doses administered were reported to the CIR. Among private noninstitutional providers, pediatric-care providers reported 93% of all doses administered and adult-care providers reported 59% of all doses administered.

Estimating Citywide pH1N1 Vaccine Administration

To assess potential discrepancies between the number of pH1N1 vaccine doses reported and the number actually administered by each surveyed provider type, we linked survey data to CIR data. The number of doses administered by survey respondents was calculated by aggregating 1) the total number of doses reported to the CIR for each provider group and 2) the total number of doses reported as administered but not reported to the CIR by that group. The reporting rate (number of doses reported to the CIR divided by the number of doses administered, both those reported to the CIR and those reported as administered but not reported by the provider) was then calculated for each provider group. To estimate the number of doses administered but not reported to the CIR by each provider group, we then applied these rates to the citywide population of providers in

Table 2. Estimates of pH1N1 vaccine administration among surveyed providers, New York, New York, USA, October 1, 2009–March 4, 2010*

Facility type	No. providers selected/no. responders	No. doses rec'd	No. doses rep'd (% of all vaccine rec'd)†	No. doses admin'd but not rep'd	Total no. (%) doses admin'd‡	No. doses remaining§	No. doses not accounted for¶	Max no. doses admin'd#	% All admin'd doses rep'd**
Private provider									
Adult care	176/108	26,800	7,117 (26.6)	5,028	12,145 (45.3)	9,270 (34.6)	5,386	17,531	58.6
Ped. care	141/81	47,000	22,244 (47.3)	1,805	24,049 (51.2)	12,267 (26.1)	10,684	34,733	92.5
Private hospital	32/17	20,800	3,905 (18.8)	1,452	5,357 (25.8)	3,128 (15.0)	12,315	17,672	72.9
Residential facility	15/9	2,700	114 (4.2)	801	915 (33.9)	880 (32.6)	905	1,820	12.5
Federally qualified health center	15/5	2,200	881 (40.0)	11	892 (40.5)	523 (23.8)	785	1,677	98.8
Health and hospitals corporation††	8/4	15,100	4,720 (31.3)	200	4,920 (32.6)	209 (1.4)	9,971	14,891	95.9
Pharmacy	4/2	600	0	0	0	600 (100)	0	0	0
College	3/1	1,000	177 (17.7)	0	177 (17.7)	800 (80.0)	23	200	100
Uniformed personnel	1/1	400	265 (66.3)	0	265 (66.3)	135 (33.8)	0	265	100
Total	395/228	116,600	39,423 (33.8)	9,297	48,720 (41.8)	27,812 (23.9)	40,069	88,789	80.9

*pH1N1, influenza A(H1N1)pdm09 virus; rec'd, received; admin'd, administered; rep'd, reported to Citywide Immunization Registry (CIR); max, maximum; ped., pediatric.
†Percentage of all doses reported to CIR = number of doses reported to CIR divided by total number of doses received.
‡Total number of doses administered = sum of doses reported to CIR and number of doses estimated administered but not reported to CIR.
§Viable or spoiled/expired.
¶Doses not unaccounted for = number of doses received minus number of doses administered and reported to CIR, number of doses administered but not reported to CIR, and doses remaining and/or expired/spoiled.
#Maximum number of doses administered = number of doses received minus number of doses remaining and/or expired/spoiled.
**Percentage of all administered doses reported to CIR = number of doses administered and reported to CIR divided by total number of doses administered.
††New York City public hospital system.

each group. These numbers were tallied for an estimate of the total number of doses administered citywide. The analysis was based on vaccine doses administered, not on the number of persons vaccinated. Citywide rates for each provider type were weighted by the number of doses received by each provider type group and then averaged to calculate an overall reporting rate. Frequencies were calculated for responses to questions about the vaccine ordering process and DOHMH communications by facility type.

We estimated that as of March 4, 2010, a total of 1,160,587 doses of pH1N1 vaccine were administered (vs. 988,298 doses reported to CIR) citywide. This number represents 42% of all doses delivered to providers. According to the survey results, an estimated 568,561 doses remained in providers' offices (viable and/or expired/spoiled) and ≈1 million (36%) of the 2.78 million doses distributed were unaccounted for. If these doses had actually been administered, the number of doses administered could be as high as 2.16 million.

Provider Satisfaction

Nearly all (96%) providers surveyed found the weekly updates on vaccine ordering and reporting sent by DOHMH to be helpful (Table 3). The percentages of providers reporting problems were 10% for enrolling in the CIR, 3% for completing and submitting the provider agreement, 9% for ordering pH1N1 vaccine, 1% for administering pH1N1 vaccine to patients, and 12% for reporting doses to the CIR (Table 4).The greatest difficulties were encountered by the 9 residential facilities surveyed; 11% reported problems with registration, and 22% reported problems with reporting doses to the CIR (Table 4).

Implications for Future Pandemic Planning

The results of the survey indicated that a large number of distributed pH1N1 doses were never administered to patients. These results are consistent with other pH1N1 vaccination efforts made in NYC during 2009–2010: only 1 of the 58 weekend point-of-dispensing clinics reached capacity, and uptake at the school-located pH1N1 vaccine program was lower than anticipated. As a result, only half of the doses received by DOHMH were administered and nearly all were reported to the CIR. In June 2010, providers who received pH1N1 vaccines from DOHMH were asked to return unused doses to the Central Vaccine Recovery Program operated by the US Department of Health and Human Services (*10*). The number of doses returned by NYC providers through this program was reported as 537,538 doses (20% of distributed doses), further supporting the conclusion that a large number of received pH1N1 vaccine doses were never administered.

Survey results support the feasibility of requiring providers to report individual-level data about vaccine administration to an Immunization Information System during a

Table 3. Provider feedback with regard to Department of Health and Mental Hygiene communications and procedures, New York, New York, USA, October 1, 2009–March 4, 2010

Provider type	No. providers	% Providers						
		Received weekly updates	Found updates helpful	Phoned for more information	Spoke with person	Received helpful information by phone	Sent email	Received helpful information by email
Private								
Adult care	108	73	92	34	92	82	15	88
Pediatric care	81	70	98	41	88	86	30	79
Private hospital	17	53	100	24	100	100	41	71
Residential	9	78	100	11	100	100	33	67
Federally qualified health center	5	20	100	40	100	100	40	50
Health and hospitals corporation†	4	25	100	0	NA	NA	25	100
Pharmacy	2	50	100	50	100	0	0	NA
College	1	100	100	100	100	100	0	NA
Uniformed personnel	1	100	100	0	NA	NA	0	NA
Total	228	69	96	35	91	85	23	79

*NA, not applicable.
†New York City public hospital system.

pandemic. Although vaccine reporting was a new requirement for adult-care providers, approximately half of adult-care providers and 44% of residential facilities successfully reported to CIR the number of pH1N1 vaccine doses administered. Although these rates may seem lower than those for other provider types, they are an accomplishment, given that nearly all of these providers were first-time CIR users and had only a short time to familiarize themselves with a new electronic system and with the routine practice of reporting vaccine doses administered.

Although the overall rate of reporting to the CIR seemed to be only 35%, survey results indicate that ≈81% of doses administered were reported. Not surprisingly, pediatric-care providers reported nearly all of the vaccine doses they administered (92.5%), and adult-care providers for whom CIR reporting was not previously required reported a smaller percentage of doses administered. We have limited information, but we can understand how providers might have had difficulty reporting if, for example, their practices were overwhelmed with patients who were ill or requesting vaccine. On the contrary, a quick-entry data screen was added to CIR to make reporting easier for providers who had not previously reported. Overall, however, few facilities reported having had difficulty meeting the requirements for obtaining pH1N1 vaccine, including the required reporting of vaccine doses administered. This result is notable, especially given that the new reporting mandate was implemented during an emergency situation.

The variable number of vaccine doses administered by different provider types was associated, in part, with when the facilities received vaccine. Most notably, residential facilities and pharmacies received vaccine late in the season, when vaccine was plentiful and readily available elsewhere and when demand was low.

Without requiring providers to report administration of pH1N1 vaccine doses, the DOHMH is unlikely to have received reports of doses administered to adults. Although CIR reporting was not complete, the vaccine data we received proved valuable in many ways. By having providers enter doses administered in real time, DOHMH was able to replenish providers' vaccine supplies to keep up with demand and make changes to the vaccine allocation plan on an ongoing basis. In addition, DOHMH was able to better monitor vaccine uptake throughout the pandemic, estimate final vaccine use, population coverage, and vaccine effectiveness (*11*). Had the pandemic been more severe and/or had there been more demand for vaccine, reporting of pH1N1 vaccine doses administered would have been even more useful and would have enabled the public health response to be more effective. The key role played by Immunization Information Systems has been affirmed by the Community Preventive Services Task Force, which identified numerous public health benefits, including increasing coverage, monitoring vaccine uptake and coverage, and evaluating public health responses to outbreaks (*12*).

Limitations

Interpretation of our survey results has limitations. First, we assumed that survey respondents were similar to nonrespondents and to providers not surveyed. It is possible that they differed; in particular, providers who responded to the survey might have been more likely to have also complied with the reporting requirement. If so, our estimates of doses administered could be high.

Second, the number of doses administered but not reported to the CIR was based on providers' self-report and was not verifiable. Providers might have intentionally underreported the number of doses they indicated

Table 4. Provider feedback with regard to problems encountered with regard to pH1N1 vaccine, New York, New York, USA, October 1, 2009–March 4, 2010*

Provider type	No. providers	% Providers who encountered problems				
		CIR registration	Provider agreement	Ordering	Vaccine admin	Reporting
Private						
Adult care	108	13	3	10	1	15
Pediatric care	81	4	1	9	0	11
Private hospital	17	6	0	12	0	0
Residential	9	11	0	0	11	22
Federally qualified health center	5	0	0	0	0	0
Health and hospitals corporation†	4	0	25	0	0	0
Pharmacy	2	50	0	0	0	0
College	1	100	0	0	0	100
Uniformed personnel	1	100	100	0	0	0
Total	228	10	3	9	1	12

*pH1N1, influenza A(H1N1)pdm09 virus; CIR, Citywide Immunization Registry; admin, administration.
†New York City public hospital system.

they gave but did not report to the CIR to appear to be in compliance with the reporting requirement. Alternatively, providers might have erroneously underestimated the number of doses administered but not reported because they might have based this estimate on the recent low demand rather than on the higher demand during the fall season. Either way, nearly all providers stated that they had not reported all doses administered despite the reporting requirement.

Third, according to the survey estimates, ≈1 million doses of vaccine were unaccounted for. Some of these doses might have been administered, resulting in an underestimate of the total number of doses administered. As mentioned previously, ≈500,000 doses were returned by NYC providers to the US Department of Health and Human Services Central Vaccine Recovery Program. Although it is not clear whether these doses were considered in our survey to be "unaccounted for," as doses of vaccine reported to us as remaining in the provider's office at the time of the survey (either viable or expired/spoiled), it supports the assertion that a large number of vaccine doses distributed to providers were never administered.

Last, we used CIR data as of March 4, 2010, to estimate reporting rates, but the survey was conducted through April 7, 2010. Doses administered during this month-long period could have led to an overestimation of doses administered but not reported. However, only 51,111 additional doses were reported to the CIR during this time by all facilities citywide, which was only 5% more doses than the number reported up to the preceding month. If these additional doses represent 81% of doses truly administered, an additional 11,989 doses were given, for a total of 63,100 doses. Adding these to the 1.16 million doses brings the total estimated doses administered to 1.22 million, an increase that does not substantially change our conclusions.

We also conducted a temporal analysis. This analysis did not indicate a difference in reporting delay in November and in February 2010, although many fewer doses were administered in 2010.

Lessons Learned

Providers who responded to the survey agreed unanimously that regular communications from DOHMH were extremely helpful. Typical communications provided information about vaccine availability, including availability of specific formulations, and which provider types could receive vaccine. Other information included emphasis on the priority groups targeted for vaccination, reporting requirements, and the need for children <10 years of age to receive 2 doses. On the basis of experience, the Bureau of Immunization has continued to send providers regular influenza season updates on vaccine recommendations and availability.

Whereas the CIR previously focused almost exclusively on pediatric-care providers, pH1N1 reporting resulted in communication and data exchange with adult-care providers. During the 2010–11 influenza season, the Bureau of Immunization used these new relationships to communicate with adult-care providers about seasonal influenza vaccine recommendations and supply. According to data matching to existing provider lists, we estimate having reached ≈50% of private adult-care providers, which might not have been possible had the reporting requirements for pH1N1 vaccine not been implemented during the 2009–10 pandemic. Moreover, these providers reported having had few problems registering for pH1N1 vaccine and using the CIR. This finding, along with the fact that about half of newly reporting providers successfully reported to the CIR the vaccine doses administered, suggests that requiring reporting of all vaccines administered, including those given to adults, is feasible.

Under the Health Information Technology for Economic and Clinical Health Act, eligible health care providers can qualify for incentive payments when they demonstrate their capacity to submit immunization data from a certified electronic health record to an immunization registry ("meaningful use" of an electronic health record) (*13*). Mechanisms established during the pH1N1 pandemic to report to the CIR the number of pH1N1 doses administered have provided a foundation for achieving meaningful use

among this provider group, which can subsequently help facilitate rapid reporting during future pandemics.

Conclusions

We found that influenza vaccine reporting was feasible across all provider types, including those not usually required to report to the CIR. We estimate that 1.16 million doses of pH1N1 vaccine were administered to NYC residents during October 2009 1,–March 4, 2010, with a minimum of 998,298 doses and a maximum of 2.16 million doses having been administered. On the basis of this experience, mandating reporting of adult vaccines should be explored because it has been shown to be feasible, even during a pandemic. Additionally, meaningful use requirements have the potential for improving reporting of vaccines and could also prove beneficial during future emergencies.

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Ms Marcello was a senior policy analyst at the New York City Department of Health and Mental Hygiene during the study period. Her research interests include translating research into public health programs and policies.

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Address for correspondence: Jane R. Zucker, Bureau of Immunization, New York City Department of Health and Mental Hygiene, 42-09 28th St, Room 5-97, Queens, New York 11101-4132, USA; email: jzucker@health.nyc.gov

Regional Variation in Travel-related Illness acquired in Africa, March 1997–May 2011

Marc Mendelson, Pauline V. Han, Peter Vincent, Frank von Sonnenburg, Jakob P. Cramer, Louis Loutan, Kevin C. Kain, Philippe Parola, Stefan Hagmann, Effrossyni Gkrania-Klotsas, Mark Sotir, and Patricia Schlagenhauf, for the GeoSentinel Surveillance Network[1]

To understand geographic variation in travel-related illness acquired in distinct African regions, we used the GeoSentinel Surveillance Network database to analyze records for 16,893 ill travelers returning from Africa over a 14-year period. Travelers to northern Africa most commonly reported gastrointestinal illnesses and dog bites. Febrile illnesses were more common in travelers returning from sub-Saharan countries. Eleven travelers died, 9 of malaria; these deaths occurred mainly among male business travelers to sub-Saharan Africa. The profile of illness varied substantially by region: malaria predominated in travelers returning from Central and Western Africa; schistosomiasis, strongyloidiasis, and dengue from Eastern and Western Africa; and loaisis from Central Africa. There were few reports of vaccine-preventable infections, HIV infection, and tuberculosis. Geographic profiling of illness acquired during travel to Africa guides targeted pretravel advice, expedites diagnosis in ill returning travelers, and may influence destination choices in tourism.

Author affiliations: University of Cape Town Groote Schuur Hospital, Cape Town, South Africa (M. Mendelson); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (P.V. Han, M. Sotir); Tokai Medicross Travel Clinic, Cape Town (P. Vincent); University of Munich, Munich, Germany (F. von Sonnenburg); University Medical Center, Hamburg-Eppendorf, Germany (J.P. Cramer); University of Geneva, Geneva, Switzerland (L. Loutan); University of Toronto, Toronto, Ontario, Canada (K.C. Kain); Assitance Publique Hôpitaux de Marseille– North University Hospital, Marseille, France (P. Parola); Yeshiva University Bronx-Lebanon Hospital Center, Bronx, New York, USA (S. Hagmann); Cambridge University Hospitals National Health Service Trust, Cambridge, UK (E. Gkrania-Klotsas); and University of Zurich Centre for Travel Medicine, Zurich, Switzerland (P. Schlagenhauf)

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Africa is a popular tourist destination and a focus for international aid work, research, and business travel. In 2011, compared with 2010, international arrivals on the African continent remained relatively stable at ≈50 million, although the number of travelers to northern Africa decreased by 1.7 million (9%) and travel to sub-Saharan Africa increased by 1.3 million (4%) travelers (*1*). Identification of the types and relative frequencies of illnesses acquired by travelers to Africa would enable targeted prevention strategies before and during travel, as well as diagnosis and management of illness in returnees.

Previously described as "the Dark Continent," referring to poor knowledge of its interior, Africa's diverse geography, ecosystems, and climate are now well defined. However, detailed understanding is lacking about the variety of illnesses experienced by travelers who visit different parts of this diverse continent. Prior studies have focused on travel-related illnesses acquired only within sub-Saharan Africa (*2*) or have concentrated on a single infection (*3*). The perception of the public and health care practitioners is that the risk of acquiring many travel-related illnesses, including malaria, is uniform throughout the continent. This misconception was particularly evident to practitioners of travel health in South Africa before the 2010 Fédération Internationale de Football Association World Cup in South Africa, an annual event that attracts hundreds of thousands of persons to the hosting country. This erroneous concept and a need for evidence-based travel advice prompted a GeoSentinel study that demonstrated marked variation in morbidity rates for malaria, African tick bite fever, and other travel-related illnesses in persons returning from South

[1]Contributing members of the GeoSentinel Surveillance Network are listed at the end of this article.

Africa compared with persons returning from neighboring countries or other countries in sub-Saharan Africa (*4*). The objective of the current analysis is to study regional variation in travel-acquired and emerging infections across Africa. Reaching this objective will better inform pretravel clinical consultations, which will help travelers recognize illnesses in the travel destination, take preventive measures, and seek treatment; and will focus differential diagnoses by clinicians among travelers returning from Africa.

Methods

The GeoSentinel Surveillance Network (www.istm.org/geosentinel/main.html) is an international network of specialized travel and tropical medicine providers housed in sites in 23 countries on 5 continents, established through the International Society of Travel Medicine (www.istm.org/) and the Centers for Disease Control and Prevention (www.cdc.gov). Details of patient recruitment, structure, and function of GeoSentinel sites that systematically report on all ill travelers have been described (*2*). Data for ill travelers are collected during or after travel. Demographics, travel history, reason for travel, clinical symptoms, and diagnostic information are recorded anonymously on a questionnaire, and GeoSentinel sites enter the information into a central database. The best available reference diagnostic tests are used at each site to categorize illness into 1 of 23 syndromic groups and >500 individual diagnoses. Representatives from the sites enter each diagnosis as laboratory confirmed or probable; both are included in the analysis. The country or region of illness acquisition is identified on the basis of itinerary, known endemicity patterns, and incubation periods.

To study regional variation in the pattern of illnesses in persons who traveled to Africa, we used the United Nations geoscheme to classify Africa into subregions: Eastern, Central, Northern, Southern, and Western Africa (Figure 1) (*5*). An ill traveler returning from a country within an African region was considered to have been exposed to the causative pathogen in that region if the GeoSentinel database had a record of its occurrence there; or if the exposure was not defined in the record but the ill person traveled only to countries within that region. We included in our study all ill travelers listed in the GeoSentinel database during March 14, 1997–May 31, 2011 (Figure 2).

Statistical Analysis

Demographic and travel characteristics of travelers to each African region were described by using frequencies and proportions for categorical variables and median and range for continuous variables. Analysis of *Plasmodium falciparum* malaria trends during 2007–2011 was calculated on the basis of monthly counts of ill returned travelers with febrile systemic illness that were aggregated over the study period. Of 54 sites in the GeoSentinel database reporting during 2007–2011, a total of 35 sites reported consistently during this period. We used only data from those 35 sites for trend analysis. Data were analyzed by using SAS version 9.2 (http://support.sas.com/software/92/index.html).

Results

We identified 16,893 ill travelers who returned from a single country or multiple countries within the same

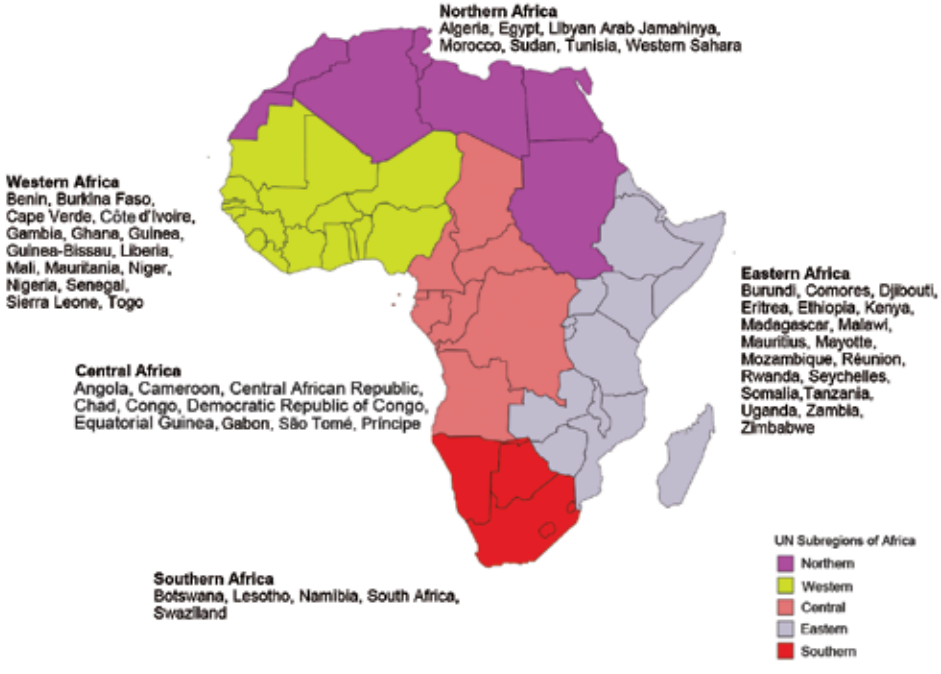

Figure 1. Regions of Africa as defined by the United Nations geoscheme (*5*). For persons whose country of exposure was unascertainable or missing but for whom all recent travel was to the same region of Africa, data were included in the final dataset.

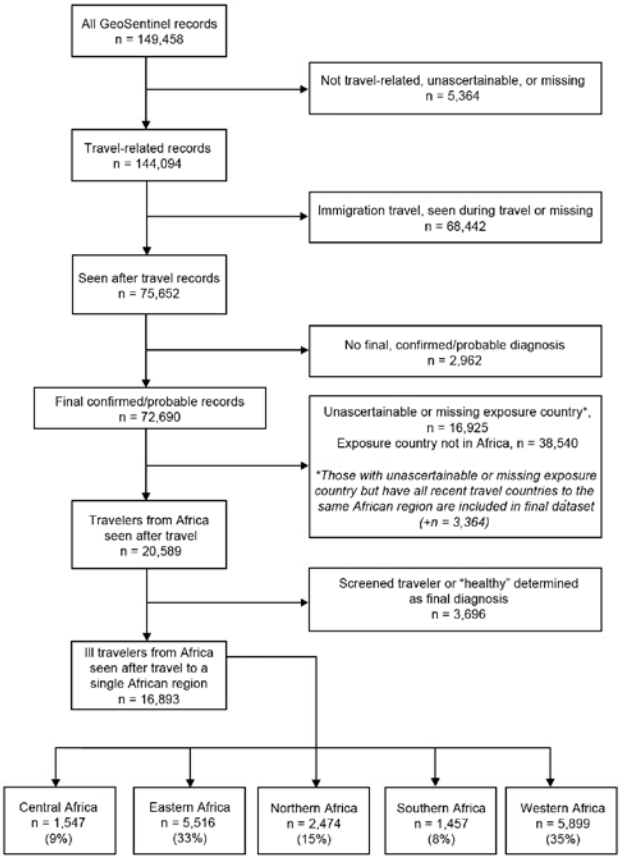

Figure 2. Flowchart for analysis of ill returned travelers from Africa reported in the GeoSentinel Surveillance Network, March 1997–May 2011. The United Nations geoscheme was used to classify Africa into subregions (*5*).

region in Africa during a 14-year period. Most acquired their illness either in Western (35%) or Eastern Africa (33%). Illness associated with travel to Southern Africa (8%) was least frequently reported (Figure 2).

Patient Characteristics

Most ill travelers returning from all regions were 18–64 years of age (Table 1). The residence of travelers spanned 72 countries; travelers were most frequently from Germany (33%), United States (12%), Canada (11%), France (10%), and Switzerland (10%).

Male travelers more commonly visited Central and Western Africa, and female travelers were more likely to visit Eastern and Northern Africa. Travelers visiting friends or relatives (VFR) more commonly visited Central (29%) and Western (33%) Africa than the other regions (2%–11%). Business travelers more frequently traveled to Central Africa (32%) than to other regions (9%–16%). Approximately three quarters of travelers to Northern (74%) and Southern Africa (78%) were tourists. Travelers to Northern Africa were less likely to have had a pretravel medical consultation (35%) than were travelers to other regions (50%–61%). Posttravel hospitalization rates were higher in travelers to Central and Western Africa (21%–25%) than in travelers to Northern and Southern Africa (5%–11%). The most frequent diagnoses among hospitalized travelers were *P. falciparum* malaria (45%), *P. vivax* malaria (4%), and unspecified febrile illness (<3 weeks) (3%).

Deaths among Travelers to Africa

Deaths of 11 travelers were recorded after travel to regions of sub-Saharan Africa (Table 2). Ten were of male sex; 10 were adults (median age 50 years). Severe *P. falciparum* malaria predominantly acquired in Western Africa was the cause of death for 9 of the 11 travelers. Two deaths occurred in expatriates, and 6 male business travelers died of *P. falciparum* malaria.

Northern Africa

Travel to Northern Africa was predominantly characterized by acquisition of gastrointestinal illnesses, comprising 66% of the 16,893 travel-related illnesses from this region and 7 of the 10 most frequent diagnoses (Table 3). In contrast, gastrointestinal disorders from regions of sub-Saharan Africa represented 27%–40% of cases. There was no difference among regions for acute or chronic diarrhea, schistosomiasis, or other gastrointestinal disorders such as intestinal strongyloidiasis. Of the reported hepatitis A cases, 28 (47%) originated in Northern Africa. Analysis of the data for individual countries in the Northern Africa region did not show variation in type of diarrheal disease or gastrointestinal disease (data not shown).

Documentation of animal bites and the need for rabies postexposure prophylaxis (PEP) showed striking geographic variation. Of the 193 reports of bites on the continent, 23% were to travelers <18 years of age. Of the 184 who received rabies PEP, 21% were travelers <18 years of age. Travelers to Northern Africa accounted for 105 (54%) of the 193 bites from dogs, cats, and others (including monkey and human) reported in Africa (Table 4); in contrast, 16 (8%) bites were reported from Southern and Central Africa combined. Similarly, 107 (58%) of the 184 exposures requiring rabies PEP were reported from Northern Africa. Although Egypt was the most commonly visited country in Northern Africa (3 times the number of visits to Morocco), travelers to Morocco received the most bites (21 dog bites, 8 cat, 3 other).

Regions within Sub-Saharan Africa

In contrast to the vast numbers of reports of gastrointestinal disease, febrile illnesses were uncommon in travelers to Northern Africa (4%). Conversely, 11%–47% of travelers returning from regions of sub-Saharan Africa had a febrile illness (Table 5). *P. falciparum* malaria was the

Table 1. Characteristics of ill travelers returning from Africa who were seen at GeoSentinel clinic sites, March 1997–May 2011*

Characteristic	Total, N = 16,893	No. (%) travelers by region: Central Africa, n = 1,547	Eastern Africa, n = 5,516	Northern Africa, n = 2,474	Southern Africa, n = 1,457	Western Africa, n = 5,899
Sex						
M	8,698 (52)	922 (60)	2,582 (47)	1,126 (46)	723 (50)	3,345 (57)
F	8,130 (48)	615 (40)	2,909 (53)	1,344 (54)	733 (50)	2,529 (43)
Age, y						
Median (range)	35 (0–92)	37 (0–92)	34 (0–89)	37 (0–87)	40 (0–87)	35 (0–84)
≤5	364 (2)	27 (2)	79 (1)	72 (3)	12 (1)	174 (3)
6–17	553 (3)	31 (2)	147 (3)	109 (4)	36 (2)	230 (4)
18–49	12,172 (72)	1,145 (75)	4,059 (74)	1,638 (67)	936 (64)	4,394 (75)
50–64	2,869 (17)	282 (18)	900 (16)	471 (19)	324 (22)	892 (15)
≥65	853 (5)	52 (4)	304 (6)	170 (7)	147 (10)	180 (3)
Expatriate	1,066 (6)	276 (18)	315 (6)	54 (2)	22 (2)	399 (7)
Travel reason						
Business	2,710 (16)	490 (32)	797 (14)	211 (9)	187 (13)	1,025 (17)
Volunteer	2,438 (14)	310 (20)	975 (18)	139 (6)	77 (5)	937 (16)
Student	419 (2)	29 (2)	162 (3)	14 (1)	28 (2)	186 (3)
Tourism	7,914 (47)	254 (16)	2,932 (53)	1821 (74)	1128 (78)	1,779 (30)
VFR†	3,304 (20)	442 (29)	622 (11)	276 (11)	34 (2)	1,930 (33)
Hospitalized	2,718 (16)	319 (21)	628 (12)	264 (11)	71 (5)	1,436 (25)
Died	11 (<1)	3 (<1)	2 (<1)	0	0	6 (<1)
Sought pretravel advice‡						
Yes	8,752 (52)	775 (50)	3,387 (61)	860 (35)	875 (60)	2,855 (48)
No	4,721 (28)	383 (25)	1,172 (21)	1,038 (42)	345 (24)	1,783 (30)
Don't know	3,420 (20)	389 (25)	957 (17)	576 (23)	237 (16)	1,261 (21)

*Values are no. (%) unless otherwise indicated. The United Nations geoscheme was used to classify Africa into subregions (*5*).
†VFR, visiting friends or relatives.
‡Sought advice from clinician pertaining to recognition of illnesses in travel destination, preventive measures, and treatment.

most common cause of fever in returning travelers from sub-Saharan Africa, a finding consistent with those of previous studies (*6*,*7*). Of travelers who had malaria, 47% were VFR. Of travelers returning with malaria, 6% were <18 years of age. The proportion of febrile illness caused by malaria differed among regions: 2% of travelers returning from Southern Africa with febrile illness had malaria, compared with 69% and 67%, respectively, from Western and Central Africa. Conversely, African tick bite fever was the leading cause of illness in 273 (47%) of 579 travelers with fever returning from Southern Africa.

P. falciparum was the most common cause of malaria from all regions, including 100% of identified malaria cases from Southern Africa. *P. vivax* was proportionately more common from Eastern Africa (8%) than from other regions (0%–4%), and most of *P. ovale* and *P. malariae* cases were acquired in Central or Western Africa. Interregional seasonal variation in malaria acquisition was noted, although Western Africa was the only region that had a recognizable pattern of malaria cases, which the GeoSentinel sites reported more frequently from July through January.

A previous GeoSentinel study identified schistosomiasis as the most frequent helminthic infection reported in travelers returning from Africa, with regional variation noted (*8*). Seventy-three percent of reported strongyloidiasis cases were acquired in travelers returning from Eastern and Western Africa; 3% were reported in travelers to Southern Africa (Table 4). Acquisition of *Strongyloides stercoralis* from previous travel to, or birth in, other countries to which this infection is endemic cannot be discounted. Loasis accounted for 86 of 236 filarial infections, 82 (94%) of which were acquired from Central Africa, consistent with known endemicity; 50 (62%) of the 82 were from Cameroon, 12 from Gabon, 10 from Central African Republic, and 3 from Congo. Female travelers (58%) were more often infected by *Loa loa* than were men; volunteers (45%) and travelers VFR (30%) were the groups most often affected. Only 10% of cases were seen in tourists. Although largely an infection diagnosed in adults, 8% of cases were diagnosed in children (median age 10 years [interquartile range 7.0–16.5 years]).

Vaccine-preventable infections (VPI; i.e., hepatitis A, influenza, measles, and *Salmonella typhi* infection [typhoid]) taken together accounted for 0.9% of illnesses in travelers returning from Africa. The proportion of VPI from each region was comparable (Table 4). Among demographic groups, tourists were most likely to have VPI, most commonly hepatitis A and typhoid acquired in Northern Africa. Sex distribution was equal, and apart from 17 (29%) of the 59 hepatitis A cases that occurred in travelers <17 years of age, most VPI were diagnosed in adults. The acquisition of a VPI was not related to whether a pretravel consult had been sought.

During the 14-year study period, 86 cases of symptomatic tuberculosis were diagnosed in travelers returning from Africa, and an additional 159 travelers had positive tuberculin skin tests. Whether the positive skin tests were caused by acquisition of infection during travel or by prior exposure is unknown because no records of pretravel test

Table 2. Deaths of ill travelers returning from Africa who were seen at GeoSentinel clinic sites, March 1997–May 2011*

Patient no.	Age, y/sex	Diagnosis	Region	Exposure country	Travel reason	Expatriate
1	66/M	Malaria, *Plasmodium falciparum*. Severe and complicated, noncerebral	Western	Burkina Faso	Business	No
2	68/M	Malaria, *P. falciparum*. Severe and complicated, cerebral	Eastern	Kenya	Tourism	No
3	50/M	Malaria, *P. falciparum*. Severe and complicated, cerebral	Western	Ghana	Business	Yes
4	61/M	Malaria, *P. falciparum*. Severe and complicated, cerebral	Western	Sierra Leone	Business	No
5	4/M	Pneumonia, bacterial, lobar	Eastern	Tanzania,	Accompanying parent on business	No
6	48/M	Malaria, *P. falciparum*. Severe and complicated, cerebral	Western	Ghana	VFR	No
7	47/M	*Mycobacterium tuberculosis*, pulmonary and extrapulmonary	Western	Unknown	VFR	No
8	57/M	Malaria, *P. falciparum*. Severe and complicated, cerebral	Western	Liberia	Missionary/volunteer/ researcher/aid worker	No
9	30/F	Malaria, *P. falciparum*. Severe and complicated, cerebral	Central	Equatorial Guinea	Business	Yes
10	53/M	Malaria, *P. falciparum*. Severe and complicated, noncerebral	Central	Angola	Business	No
11	40/M	Malaria, *P. falciparum*. Severe and complicated, cerebral	Central	Angola	Business	No

*Three patients with malaria who died did not receive chemoprophylaxis, 1 received mefloquine, and data were missing for the remaining 5 travelers. The United Nations geoscheme was used to classify Africa into subregions (*5*). VFR, visiting friends or relatives.

results are available. Travelers returning from Central and Southern African regions, which are among countries with the highest rates of tuberculosis worldwide (*9*), accounted for 6 cases of symptomatic tuberculosis (Table 4).

Acute HIV infection was recorded for 44 persons. As was the case for tuberculosis, the number of acute HIV infections from Central and Southern Africa was lower than that from Eastern and Western Africa. No cases were diagnosed in travelers to Northern Africa.

Dengue is a common cause of illness in travelers to the Asia–Pacific region and Latin America (*2*); however, in our study, dengue acquired in Africa was diagnosed in as few as 113 travelers with febrile illness during the 14-year study period. Our data suggest that 81% of cases were acquired during travel to either Eastern or Western Africa (Table 4). Dengue was diagnosed equally in both sexes, and infection in travelers 18–49 years of age accounted for 81% of cases. Tourists were the major risk group for this illness.

Discussion

Our study provides an evidence base of regional infectious disease exposures among travelers returning from Africa. These data show a profile of travel-related illness that differs with that of resident populations in these regions; this knowledge is essential in prioritizing preventive measures for the approximately 50 million travelers to Africa each year. Diarrheal and other gastrointestinal illnesses, hepatitis A, dog bites, and a very low proportion of febrile illnesses characterized the health of travelers returning from Northern Africa. In contrast, febrile illnesses were the predominant cause of clinic visits in travelers returning from sub-Saharan Africa, although considerable differences were evident in the etiology of fever in travelers from different regions. Malaria, which was most common in travelers returning from Central and Western Africa, was seen infrequently in travelers to Southern and Northern Africa. The incidence of helminthic infections also varied considerably: schistosomiasis and strongyloidiasis predominated in travelers returning from Eastern and Western Africa, but 82 of 86 *L. loa* infections reported in this study were diagnosed in travelers returning from Central Africa (Table 4). HIV infection and tuberculosis dominate the incidence of disease for much of sub-Saharan Africa and are an increasing concern for travelers, but our results show that these infections were rarely diagnosed in travelers at GeoSentinel Sites (Table 4).

Several factors likely contributed to our observations. Certain illnesses related to travel in Africa are more common in particular demographic groups. For instance, malaria is more common in travelers VFR than in tourists (*9*). In our study, malaria-related deaths occurred most often in men who traveled for business, a finding that may have implications for companies with expanding business interests in Africa. Pretravel medical advice and use of effective malaria prevention measures and chemoprophylaxis are essential for business travelers to areas of risk. Quantifying malaria risk is difficult, but the regional profiles presented here in which malaria predominated as a diagnosis are useful indicators (Tables 3, 5). High rates of malaria in Western and Central Africa reflect high malaria

Table 3. Diagnoses in descending order of frequency, by region of origin, for ill travelers returning from Africa who were seen at GeoSentinel clinic sites, March 1997–May 2011*

	Illness and no. (%) travelers				
Total, N = 16,893	Central Africa, n = 1,547	Eastern Africa, n = 5,516	Northern Africa, n = 2,474	Southern Africa, n = 1,457	Western Africa, n = 5,899
Malaria, *Plasmodium falciparum*. 2,118 (13)	Malaria, *P. falciparum*, 313 (20)	Viral syndrome, no rash, 444 (8)	Diarrhea, acute unspecified, 419 (17)	Rickettsia, tick-borne spotted fever, *R. africae, R. conorii, R. rickettsii* and other, 273 (19)	Malaria, *P. falciparum*, 1,484 (25)
Diarrhea, acute unspecified, 1,373 (8)	Viral syndrome, no rash, 114 (7)	Diarrhea, acute unspecified, 414 (8)	Diarrhea, chronic unknown, 262 (11)	Viral syndrome, no rash, 177 (12)	Viral syndrome, no rash, 396 (7)
Viral syndrome, no rash, 1200 (7)	Filaria, *Loa Loa*, 81 (5)	Malaria, *P. falciparum*, 291 (5)	Diarrhea, acute bacterial, 145 (6)	Diarrhea, acute unspecified, 95 (7)	Diarrhea, acute unspecified, 365 (6)
Diarrhea, chronic unknown, 791 (5)	Diarrhea, acute unspecified, 80 (5)	Diarrhea, chronic unknown, 254 (5)	Gastroenteritis, 107 (4)	Diarrhea, chronic unknown 69 (5)	*Giardia*, 209 (4)
Diarrhea, acute bacterial, 638 (4)	Diarrhea, acute bacterial, 48 (3)	Diarrhea, acute bacterial, 219 (4)	Rabies, postexposure prophylaxis, 107 (4)	Febrile illness, unspecified (<3 wk), 53 (4)	Diarrhea, acute bacterial, 207 (4)
Febrile illness, unspecified, <3 wk, 495 (3)	Febrile illness, unspecified, <3 wk, 46 (3)	Febrile illness, unspecified, <3 wk, 180 (3)	Irritable bowel syndrome, post infectious, 89 (4)	Respiratory tract infection (upper), 40 (3)	Febrile illness, unspecified, <3 wk, 184 (3)
Giardia, 491 (3)	Diarrhea, chronic unknown, 42 (3)	Respiratory tract inf (upper), 167 (3)	*Giardia*, 87 (4)	Bite or sting, insect, 33 (2)	Diarrhea, chronic unknown, 164 (3)
Respiratory tract infection (upper), 387 (2)	*Giardia*, 41 (3)	Bite or sting, insect, 142 (3)	Viral syndrome, no rash, 69 (3)	Rash, unknown etiology (non-febrile), 33 (2)	Respiratory tract infection (upper), 124 (2)
Irritable bowel syndrome, post infectious, 375 (2)	*Strongyloides*, simple intestinal, 34 (2)	Giardiasis, 133 (2)	Bite, dog, 52 (2)	Bite, insect; superinfected, 25, (2)	Irritable bowel syndrome, post-infectious, 110 (2)
Rickettsia, tick-borne spotted fever, 336 (2)	Malaria, severe and complicated, noncerebral, 27 (2)	Irritable bowel syndrome, post-infectious, 131 (2)	*Campylobacter*, 42 (2)	Bite, Tick 23 (2)	Malaria, species unknown 103 (2)

*The United Nations geoscheme was used to classify Africa into subregions (*5*).

transmission levels in these areas. Entomologic inoculation rates (the number of infectious mosquito bites that a person receives in a certain time period) are a useful indicator of malaria risk. For example, the entomological inoculation rate for Bayma in Sierra Leone (Nov 1990–Oct 1991) was 884, compared with 1.5 for Kilifi Town in Kenya during the same period (*10*).

For some infections, such as the helminthic infections loaisis and onchocerciasis, vector distribution affects acquisition. Seasonal and climatic factors and degree of endemicity influence the likelihood of malaria and dengue transmission, whereas food and water hygiene, along with differences in sanitation, influence acquisition of diarrheal diseases. Illnesses that are commonly self-limiting and often have mild symptoms, such as influenza and dengue, are less likely to result in a visit to a clinic. Furthermore, travelers in a sub-Saharan Africa country who have fever may be examined by clinicians who will treat them empirically for malaria and thus misdiagnose another infection.

The finding that travelers were rarely seen in GeoSentinel site clinics for VPI, irrespective of the region visited, extends previous observations from Southern Africa (*4*) and remains a paradox, considering that rates of vaccination against these illnesses are historically <45% (*11–13*). We hypothesize that subclinical infection of children with hepatitis A or the low likelihood of adults or children with mild influenza-like illness to seek medical attention may account for low numbers of these 2 VPI reported from GeoSentinel sites. Most hepatitis A cases were in travelers to Northern Africa, which reflects the high rate of gastrointestinal infections from that region (Table 3). Our findings emphasize the importance of hepatitis A vaccination, which should be emphasized to health care providers and travelers alike, particularly when Northern Africa is the intended travel destination.

The high number of animal bites and the subsequent need for rabies PEP in travelers returning from Northern Africa are likely to reflect bias because 97% of GeoSentinel-reported cases in travelers returning from Northern Africa who sought rabies PEP were reported to the Marseille, France, site, which is a reference center for management of suspected rabies exposures. However, the paucity of reported bites and need for rabies PEP at GeoSentinel sites among travelers returning from sub-Saharan Africa may in part reflect that travelers to sub-Saharan Africa were more often travelers VFR or long-term travelers, who may be more likely than tourists to Northern Africa to seek treatment at the time of exposure (*14*).

Sixty-seven percent of the world's HIV-infected population resides in sub-Saharan Africa (*15*), and tuberculosis

Table 4. Nonmalarial illness among travelers returning from Africa who were seen at GeoSentinel clinic sites, March 1997–May 2011*

Illness/incident	No. travelers					
		Region visited before illness				
	Total	Central Africa	Eastern Africa	Northern Africa	Southern Africa	Western Africa
Parasitic infection by helminths						
Schistosomes	530	42	278	41	22	147
Unknown spp.	274	27	129	22	13	83
Schistosoma mansoni	147	9	89	14	5	30
S. haematobium	118	7	66	5	5	35
Filaria						
Strongyloides	195	34	78	13	6	64
Simple intestinal	191	34	78	12	6	61
Hyperinfection	4	0	0	1	0	3
Non-*Strongyloides*	140	102	6	1	1	30
Loa loa	86	82	1	0	0	3
Onchocerca volvulus	21	12	0	0	0	9
Other	31	9	4	0	1	17
Wucheria bancrofti	4	1	1	1	0	1
Vaccine-preventable disease	146	9	47	37	7	46
Hepatitis A	59	3	14	28	1	13
Influenza	24	0	11	4	2	7
Measles	5	0	0	1	3	1
Typhoid fever†	58	6	22	4	1	25
Bite wounds‡	193	5	47	105	11	25
Bite wounds necessitating rabies prophylaxis	184	4	38	107	13	22
Source of bite						
Dog	91	4	17	52	7	11
Cat	46	0	8	36	0	2
Other¶	56	1	22	17	4	12
Dengue (uncomplicated)	113	6	46	5	8	48
Tuberculosis	86	2	33	16	4	31
Pulmonary	43	2	14	14	2	11
Extrapulmonary	24	0	13	0	0	11
Miliary, disseminated	13	0	4	1	2	6
Meningitis	5	0	2	1	0	2
Multidrug resistant#	1	0	0	0	0	1
Acute HIV infection	44	4	21	0	5	14

*The United Nations geoscheme was used to classify Africa into subregions (*5*).
†*Salmonella enterica* serotype Typhi.
‡Two travelers returning from Northern Africa and 2 returning from Southern Africa for whom bite wounds were not registered received rabies postexposure prophylaxis.
¶Other bites: monkey (23 bites); snake (3); rat (2); human, horse, puff adder, rabbit arthropoda, baboon, bat, hamster, leech, mangout, mouse, scolopendra, squirrel (1 each), missing data (14).
#Resistance to rifampin and isoniazid.

prevalence in some regions of Africa approaches 1% (*16*). Because 5%–50% of travelers report casual sexual experiences while traveling (*17*), it is surprising that so few cases of acute HIV infection were documented in travelers examined in GeoSentinel sites if risk behavior and endemicity of infection are high in many regions (*18*). Travelers who have symptoms of HIV and other sexually transmitted infections may seek care at specialty clinics rather than at GeoSentinel sites. However, because symptoms of acute HIV infection are commonly protean, and often manifest as a nonspecific febrile illness, travelers are as likely to go to GeoSentinel clinic sites as to specialist clinics. HIV should always be considered as a differential diagnosis in febrile returning travelers or in travelers who have clinical features compatible with HIV seroconversion illness. Regarding tuberculosis, although reactivation of latent infection may occur many years after acquisition, it is noteworthy that despite the large number of tuberculosis cases in most regions of sub-Saharan Africa, so few symptomatic cases occurred in travelers to Africa during the prolonged period of this study.

Previous studies of illnesses acquired by travelers to Africa have focused on illnesses acquired from sub-Saharan Africa as a whole (*2*,*19*), have been conducted at a single center (*20*,*21*), or have had analyses limited to a single disease or infection (*2*,*5*). Strengths of the current study are data capture from 54 international surveillance sites and >500 different diagnoses, enabling us to show regional patterns of illness in Africa. In addition, data were collected during an extended time period, which may offset acute spikes in reporting particular diagnoses that could skew the data.

Conclusions

This study confirms that the likelihood of acquiring a specific infection or disease is not homogenous throughout the African continent. Medical practitioners examining

Table 5. Malaria in ill returned travelers from Africa seen at GeoSentinel clinic sites, March 1997–May 2011*

Illness	Total, N = 16,893	No. (%) travelers: Region visited before illness				
		Central Africa, n = 1,547	Eastern Africa, n = 5,516	Northern Africa, n = 2,474	Southern Africa, n = 1,457	Western Africa, n = 5,899
Febrile/systemic illness	5,505 (33)	626 (40)	1,474 (27)	219 (9)	579 (23)	2,607 (44)
Malaria†	2,789 (50.7)	416 (66.5)	515 (34.9)	37 (16.9)	12 (2.1)	1,809 (69.4)
Plasmodium falciparum‡	2,230 (40.5)	338 (54.0)	316 (21.4)	22 (10.0)	9 (1.6)	1,545 (59.3)
Uncomplicated	2,118 (38)	313 (50.0)	291 (19.7)	22 (10.0)	8 (1.4)	1,484 (56.9)
Severe noncerebral	104 (1.9)	27 (4.3)	23 (1.6)	1 (0.5)	1 (0.2)	52 (2.0)
Severe cerebral	61 (1.1)	13 (2.1)	12 (8)	0	1 (0.2)	35 (1.3)
P. vivax	197 (3.6)	19 (3.0)	122 (8.3)	9 (4.1)	0	47 (1.8)
P.ovale	138 (2.5)	21 (3.4)	26 (1.8)	2 (1)	0	89 (3.4)
P.malariae	84 (1.5)	20 (3.2)	17 (1.2)	2 (1)	0	45 (1.7)
Unknown species	179 (3.3)	26 (4.2)	44 (3.0)	3 (1.4)	3 (0.5)	103 (4.0)

*The United Nations geoscheme was used to classify Africa into subregions (*5*).
†Sudan and Western Sahara are malaria-endemic countries.
‡Row totals indicate unique patients.

returning febrile travelers from any region in sub-Saharan Africa must consider malaria as the first diagnosis. However, febrile patients, particularly those from Southern Africa, infrequently have malaria; other illnesses such as rickettsial infections are more common and should be strongly suspected. Concern about risk and possible damage to health is a determinant of whether a person will travel to a particular destination (*22*), so misconceptions should be corrected. However, because outbreaks reported, such as the recent reports of dengue virus in Angola (*23*) and Kenya (*24*) can rapidly alter exposure risk, up-to-date knowledge of patterns of disease is crucial.

During pretravel consultation, prevention of illness in travelers to different regions within Africa should be prioritized on the basis of the regional profile of diagnoses. For Northern Africa, advice should include detailed information about food hygiene and rabies prophylaxis. For countries in the Southern African region, malaria chemoprophylaxis and mosquito bite prevention measures should be used to prevent malaria acquisition during travel to regions in which malaria is endemic. The travel medicine advisor should be familiar with malaria-endemic and malaria-free areas in Africa to be able to provide suitable recommendations. Special attention should be paid to travelers VFR, who are most likely to acquire malaria, and to business travelers, whose malarial infection may be more likely to be fatal. Advice about preventing tick-borne rickettsial infections is essential for travelers to Southern Africa. Helminthic infections should be discussed. Preexposure and postexposure rabies vaccination, travelers' diarrhea, and vaccine-preventable diseases should be discussed with travelers to destinations on the entire African continent.

Our analysis has some limitations: GeoSentinel Sites see a subset of all travelers to a particular country or region, and a main limitation of our study is an inability to calculate absolute risk for or true incidence of a disease from that country or region. Furthermore, not all returning travelers who are ill will seek care at a GeoSentinel site but instead will visit infectious diseases specialists, pediatricians, or nonspecialists. Children and adults with mild illness, such as an influenza-like illness, may not seek care at all. For 16,925 travelers in the GeoSentinel database, the name of the country in which they were exposed was missing or unascertainable. Of these, 3,364 persons had traveled to countries within the same African region and nowhere else and were therefore included in the dataset. The remaining 13,561 records were excluded from analysis. The effect of this removal of records on introducing bias is unknown. However, including them in the analysis would have risked including illnesses ascribed to 1 region but acquired in another. One GeoSentinel site contributed 25% of the final dataset, and these travelers were more likely to have traveled to Northern Africa (40%), be female (52%), and be a tourist (36%). Excluding this group did not substantially alter the profile of the final dataset. Northern Africa is viewed increasingly by European tourists as a Mediterranean-like destination in which risk for illness is lower than that in other parts of Africa, which may partially explain the low rate of pretravel consultation for travelers to Northern Africa.

The United Nations geoscheme for dividing Africa into 5 constituent regions is limited in that it was originally devised to enable statistical analysis of various parameters. Therefore, assignment of any 1 country into a particular area was for statistical convenience and was not meant to imply assumptions regarding health data. Thus, some countries, particularly those that border each other but are in different regions by the United Nations definition, may share health exposure risks for travelers, which therefore limits our study's findings. However, because no division of Africa has been performed on health grounds, devising an arbitrary regional division would be equally as speculative and open to bias.

In conclusion, our study shows that health risks of travel to Africa are not uniform. Geographic profiling of

illness acquired during travel to Africa facilitates targeted pretravel advice, expedites diagnosis in ill returnees, and may influence destination choices in tourism.

Members of the GeoSentinel Surveillance Network who contributed data to this study are: Rahul Anand, University of Utah, Salt Lake City, Utah, USA; Hilmir Ásgeirsson, Karolinska University Hospital, Stockholm, Sweden; Elizabeth D. Barnett, Boston University, Boston, Massachusetts, USA; Sarah Borwein, TravelSafe Medical Centre, Hong Kong SAR, China; Gerd-Dieter Burchard, Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany; John D. Cahill, St. Luke's-Roosevelt Hospital Center, New York, New York, USA; Daniel Campion, London InterHealth, London, UK; Francesco Castelli, University of Brescia, Brescia, Italy; Eric Caumes, Hôpital Pitié-Salpêtrière, Paris, France; Lin H. Chen, Mount Auburn Hospital, Harvard University, Cambridge, Massachusetts; Bradley A. Connor, Cornell University, New York; Christina M. Coyle, Albert Einstein School of Medicine, Bronx, New York; Jakob Cramer, Bernhard-Nocht-Institute for Tropical Medicine, Hamburg; Jane Eason, Beijing United Family Hospital and Clinics, Beijing, China; Cécile Ficko, Hôpital d'instruction des armées Bégin, Saint-Mandé, France; Vanessa Field, London InterHealth, London; David O. Freedman, University of Alabama at Birmingham, Alabama, USA; Abram Goorhuis, University of Amsterdam, Amsterdam, the Netherlands; Martin P. Grobusch, University of Amsterdam, Amsterdam; Alejandra Gurtman, Mount Sinai Medical Center, New York; Devon C. Hale, University of Utah, Salt Lake City, Utah, USA; Annemarie Hern, Worldwise Travellers Health and Vaccination Centre, Auckland, New Zealand; Noreen Hynes, Johns Hopkins University, Baltimore, Maryland, USA; Mogens Jensenius, Oslo University Hospital, Oslo, Norway; Robert Kass, Travellers Medical and Vaccination Centres of Australia, Adelaide, Australia; Amy D. Klion, National Institutes of Health, Bethesda, Maryland; Phyllis E. Kozarsky, Emory University, Atlanta, Georgia, USA; Karin Leder, Royal Melbourne Hospital, Melbourne, Australia; Carmelo Licitra, Orlando Regional Health Center, Orlando, Florida, USA; Rogelio López-Vélez, Hospital Ramon y Cajal, Madrid, Spain; Michael W. Lynch, Fresno International Travel Medical Center, Fresno, California, USA; Alberto Matteelli, University of Brescia; Anne McCarthy, University of Ottawa, Ottawa, Canada; George McKinley, St. Luke's-Roosevelt Hospital Center, New York; Susan McLellan; Tulane University, New Orleans, Louisiana, USA; José Antonio Pérez Molina, Hospital Ramon y Cajal; Robert Muller, Travel Clinic Services, Johannesburg, South Africa; Thomas B. Nutman, National Institutes of Health; Alice Pérignon, Hôpital Pitié-Salpêtrière; Phi Truong Hoang Phu, International SOS Clinic, Ho Chi Minh City, Vietnam; Watcharapong Piyaphanee, Mahidol University, Bangkok, Thailand; Christophe Rapp, Hôpital d'instruction des armées Bégin, Saint-Mandé, France; David Roesel, University of Washington and Harborview Medical Center, Seattle, Washington, USA; Eli Schwartz, Chaim Sheba Medical Center, Tel Hashomer, Israel; Marc Shaw, Worldwise Travellers Health and Vaccination Centre, Auckland; Udomsak Silachamroon, Mahidol University; William Stauffer, Regions Hospital, St. Paul, Minnesota, USA; Natsuo Tachikawa, Yokohama Municipal Citizen's Hospital, Yokohama, Japan; Joseph Torresi, Royal Melbourne Hospital; Johan Ursing, Karolinska University Hospital; Jean Vincelette, Centre Hospitalier de l'Université de Montréal, Montréal, Québec, Canada; Patricia Walker, HealthPartners, St. Paul; Andy Wang, Beijing United Family Hospital and Clinics; Mary E. Wilson, Harvard University; Henry Wu, Emory University; Yukihiro Yoshimura, Yokohama Municipal Citizen's Hospital.

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Dr Mendelson is principal specialist and head of the Division of Infectious Diseases and HIV Medicine at University of Cape Town. He is director of the Cape Town Geosentinel Travel Surveillance Network Site. His research interests include travel medicine, antibiotic stewardship, HIV–tuberculosis co-infection, and neglected tropical diseases.

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Address for correspondence: Marc Mendelson, Division of Infectious Diseases & HIV Medicine, Department of Medicine, University of Cape Town, G16/68 Groote Schuur Hospital, Observatory 7925, Cape Town, South Africa; email: marc.mendelson@uct.ac.za

Active Surveillance for Avian Influenza Virus, Egypt, 2010–2012

Ghazi Kayali, Ahmed Kandeil, Rabeh El-Shesheny, Ahmed S. Kayed, Mokhtar M. Gomaa, Asmaa M. Maatouq, Mahmoud M. Shehata, Yassmin Moatasim, Ola Bagato, Zhipeng Cai, Adam Rubrum, Mohamed A. Kutkat, Pamela P. McKenzie, Robert G. Webster, Richard J. Webby, and Mohamed A. Ali

Continuous circulation of influenza A(H5N1) virus among poultry in Egypt has created an epicenter in which the viruses evolve into newer subclades and continue to cause disease in humans. To detect influenza viruses in Egypt, since 2009 we have actively surveyed various regions and poultry production sectors. From August 2010 through January 2013, >11,000 swab samples were collected; 10% were positive by matrix gene reverse transcription PCR. During this period, subtype H9N2 viruses emerged, cocirculated with subtype H5N1 viruses, and frequently coinfected the same avian host. Genetic and antigenic analyses of viruses revealed that influenza A(H5N1) clade 2.2.1 viruses are dominant and that all subtype H9N2 viruses are G1-like. Cocirculation of different subtypes poses concern for potential reassortment. Avian influenza continues to threaten public and animal health in Egypt, and continuous surveillance for avian influenza virus is needed.

In 2008, highly pathogenic avian influenza (HPAI) A(H5N1) virus became enzootic among poultry in Egypt, and the country became an epicenter for virus activity (*1*). As the established viruses drifted over time, viral genetic and antigenic diversity was generated. During 2010–2011, subclade 2.2.1 viruses (direct-drift progeny of the initially introduced virus) and 2.2.1.1 viruses (which might have emerged because of vaccine pressure) were cocirculating among poultry in Egypt (*2*). These subclades differed genetically and antigenically, hence complicating control efforts, especially vaccination (*3*). Subclade 2.2.1 viruses, commonly isolated from backyard flocks that are not vaccinated, caused all of the human cases in Egypt; from 2006 through September 2013, the toll rose to 173 cases and 63 deaths (*4*,*5*). Subclade 2.2.1.1 viruses were more prevalent on commercial farms, where vaccines are more frequently used (*6*). Furthermore, recent reports have indicated that very few mutations are needed for subtype H5N1 to become transmissible among ferrets, the best mammalian model of human influenza infection (*7*,*8*). In Egypt, a subtype H5N1 virus was found to have 2 of the 4 mutations needed to gain the transmissibility function, thereby underlying the need and urgency for surveillance among poultry (*8*). The Nile Delta region of Egypt was also identified as an area where substantial reassortment of influenza viruses can take place (*9*). As a further complication, in 2011, subtype H9N2 viruses were detected in poultry from areas in Egypt where subtype H5N1 viruses circulate (*10*).

Since 2009, we have been conducting systematic, active surveillance of avian influenza virus (AIV) among poultry in Egypt; the same locations are sampled over time, regardless of whether a clinical outbreak of disease is present. We previously reported that the threat of HPAI (H5N1) virus is widespread beyond rural areas and that the commercial sector is a key reservoir for virus transmission (*11*). Here we provide an update on the changing epizootiology and genetic features of AIV in Egypt and report coinfection of poultry in Egypt with influenza virus subtypes H5N1 and H9N2.

Materials and Methods

Sample Collection and Processing

A team of veterinarians collected cloacal and oropharyngeal swab samples from 11,452 birds from 4 poultry

Author affiliations: St. Jude Children's Research Hospital, Memphis, Tennessee, USA (G. Kayali, A. Rubrum, P.P. McKenzie, R.G. Webster, R.J. Webby); National Research Center, Giza, Egypt (A. Kandeil, R. El-Shesheny, A.S. Kayed, M.M. Gomaa, A.M. Maatouq, M.M. Shehata, Y. Moatasim, O. Bagato, M.A. Kutkat, M.A. Ali); and Georgia State University, Atlanta, Georgia, USA (Z. Cai)

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production sectors: commercial farms, backyard flocks, live-bird markets, and abattoirs. One swab sample was collected per bird, and depending on the size of the population, as many as 5 birds were sampled per flock. Birds were not randomly selected; samples were also collected from sick or dead birds found on site. From August 2010 through January 2013, a total of 6,904 cloacal and 4,548 oropharyngeal samples were collected from 63 sites in 7 governorates in Egypt, including Cairo (4 neighborhoods); 4 Nile Delta governorates (Qalubiya [12 villages], Menofiya [9 villages], Sharqiya [3 towns], and Daqahliya [4 towns]); and 2 mid-Egypt governorates (Fayyoum [22 villages] and BeniSuef [9 villages]) (Figure 1). The selected governorates represent the main foci of the poultry industry in Egypt and sites of previous AIV detection (*11*). The selected sampling sites were areas at which the veterinarian was known to the local population and thus had access to the poultry. The sites were routinely visited on a monthly basis regardless of the occurrence of clinical signs or poultry deaths. Study veterinarians subjectively recorded their field observations. Swab samples were collected in medium containing 50% glycerol, 50% phosphate-buffered saline (PBS), penicillin (2×10^6 U/L), streptomycin (200 mg/L), and amphotericin B (250 mg/L) (antimicrobial drugs from Lonza, Walkersville, MD, USA). Samples were chilled on ice until delivered to the laboratory (within 24 hours). All samples were stored at –80°C until used.

Screening for AIV

For detection of AIV, 100 µL from 5 samples was pooled; RNA extracted from pools collected up to July 2012 was subjected to reverse transcription PCR (RT-PCR) to amplify 244 bp of the matrix segment of AIV, according to World Health Organization (WHO) protocol (*12*). Samples with amplified M segments were subjected to H5, H7, and H9 hemagglutinin subtype determination by RT-PCR according to the same WHO protocol, except the annealing temperature for H5 primers was 58°C (*12*,*13*). As of August 2012, quantitative RT-PCR (qRT-PCR) was used for typing and subtyping AIV. Typing and subtyping primers are listed in Table 1.

Virus Isolation

Samples that showed a positive reaction in the partial M segment RT-PCR were grown in the allantoic cavities of 10-day-old specific pathogen–free embryonated chicken eggs. Virus titers were determined by chicken red blood cell hemagglutination assays (*12*).

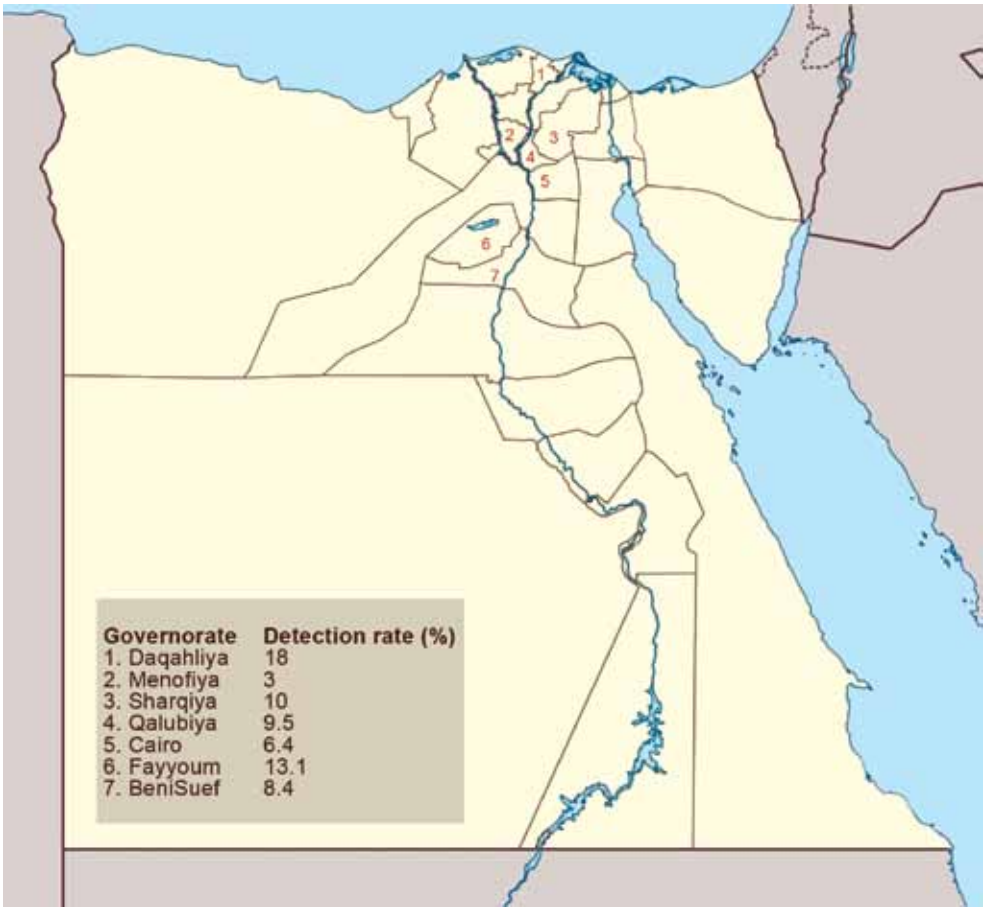

Figure 1. Location of surveillance governorates and percentage of avian influenza virus detection in each governorate, Egypt, 2010–2012.

Table 1. Primers used for H5 and H9 subtyping of avian influenza viruses from Egypt, 2010–2012*

Primer	Sequence, 5′→3′	Reference
M30F2/08	ATGAGYCTTYTAACCGAGGTCGAAACG	(*12*)
M264R3/08	TGGACAAANCGTCTACGCTGCAG	
H5–155f	ACACATGCYCARGACATACT	(*13*)
H5–699r	CTYTGRTTYAGTGTTGATGT	
H9–151f	CTYCACACAGARCACAATGG	
H9–638r	GTCACACTTGTTGTTGTRTC	
BDH9–4F2	CAAGCGTGACAACAGAAAATTTGG	Designed in-house†
BDH9–2R2	CTCCTGAGAGAACGTGTCCATACC	
H9PROB	FAM CTTACTCGCAATGTCTGGCCTGGTTTTAG BHQ1	
AH5b_Forward	GGA ATGYCCCAAATATGTGAAATCAA	(*14*)
AH5b_R	CCACTCCCCTGCTCRTTGCT	
H5PROB	FAM TACCCATACCAACCATCTACCATTCCC BHQ1	
Inf-A F	ACCRATCCTGTCACCTCTGAC	
Inf-A R	AGGGCATTYTGGACAAAKCGTCTA	
Inf-A POB	FAM TGCAGTCCTCGCTCACTGGGCACG BHQ1	

*Typing by reverse transcription PCR and quantitative reverse transcription PCR.
†St. Jude Children's Research Hospital, Memphis, TN, USA.

Hemagglutinin Gene Sequencing and Sequence Analyses

The purified amplicons of 26 H5 and 15 H9 segments, selected to represent time and species, were sequenced as described (*15*). Phylogenetic analyses were performed by using MEGA version 4.0.2 (www.megasoftware.net) with the neighbor-joining method and Poisson correction (*16*). The sequences were submitted to GenBank under accession nos. KF258174–91, CY099582–8, CY099591–3, JX912982–6, JX912988, JX912990–2, and JX912994–7.

Antigenic Cartography

Chicken red blood cell hemagglutination inhibition (HI) assays were conducted in accordance with the WHO protocol (*12*) and with monoclonal antibodies against influenza virus subtype H5N1. Antigenic maps were constructed by using virus titers and AntigenMap software (*17*).

Influenza Virus Subtype H5N1 and H9N2 Co-infection

Because co-infection with influenza virus subtypes H5N1 and H9N2 can give way to viral reassortment and production of viral progeny with unpredictable phenotypes, we studied co-infected samples in more detail. The presence of the 2 viruses in 3 selected samples collected in 2012 (Q5018B, D5809C, D5809D) was detected by RT-PCR, qRT-PCR, hemagglutinin sequence analysis, immunofluorescence, and Western blotting. The samples were propagated in specific pathogen–free embryonated chicken eggs, and the allantoic fluid was subjected to qRT-PCR. To separate the 2 viruses, we then conducted a plaque purification assay. Individual plaques were picked and injected into specific pathogen–free embryonated chicken eggs and MDCK cells (in the presence or absence of L-1-tosylamide-2-phenylethyl chloromethyl ketone [TPCK]–treated trypsin). After incubation, the allantoic fluids and cell culture supernatant were harvested and subjected to qRT-PCR.

Plaque Purification

A 100-μL sample of the original decontaminated samples and 10-fold serial dilutions from each sample were inoculated into wells of a 6-well plate containing confluent MDCK cells with 400 μL serum-free medium. The plates were incubated at 37°C for 1 hour. The wells were aspirated to remove residual viral solution. Each well was then immediately covered with 2 mL 1× agarose overlay mixture (final concentration 1% agarose type 1, 1× Dulbecco modified Eagle medium, 10% antibiotic/antimycotic solution). Plates were then incubated at 37°C under 5% CO_2 for 2 days. Plaques were picked, and each plaque was inoculated into specific pathogen–free embryonated chicken eggs for propagation of purified plaques.

Immunofluorescence

MDCK cells were inoculated with original co-infected specimens. At 1 day after inoculation, the cells were fixed with 1 mL 3.7% formalin in PBS for 5 min, and then 1 mL cold methanol was added for 5 min. Cells were blocked by using 1 mL 1% bovine serum albumin (Serva, Heidelberg, Germany) in PBS-Tween 20 at 37°C for 1 hour. Rat and chicken antiserum against H5N1 and H9N2 viruses, respectively, were incubated with the fixed cells. Fluorescein isothiocyanate–conjugated goat anti–chicken IgG and goat anti–rat IgG diluted 1:2000 (KPL, Gaithersburg, MD, USA) were then added. Fluorescently labeled cells were examined by using fluorescence microscopy.

Western Blotting

Viruses propagated in specific pathogen–free embryonated chicken eggs were analyzed by SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as described (*18*); the only modification was that 1% bovine serum albumin in PBS–0.3% Tween20 was used to block the protein-free binding sites on the nitrocellulose membrane. Immunorecognition was performed on cut membrane strips

carrying chicken anti-H9N2 serum (dilution 1:50) or mouse anti-H5 monoclonal antibody (dilution 1:100). Immune detection was conducted by using peroxidase-conjugated goat anti–chicken IgG and goat anti–mouse IgG (KPL) diluted 1:2000 in PBS–0.3% Tween20.

Propagation Rates

Equal titers of influenza virus subtypes H5N1 and H9N2 were separately or jointly inoculated into specific pathogen–free embryonated chicken eggs and MDCK cells. The amount of virus propagated was measured by qRT-PCR at 24 hours after inoculation.

Statistical Analysis

The proportion of samples that were positive according to the different study variables was determined. The Pearson χ^2 test was used

Results

AIV in Poultry

Test results were positive for 1,144 birds (Table 2); the overall percentage of AIV detected was 10% (95% CI 9.5%–10.5%). This percentage differed significantly according to governorate, species, production sector, health status, and age (Table 2). The detection percentage by governorate ranged from 3% in Menofiya to 18% in Daqahliya (Figure 1). Detection percentage for urban Cairo was 6.4%. Of the swab samples collected, 84.2% were from chickens, 10.3% were from ducks, and 5.5% were from other species of domestic birds (Table 2). The detection percentage was highest among turkeys (15.3%); followed by ducks (12.3%); and then chickens, pigeons, and geese (≈9%). Among swab samples collected from the different poultry sectors, the highest percentage positive for AIV (≈12%) came from commercial farms and backyard flocks, followed by live-bird markets (6.7%) and abattoirs (5.1%). Most (88.3%) swab samples were collected from apparently healthy birds; of those, 8.3% were positive for AIV. Influenza A viruses were detected in 20.8% of sick and 42.4% of dead birds. The detection percentage among poultry >1 year of age was 19.4%; that among birds <1 year of age was 9.9%.

More AIV was detected in poultry during colder months, and none was detected in August (Figure 2; online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/20/4/13-1295-Techapp1.pdf). Among poultry, the detection percentage for AIV was <5% until September 2012, when we recorded a sudden increase of >15%. This outbreak peaked at ≈25% in October 2012 and continued into January 2013 (Figure 2). This outbreak was detected at all of our sampling sites and was more pronounced in Cairo, Fayyoum, and BeniSuef, where we did not detect AIV before this outbreak (online Technical Appendix Figure 1).

Of the 1,144 influenza virus–positive samples, we subtyped 897. From August 2010 through November 2011, all

Table 2. Epizootiologic data for avian influenza virus isolated from poultry in 7 governorates in Egypt, 2010–2012

Variable	Samples collected, no. (%)	Influenza A–positive samples, no. (%)	p value
Sample type			Not significant
Cloacal	6,904 (60.3)	686 (9.9)	
Oropharyngeal	4,548 (39.7)	458 (10.1)	
Governorate			<0.001
Cairo	2,690 (23.4)	173 (6.4)	
Daqahliya	1,440 (12.6)	259 (18)	
Qalubiya	1,478 (12.9)	141 (9.5)	
Menofiya	935 (8.2)	28 (3)	
Sharqiya	2,365 (20.7)	236 (10)	
Fayyoum	2,006 (17.5)	262 (13.1)	
BeniSuef	538 (4.7)	45 (8.4)	
Species			0.023
Chickens	9,639 (84.2)	938 (9.7)	
Ducks	1,179 (10.3)	145 (12.3)	
Geese	139 (1.2)	12 (8.6)	
Pigeons	410 (3.6)	36 (8.8)	
Turkeys	85 (0.7)	13 (15.3)	
Production sector			<0.001
Abattoir	992 (8.7)	51 (5.1)	
Commercial farm	6,398 (55.8)	745 (11.6)	
Backyard flock	1,261 (11)	159 (12.6)	
Live-bird market	2,801 (24.5)	189 (6.7)	
Bird health status			<0.001
Healthy	10,117 (88.4)	841 (8.3)	
Sick	1,217 (10.6)	35 (20.8)	
Dead	118 (1)	50 (42.4)	
Bird age, y			<0.001
0–1	11,328 (98.9)	1120 (9.9)	
>1	124 (1.1)	24 (19.4)	

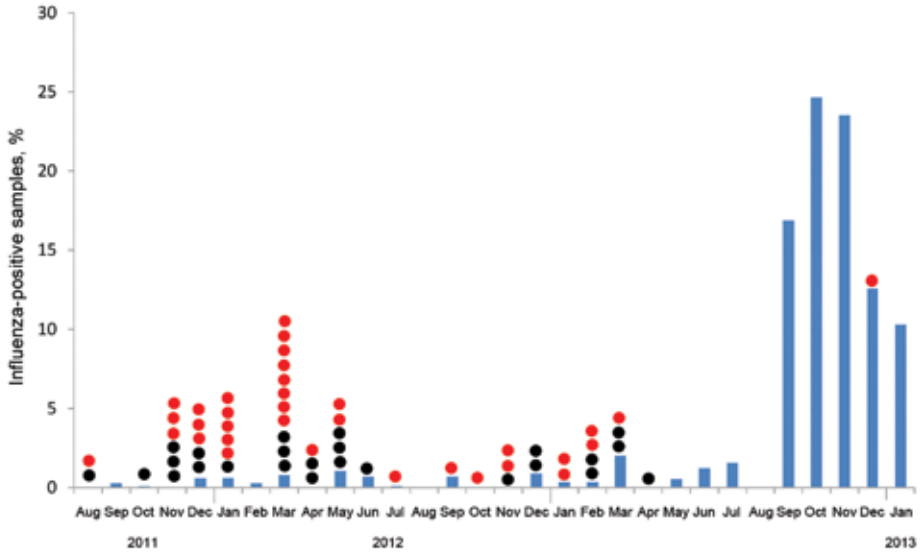

Figure 2. Avian influenza virus infections, by month, Egypt, 2011–2013. Blue bars, detection of the virus in birds; red dots, cases of influenza A(H5N1) virus infections in humans; and black dots, human deaths from influenza A(H5N1) virus infection.

59 AIV samples were subtype H5N1. Figure 3 shows the percentage of each subtype identified from December 2011 through January 2013. In December 2011, we detected the first subtype H9N2 virus in our surveillance program. In March 2012, we detected the first subtype H5N1 and H9N2 co-infections. We then detected 151 incidences of co-infection throughout the reporting period. During the September 2012–January 2013 outbreak, detection of AIV increased dramatically; thus, we randomly selected positive samples from that period for subtyping. During September–November 2012, subtype H5N1 and co-infections constituted most (92%) subtypes detected. In December 2012, detection of subtype H5N1 decreased. Overall, subtype H5N1 was the dominant subtype by governorate, species, production sector, health status, and age (Table 3). Throughout this period, we isolated 112 viruses in specific pathogen–free embryonated chicken eggs.

Phylogenetics

We constructed a phylogenetic tree of the hemagglutinin gene of influenza A(H5N1) viruses from Egypt (Figure 4). Clade 2.2 viruses circulated during 2006–2007 and were distinct from those that prevailed during 2008–2009, when the virus was declared enzootic. Clades 2.2.1 and 2.2.1.1 viruses cocirculated from 2010 through mid-2011. Clade 2.2.1.1 then receded, and all viruses isolated from late 2011 through 2013 were from clade 2.2.1. The subtype H5N1 virus sequence obtained from samples that were co-infected with subtype H9N2 virus did not differ from the sequences of clade 2.2.1 viruses. However, 1 subtype H5N1 virus from a co-infected sample (A/chicken/Egypt/Q5013B/2012) clustered with the extinct clade 2.2 viruses. No other virus from recent years has had a similar sequence.

Phylogenetic analysis of the hemagglutinin gene of subtype H9N2 viruses that reemerged in Egypt in 2011

Figure 3. Subtypes of influenza A viruses detected in poultry, by month, by using reverse transcription PCR, Egypt, 2011–2013.

Table 3. Epizootiologic data for avian influenza virus subtypes H5N1, H9N2, and H5/H9 , Egypt, 2010–2012

Variable	No.	H5N1, %	H9N2, %	H5/H9, %
Governorate				
Cairo	132	99.2	0.8	0
Daqahliya	175	50.9	17.7	31.4
Qalubiya	92	71.7	5.4	22.8
Menofiya	28	96.4	3.6	0
Sharqiya	200	54.5	28.0	17.5
Fayyoum	225	72	13.8	14.2
BeniSuef	45	82.2	0	17.8
Species				
Chickens	709	62.9	17.5	19.6
Ducks	139	90.6	0.7	8.6
Geese	12	100	0	0
Pigeons	25	100	0	0
Turkeys	12	100	0	0
Production sector				
Abattoir	42	100	0	0
Commercial farm	572	59.6	18.7	21.7
Backyard flock	145	85.5	7.6	6.9
Live-bird market	138	82.6	5.1	12.3
Bird health status				
Healthy	660	66.8	14.2	18.9
Sick	147	75	15.8	9.2
Dead	41	80.5	0	19.5
Bird age, y				
0–1	608	69.6	14.3	16
>1	13	54.2	0	45.8

indicated that only viruses with a G1-like lineage circulated among poultry in Egypt (Figure 5). Influenza A(H9N2) viruses from Egypt clustered together, thus showing minor evolution during the past 2 years. Sequences obtained from samples co-infected with subtype H5N1 also showed no significant differences from the sequences of subtype H9N2 viruses that were not from co-infected samples.

Antigenic Characterization of Influenza A(H5N1) Viruses

Results of HI assay of the 2012–2013 viruses conducted against a panel of monoclonal antibodies were used to update a previously published antigenic cartograph (*3*). Our results indicate that antigenically, subtype H5N1 viruses from Egypt have drifted over time; in 2010, two clusters of viruses (clades 2.2.1 and 2.2.1.1) cocirculated. In 2011–2013, clade 2.2.1 viruses dominated (online Technical Appendix Figure 2).

Clinical Signs, 2006–2013

We previously reported our field observations of illness and death caused by HPAI viruses in poultry in Egypt (*19*). Briefly, during 2006–2007, when clade 2.2 viruses were circulating, mortality rates were up to 100%. During 2008–2009, when the virus became enzootic, mortality rates dropped to 30%–40%. Here we show that in 2010, when viruses from clades 2.2.1 and 2.2.1.1 cocirculated, mortality rates were 20%–60%, and this trend continued into 2011. Subsequently, the commercial farms we surveyed decreased their vaccine use. By 2012, as clade 2.2.1.1 viruses receded, mortality rates started to increase. Samples that indicated co-infection with subtypes H5N1 and H9N2 came from flocks that were killed because of the infection. Furthermore, the September 2012 outbreak was caused by viruses that caused high mortality rates among commercial and backyard flocks. In fact, most (96%) influenza-positive samples that were collected from sick or dead poultry were collected during this outbreak.

Co-infections

The presence of influenza virus subtypes H5N1 and H9N2 in the 3 selected samples was confirmed by RT-PCR, qRT-PCR, hemagglutinin sequence analysis, immunofluorescence, and Western blotting (online Technical Appendix Figure 3). In egg culture, subtype H9 virus grew faster than did subtype H5 after 1 passage in eggs, although the cycle thresholds for both viruses were the same for the original swab sample (online Technical Appendix Figure 4). To separate the 2 viruses, we then conducted a plaque purification assay. All plaques that were individually picked from the plaque assay and propagated on egg or cell culture were subtype H9N2 viruses.

To understand our inability to isolate subtype H5N1 virus by plaque purification, we conducted an experimental co-infection analysis of both viruses at an equal dose of 100 PFU/mL in MDCK cells and in specific pathogen–free embryonated chicken eggs. Our results showed that 24 hours after inoculation, both viruses grew to similar titers in MDCK cells in the presence of TPCK-treated trypsin, and the presence of the other virus in the culture did not

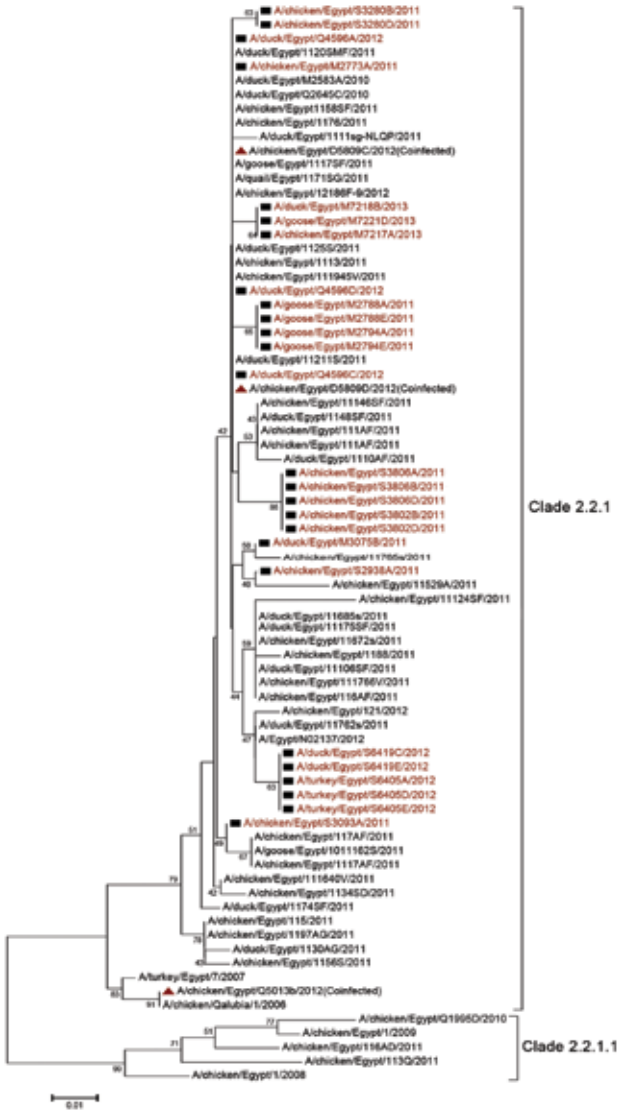

Figure 4. Phylogenetic tree of the hemagglutinin gene of influenza A(H5N1) viruses from Egypt, 2010–2012. Squares and red text indicate viruses that were isolated and sequenced as part of the study. Black text indicates sequences available on GenBank from previous years or other groups. Triangles indicate co-infection with influenza virus subtypes H5N1 and H9N2. Scale bar indicates phylogenetic distance (1 base substitution/100 positions).

affect propagation (online Technical Appendix Figure 5). In eggs, subtype H9N2 virus grew more efficiently than did subtype H5N1 virus, but the presence of the other virus did not affect the propagation rate of either virus (online Technical Appendix Figure 5).

Discussion

AIV subtypes H5N1 and H9N2 were very common among domestic poultry in Egypt. The highest percentage of AIV detected was among turkeys and ducks that appeared to be healthy. Ducks in Egypt, like those in other regions, play a key role in AIV transmission (*20*).We also detected AIV in chickens in all sectors of production. Virus detection among pigeons sampled at markets and abattoirs was 8.8%, but no viruses were isolated from these birds. Therefore, pigeons might have become incidental carriers while coming in contact with other infected poultry in live-bird markets at which all the pigeons included in our surveillance were swabbed. Percentage of AIV detection was high (≈12%) at commercial farms and backyard flocks, where chickens, ducks, geese, and turkeys were sampled. In our previous analysis of surveillance data obtained from August 2009 through July 2010, the commercial farm sector was a more common reservoir of AIV than was the backyard sector (*11*). The continuous evolution of the virus and the disappearance of clade 2.2.1.1 viruses that predominantly circulated on commercial farms might explain the findings in our current analysis. AIV detection rates at abattoirs in Cairo and live-bird markets at different sites were ≈6%. This finding suggests that the threat of bird-to-human transmission might extend beyond the backyard setting, where most cases of subtype H5 infection in humans in Egypt were reported. Our previous analysis had the same result (*11*). Influenza virus infection in apparently health poultry increased from 4.5% in the previous period to 8.3%. This finding might be caused by subtype H9N2 virus infections that are mainly asymptomatic in poultry.

During our surveillance period, influenza A(H5N1) virus infections among humans occurred in a seasonal pattern that peaked during February and March (Figure 2). These cases in humans occurred mostly during months when influenza activity was detected in poultry. Globally, most humans with influenza A(H5N1) virus infection reported having had contact with sick or dead poultry (*21*). The decrease in the number of cases in humans in late 2012, when infections in poultry increased, remains unexplained, although this decrease occurred at a time when human cases usually peak. The political situation during this period might have affected case detection and reporting.

Previous studies have documented the presence of other influenza virus subtypes in migratory birds in Egypt, although none have reported isolating those subtypes from domestic poultry (*22–24*). In contrast, subtype H9N2 viruses have been detected in domestic poultry in several neighboring Middle Eastern countries (*25–29*). As of May 2011, subtype H9N2 viruses were detected on quail and chicken farms in Egypt by another group (*10*), and in December 2011, they were detected by our surveillance. However, how influenza virus subtype H9N2 was introduced into Egypt remains unclear.

Using RT-PCR, we detected a substantial rate of coinfection with influenza virus subtypes H9N2 and H5N1.

This cocirculation and co-infection of multiple influenza viruses increases the chances of subtype H5N1 virus reassortment. Our phylogenetic and antigenic analyses of subtype H5N1 viruses indicated that clade 2.2.1.1 viruses receded and that 2.2.1 viruses are widely circulating. The emergence and recession of clade 2.2.1.1 viruses warrants further investigation of the role that vaccine use plays in emergence of variants. During September 2012–January 2013, increased detection (as much as 24%) of clade 2.2.1 virus infection was noticed. These viruses were associated with high mortality rates and were responsible for the September 2012 outbreak. Furthermore, this shift from the ≤5% that was detected before that time was substantial. Subtype H9N2 viruses might have played a role in this increased spread and severity through an unknown mechanism. However, subtype H9N2 virus did not mask infection with subtype H5N1 virus, as previously suggested (*30*). Yet in co-infected samples, subtype H9N2 might be more easily detected, especially if culture or serologic assays were used, because our data indicate that subtype H9N2 virus grows faster than subtype H5N1 virus in co-infected field samples. Subtype H9N2 virus was circulating in neighboring Middle Eastern countries since at least 2000 but was not detected in Egypt until May 2011; therefore, we hypothesized that subtype H9N2 virus emerged when the pathogenicity of subtype H5N1 virus decreased. Accordingly, we repeated our co-infection experiments with subtype H5N1 viruses isolated annually during 2006–2011. The results were similar to the subtype H9N2/H5N1 virus co-infections in 2012, showing that varying subtype H5N1 virus pathogenicity over time was not a factor in the emergence of subtype H9N2 virus in Egypt (data not shown). Factors that led to the emergence of subtype H9N2 virus and the consequences of its co-infection with subtype H5N1 virus remain unclear.

Through our systematic surveillance program of AIV in poultry in Egypt, we were able to detect 3 major events: emergence of subtype H9N2 virus, co-infection of single hosts with subtypes H9N2 and H5N1 viruses, and increased detection of AIV as of September 2012. We determined that the reservoir for AIV is not localized to a specific sector of poultry production in Egypt, is not specific to a single species, and is geographically widespread. Although our findings indicate that cocirculation and co-infection with AIVs are low, these events are of major concern because of their high potential for reassortment, which can lead to virus progeny with novel characteristics that threaten not only avian health but also human health.

Our study had several limitations. First, our surveillance did not include all geographic areas, and southern Egypt was not adequately represented. The climate in southern Egypt differs from that in the Delta, and the presence of the Aswan Dam and Nasser Reservoir might affect the epizootiology of influenza viruses given the presence of wild bird species.

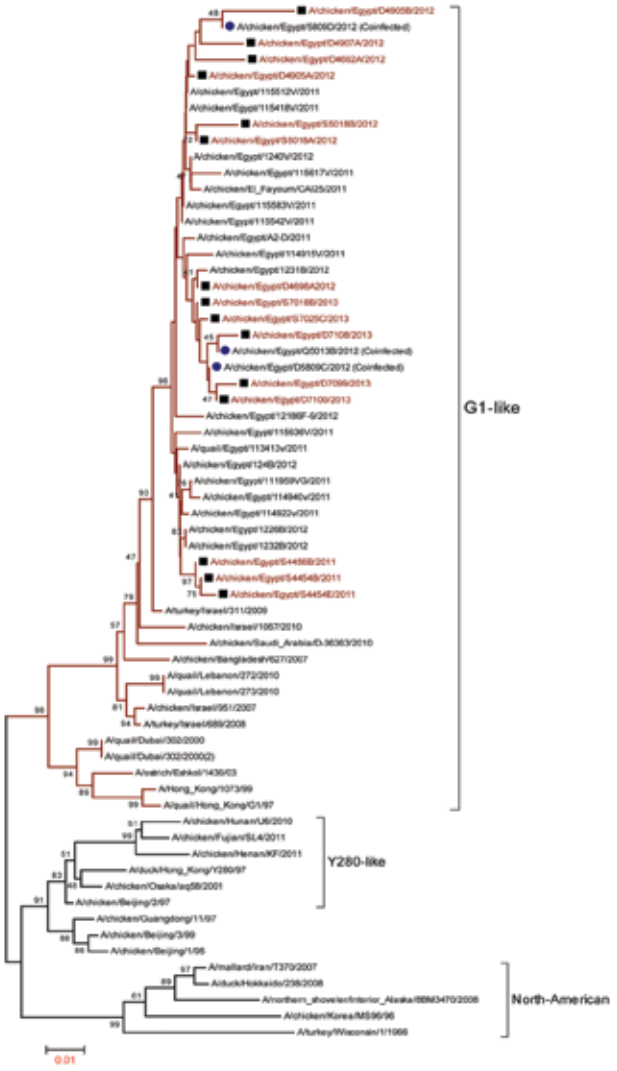

Figure 5. Phylogenetic tree of the hemagglutinin gene of influenza A(H9N2) viruses from Egypt, 2010–2012. Squares and red text indicate viruses that were isolated and sequenced as part of the study. Black text indicates sequences available on GenBank from previous years or other groups. Circles indicate co-infection with influenza virus subtypes H5N1 and H9N2. Scale bar indicates phylogenetic distance (1 base substitution/100 positions).

Furthermore, we did not sample any wild or migratory birds, thus limiting our findings, especially those associated with the emergence of subtype H9N2 virus. In areas where we conducted our surveillance, we did not randomly select sites; rather, we sampled sites that were accessible to our veterinarians. Although the possibility is small, given our sampling scheme, selection bias might have occurred.

Our findings showed that influenza viruses continue to threaten animal and human health in Egypt. In the poultry industry, HPAI A(H5N1) viruses usually lead to major economic losses. Subtype H9N2 viruses, although of low

pathogenicity, are correlated with increased severity because of co-infection with other poultry viruses; thus, they indirectly might lead to economic losses for the industry. On the public health side, our findings that AIVs are widespread throughout poultry sectors and geographic regions indicate that a large segment of the population of Egypt is at risk. Subtype H9N2 viruses also infect humans, thereby adding to the risk for infection with subtype H5N1 virus. Our results can be used to better focus and target animal health and public health policy in Egypt. Indeed, Egypt remains an epicenter for AIV circulation, and vigilant surveillance remains the single-most effective tool for keeping track of these viruses.

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Dr Kayali is a postdoctoral research associate at the St. Jude Children's Research Hospital, Memphis, Tennessee. His research interests are the epidemiology of influenza and viral zoonotic diseases.

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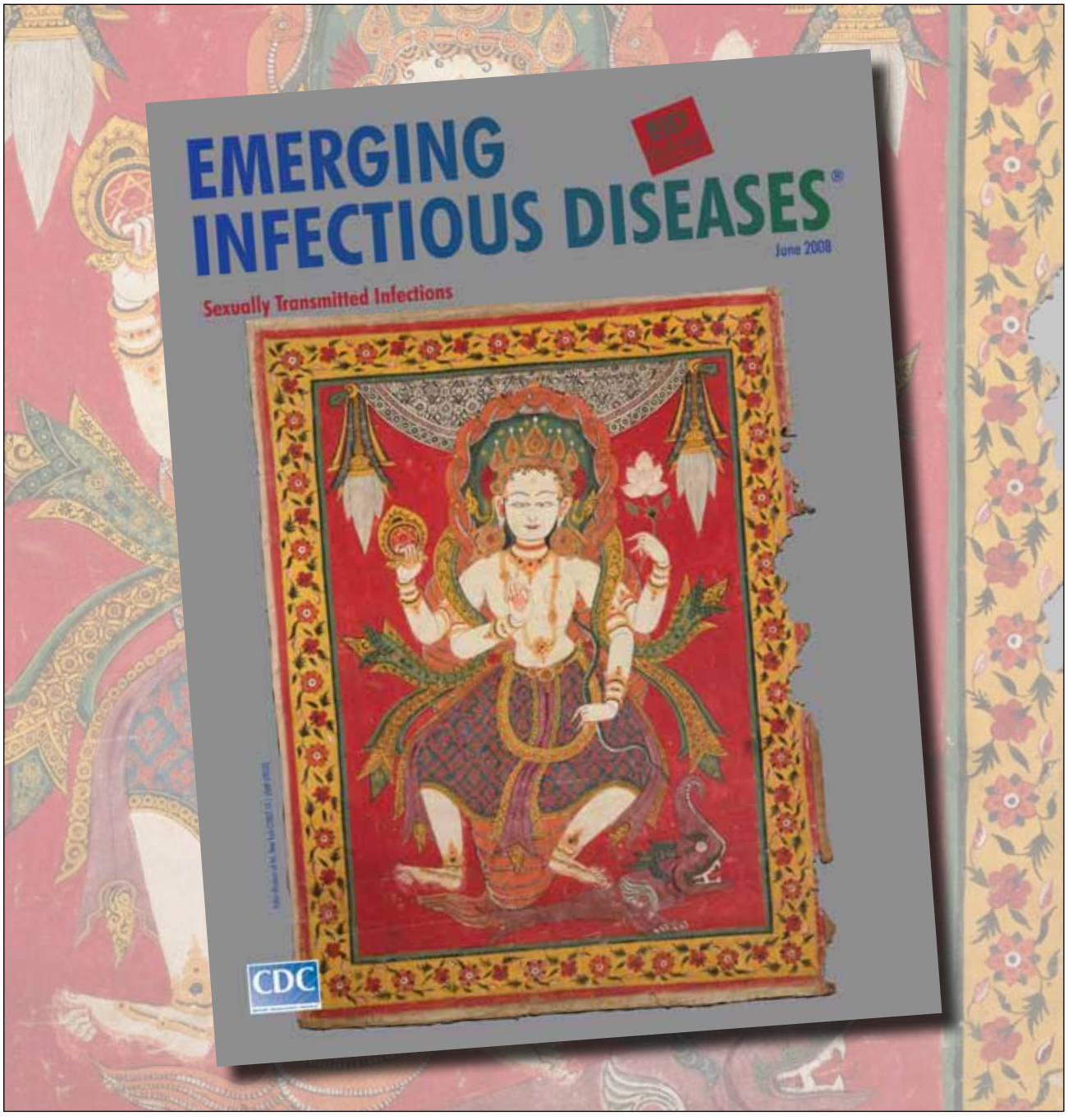
Address for correspondence: Ghazi Kayali, Division of Virology, Department of Infectious Diseases, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA; email: ghazi.kayali@stjude.org



Antibodies against MERS Coronavirus in Dromedaries, United Arab Emirates, 2003 and 2013

Benjamin Meyer, Marcel A. Müller, Victor M. Corman, Chantal B.E.M. Reusken, Daniel Ritz, Gert-Jan Godeke, Erik Lattwein, Stephan Kallies, Artem Siemens, Janko van Beek, Jan F. Drexler, Doreen Muth, Berend-Jan Bosch, Ulrich Wernery, Marion P.G. Koopmans, Renate Wernery, and Christian Drosten

Middle East respiratory syndrome coronavirus (MERS-CoV) has caused an ongoing outbreak of severe acute respiratory tract infection in humans in the Arabian Peninsula since 2012. Dromedaries have been implicated as possible viral reservoirs. We used serologic assays to analyze 651 dromedary serum samples from the United Arab Emirates; 151 of 651 samples were obtained in 2003, well before onset of the current epidemic, and 500 serum samples were obtained in 2013. Recombinant spike protein–specific immunofluorescence and virus neutralization tests enabled clear discrimination between MERS-CoV and bovine CoV infections. Most (632/651, 97.1%) dromedaries had antibodies against MERS-CoV. This result included all 151 serum samples obtained in 2003. Most (389/651, 59.8%) serum samples had MERS-CoV–neutralizing antibody titers >1,280. Dromedaries from the United Arab Emirates were infected at high rates with MERS-CoV or a closely related, probably conspecific, virus long before the first human MERS cases.

Middle East respiratory syndrome coronavirus (MERS-CoV) is an emerging pathogen associated with severe respiratory symptoms and renal failure in infected patients (*1*,*2*). Globally, 156 laboratory-confirmed cases of infection with MERS-CoV, including 65 deaths, were reported as of early November 2013. All human cases were linked to the Arabian Peninsula (Saudi Arabia, Jordan, Oman, Qatar, Kuwait, and the United Arab Emirates). Imported cases were detected in countries in Europe and Africa (United Kingdom, Germany, Italy, France, and Tunisia) (*3*).

Transmission patterns, including the putative zoonotic source of the virus, remain unclear. Hypotheses include frequent zoonotic infections with limited subsequent human-to-human transmission chains and existence of a self-sustained epidemic in humans (*4*). A recent study found evidence to support the existence of epidemiologically unlinked cases in a large outbreak in the al-Hasa region, Saudi Arabia (*5*). It was speculated that zoonotic introductions of MERS-CoV from an unknown reservoir might occur at high rates, in addition to obvious human-to-human transmission.

Coronaviruses (CoV) are positive-sense RNA viruses. Viruses in the genera *Alphacoronavirus* and *Betacoronavirus* are associated with mammals and show a particularly high level of diversification in bats. Viruses in the genera *Gammacoronavirus* and *Deltacoronavirus* are mostly avian-associated viruses (*6*,*7*). MERS-CoV belongs to *Betacoronavirus* phylogenetic lineage C that, in addition to MERS-CoV, contains 2 distinct bat-associated CoV species (HKU4 and HKU5) (*1*,*8*).

Insectivorous bats of the family Vespertilionidae were recently shown to carry viruses that are probably conspecific with MERS-CoV (*9*). However, the limited rate of contact between humans and insectivorous bats makes a continuous and frequent acquisition of MERS-CoV from bats an unlikely scenario. In a manner similar to observations regarding severe acute respiratory syndrome CoV (SARS-CoV), an intermediate reservoir host might exist from which human infections are acquired. Dromedaries from

Author affiliations: University of Bonn Medical Centre, Bonn, Germany (B. Meyer, M.A. Müller, V.M. Corman, D. Ritz, S. Kallies, A. Siemens, J.F. Drexler, D. Muth, C. Drosten); National Institute for Public Health and the Environment, Bilthoven, the Netherlands. (C.B.E.M. Reusken, G.-J. Godeke, J. van Beek, M.P.G. Koopmans); Erasmus Medical Centre, Rotterdam, the Netherlands (C.B.E.M. Reusken, J.F. Drexler, M.P.G. Koopmans); EUROIMMUN AG, Lübeck, Germany (E. Lattwein); Utrecht University, Utrecht, the Netherlands (B.-J. Bosch); and Central Veterinary Research Laboratory, Dubai, United Arab Emirates (U. Wernery, R. Wernery)

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different regions in Africa and the Arabian Peninsula have been shown to have antibodies against MERS-CoV (*10*,*11*). Animals from the Arabian Peninsula had high neutralizing serum activities overall and reciprocal antibody titers ≤320–1,280, which support recent infection with MERS-CoV or a highly related virus. Thus, dromedaries might serve as intermediate hosts. However, detailed serologic studies in countries with actual incidence of MERS-CoV infections in humans have not been conducted.

Serologic analysis of CoVs is challenging because of cross-reactivity between CoVs infecting the same host and the broad distribution of CoVs in diverse mammalian species (*6*,*7*,*12*–*14*). Antibodies directed against some of the major antigens of different CoVs are known to cross-react in standard serologic assays (*15*,*16*). Potential cross-reactivity is a diagnostic challenge because camelids are known to be infected with bovine CoV (BCoV), a distinct betacoronavirus of phylogenetic lineage A unrelated to the MERS-CoV (*17*,*18*). As an additional challenge, camel immunoglobulins lack a light chain peptide, which affects specific physical properties, such as altered size and stability, compared with immunoglobulins of other mammals (*19*,*20*). The influence of this feature on serologic assays has not been thoroughly investigated. Thus, serologic assays should be applied with caution, and different assay formats should be tested concurrently.

We reported a 2-staged approach for MERS-CoV serologic analysis in humans (*15*,*16*). Expanding upon these studies, we used in the present study a recombinant MERS-CoV spike protein immunofluroescence assay (rIFA) augmented by a validated protein microarray (*10*,*21*), followed by MERS-CoV–specific neutralization assay, to screen 651 dromedary serum samples from the United Arabian Emirates. Cross-reactivity against clade A betacoronaviruses was assessed by using a immunofluorescence assay (IFA) and a BCoV-specific neutralization assay. Serum samples obtained in 2003 and 2013 were compared to obtain information for the time in which MERS-related CoV has been circulating in camels.

Methods

Sampling

A total of 651 dromedary (*Camelus dromedarius*) serum samples were systematically sampled in Dubai, United Arab Emirates and the surrounding area in 2003 (collection 4, n = 151) and in 2013 (collections 1A, 1B, 2, and 3; n = 500). The total number of camels in that area was 360,000 in 2010 (*22*). Fecal samples were also available for collections 1A and 1B (n = 182), all obtained in 2013. Animals in collection 1B were born and raised at the Dubai Central Veterinary Research Laboratory, which tests ≈70,000 camels per year (*23*) and had no contact with other camels. Camels in collection 2 were racing camels (age range 2–8 years), and camels in collection 3 were adult livestock camels originally purchased from Saudi Arabia, Sudan, Pakistan and Oman.

Dromedary blood was obtained for routine health screening by jugular vein puncture according to standard veterinary procedures by trained personnel. For most serum samples, animal owners requested sample codes to be anonymous. All samples obtained during 2003 and 2013 were stored at –80°C until further analysis. For comparison, 16 serum samples from *C. bactrianus* camels in zoologic gardens in Germany were included in the study. All serum samples were shipped in agreement with German import regulations.

Recombinant Spike IFA

For screening purposes, an rIFA was used (*15*,*24*). In brief, Vero B4 cells were transfected with pCG1 eukaryotic expression vector that contained the complete spike sequence of MERS-CoV or human CoV-OC43. Cells were fixed 24-h post-transfection with ice-cold acetone/methanol and stored dry at 4°C. Serum samples were applied at a dilution of 1:80 for 1 h at 37°C, which was optimal for reducing nonspecific reactions and maintaining sensitivity. Secondary detection was conducted by using a goat anti-llama IgG fluorescein isothiocyanate–conjugated antibody. For some negative serum samples, dilutions of 1:20 and 1:40 were also tested.

Spike Protein Microarray

A confirmatory assay based on a protein microarray was performed as described (*10*,*21*) by using the spike S1 subunits of MERS-CoV, human CoV-OC43, and SARS-CoV. Serum samples were used at 1:20 dilutions on microarray chips. Relative light units were determined by using secondary cyanine 5–conjugated goat anti-llama IgG.

MERS-CoV Conventional IFA

A MERS-CoV IFA with infected Vero cells was conducted as described (*15*) by using commercially available MERS-CoV IFA slides (EUROIMMUNAG, Lübeck Germany). Serum samples were used at dilutions of 1:20–1:5,120. Secondary detection was conducted by using goat anti-llama fluorescein isothiocyanate–labeled IgG (1:200 dilution; Agrisera, Vännas, Sweden).

Serum Neutralization Test

Serum neutralization tests were conducted as described (*10*) by using Vero B4 (MERS-CoV) or PT (BCoV) cells. To reduce volumes of serum needed, all neutralization tests were performed in a 96-well format. Reactions contained 50 PFUs of MERS-CoV (EMC/2012 strain) or BCoV (Nebraska strain) in 25 μL of medium mixed 1:1

with camel serum diluted in 25 μL serum-free Dulbecco minimum essential medium. The starting dilution was 1:40. After incubation for 1 h at 37°C, each well was infected for 1 h at 37°C with a 50 μL virus–serum mixture. Supernatants were removed and fresh complete Dulbecco minimum essential medium was added. Assays were terminated by fixation with 8% paraformaldehyde for 30 min and stained with crystal violet after 3 days. Neutralization titers were defined as serum dilutions reducing cytopathic effects in 2 parallel wells.

Detection of Virus Nucleic Acid

Viral RNA was extracted from serum and fecal samples by using the MagNA Pure System (Roche, Basel, Switzerland) and an input volume of 100 μL of serum or fecal material suspended 1:10 in phosphate-buffered saline buffer. The elution volume was 100 μL for serum and fecal suspensions. To identify CoV-specific nucleic acids, 2 generic CoV PCRs were performed as described (*25*–*27*), followed by subsequent Sanger sequencing of the amplified DNA.

Results

To characterize reactivity of camel serum samples with MERS-CoV in different assay formats, we chose 11 camel serum samples with weak and strong reactivity predetermined by using a simple IFA. The 11 serum samples were titrated in a 2-fold dilution series in all applied assays. The reactivity pattern of the MERS-CoV spike protein (MERS-S) was compared against that of the human CoV-OC43 spike protein (OC43-S). As in our previous study (*10*), human CoV-OC43 was used instead of BCoV in these initial experiments because it is serologically indistinguishable from BCoV and is not subject to handling restrictions of German Animal Diseases Protection Act (*28*). Overall titers against MERS-S were higher than those against OC43-S, and several serum samples reacted exclusively against 1 of the 2 viruses (Table 1), suggesting the absence of general cross-reactivity between spike proteins of both viruses by IFA. Typical patterns of reactivity observed for camel serum samples are shown in the Figure, panel A.

A previously published microarray-based assay that used the receptor-binding S1 spike subunit of MERS-CoV (MERS-S1), human CoV-OC43 (OC43-S1), and SARS-CoV (SARS-S1) was also evaluated. In contrast to our previous studies (*10*,*21*) we chose a lower fluorescence intensity cutoff of 4,000 instead of 20,000 relative fluorescence units (RFU) to maximize the sensitivity and thereby challenge the target specificity. All 3 MERS IFA-negative serum samples had signal intensities <4000 RFU at serum dilutions of 1:20 (Table 1). All rIFA-positive serum samples had saturated signals >65,535 RFU. The OC43-S1 reactivity pattern across the serum panel was comparable with that for the OC43-S rIFA. As expected, all serum samples were negative against the SARS-S1 control antigen. A comparison of typical reactivity patterns in the microarray with those of the IFA is shown in the Figure, panel B. Results for the rIFA and protein microarray were highly congruent.

The panel of camel serum samples was additionally tested in a commercially available IFA that used cells infected with MERS-CoV (vIFA) (EUROIMMUN AG). The use of whole virus provides additional structural and nonstructural protein antigens, including envelope, membrane, nucleocapsid, and diverse replicase proteins. However, because of conserved features of nonstructural proteins among even distantly related CoVs (*7*,*12*),

Table 1. Validation of serologic assays for coronaviruses with differentially reactive dromedary serum samples, United Arab Emirates, 2013*

	rIFA titer†‡		Protein array (RFU) ‡§			vIFA titer†‡	Neutralization test titer¶#	
Serum no.	MERS-S	OC43-S	MERS-S1	OC43-S1	SARS-S1	MERS-CoV	MERS-CoV	BCoV
1	–	–	2,555	3,868	2,606	–	–	40
2	–	320	2,770	18,896	2,776	–	–	80
3	–	640	3,950	65,535	2,751	–	–	160
4	320	–	65,535	3,921	1,726	640	40	–
5	>10,240	320	65,535	7,247	2,306	>5,120	2,560	160
6	5,120	640	65,535	5,069	2,098	2,560	640	160
7	>10,240	160	65,535	7,179	2,198	>5,120	640	40
8	5,120	320	65,535	55,826	2,412	>5,120	1,280	160
9	5,120	>5,120	65,535	65,535	2,087	>5,120	1,280	320
10	>10,240	320	65,535	22,695	2,303	>5,120	1,280	320
11	5,120	1,280	65,535	28,391	2,858	>5,120	640	40

*rIFA, recombinant immunofluorescence assay (antigen used was complete spike protein); RFU, relative fluorescence units; vIFA, Middle East respiratory syndrome coronavirus–based immunofluorescence assay (antigen used complete virus); MERS-S, spike protein from Middle East respiratory syndrome coronavirus; OC43-S, spike protein from human coronavirus OC34; SARS-S, spike protein from severe acute respiratory syndrome virus; MERS-CoV, Middle East respiratory syndrome coronavirus; BCoV, bovine coronavirus; –, negative.
†Serum dilutions started at 1:20.
‡Assay was used for screening purposes.
§RFU <4,000 were considered negative. The serum dilution used in this assay was 1:20 (antigen used was S1 subunit of spike protein).
¶Serum dilutions started at 1:40.
#Assay applied for confirmation purposes.

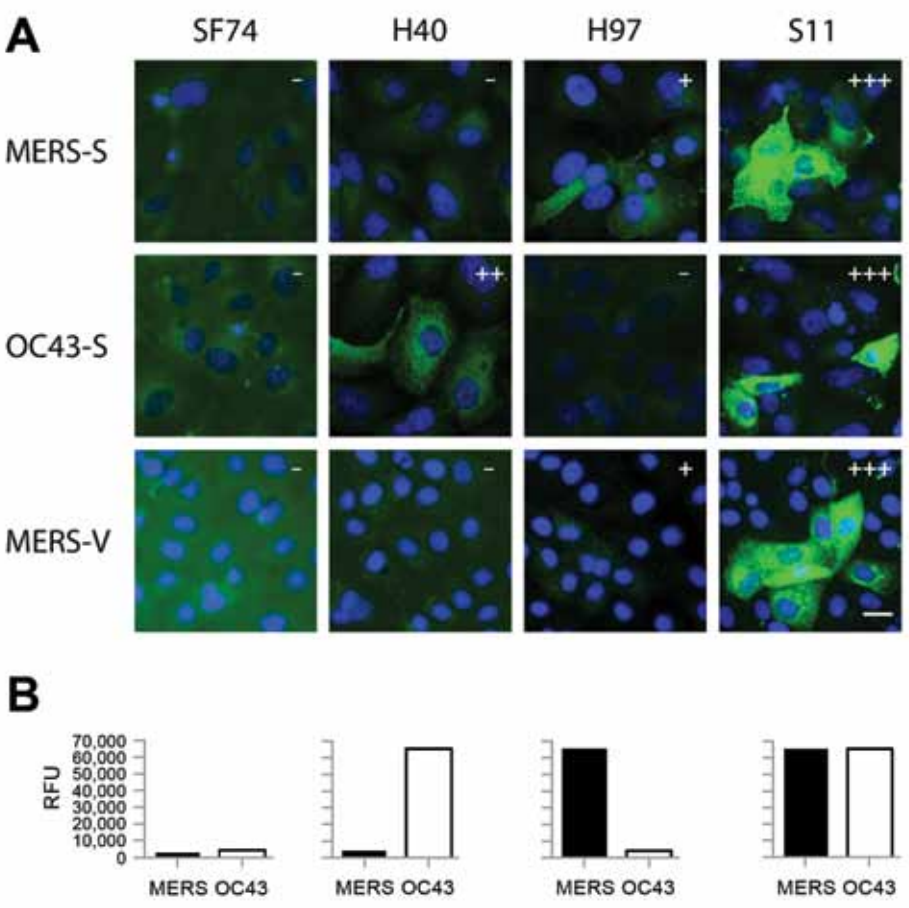

Figure. Immunofluorescence and microarray reactivity patterns for antibodies (SF74, H40, H97, and S11) against Middle East respiratory syndrome coronavirus (MERS-CoV) in serum samples from dromedaries, United Arab Emirates, 2013. A) Serum samples tested against overexpressed MERS-CoV spike protein (MERS-S), overexpressed human CoV-OC43 spike protein (OC43-S), and Vero cells infected with MERS-CoV (MERS-V). Fluorescence intensities were evaluated as follows: –, negative; +, weakly reactive; ++, reactive; +++, strongly reactive. Scale bar indicates 20 μm. B) Relative fluorescence units (RFU) were determined for the same serum samples by microarray using S1 domains of MERS-CoV and human CoV-OC43.

cross-reactivity was possible with this assay (*15*). In the tested panel of camel serum samples, vIFA titers corresponded well to titers determined by rIFA and generally equal to or higher than titers in the rIFA (Table 1). Despite the absence of cross-reactivity between MERS-S–positive and OC43-S–positive serum samples in this test (Figure, panel A), in previous studies the vIFA showed false-positive results with human CoV-OC43–positive serum samples, in particular if used at lower dilutions, such as 1:10 or 1:20 (*15*,*16*).

To confirm results from affinity assays with results from a functional test, we determined endpoint virus neutralization titers by using a microneutralization test against MERS-CoV and BCoV. In most animals MERS-CoV serum neutralization titers were higher than titers against BCoV (serum samples 4–11) (Table 1). High IFA titers generally corresponded with high neutralization titers, with exceptions for some BCoV antibody–positive serum samples. Divergence between affinity and neutralization assays can result from waning neutralizing antibody activity for infections that occurred long ago. Neutralization assays confirmed the absence of cross-neutralization between MERS-CoV and BCoV antibodies in either direction even at low dilutions, such as 1:40. However, sample no. 1 (Table 1) neutralized BCoV at a dilution of 1:40 despite showing negative results in all other serologic assays. This finding indicates that nonspecific neutralization activities might be encountered with camel serum samples, suggesting that higher serum dilutions should be used when conducting critical investigations such as viral reservoir studies.

On the basis of the validation studies, we investigated 4 collections of serum samples from dromedaries from the United Arab Emirates that were sampled in 2003 and 2013. For initial screening, we chose the rIFA because of its proven sensitivity and decreased chances of generating false-positive results. All 667 camel serum samples from the United Arab Emirates and Germany were initially screened at dilutions of 1:80. A total of 89.0%–100.0% of serum samples in 4 collections showed positive results (Table 2). Seroprevalence was higher for collections from exclusively adult animals (collections 3 and 4) than for a collection from young racing camels (2–8 years of age, collection 2). Clear seropositive results included 151 dromedary serum samples obtained in 2003 (collection 4). All 16 serum samples from German zoologic gardens were tested at the same dilution and showed no reactivity

Table 2. MERS-CoV serologic results for dromedary serum and fecal samples, United Arab Emirates, 2003 and 2013*

Collection	Year	No. camels/ sex	Camel age	Feature	No. samples	Serum dilution, no. (%) positive			
						rIFA, MERS-S†	Neutralization test, MERS-CoV		
						80	<640	640–1,280	>1,280
1A	2013	2/M, F	A, J	Paired serum and fecal samples	177	175 (98.9)	24 (13.6)	74 (41.8)	79 (44.6)
1B	2013	2/M, F	A, J	Animals raised at CVRL	5	0	5 (100.0)	0	0
2	2013	2/M, F	2–8 y	Racing camels	100	89 (89.0)	55 (55.0)	3 (3.0)	42 (42.0)
3	2013	2/M, F	A	Livestock camels‡	218	217 (99.5)	23 (10.6)	13 (6.0)	182 (83.5)
4	2003	1/F	A	Systematically sampled	151	151 (100.0)	35 (23.2)	30 (19.9)	86 (57.0)
Total					651	632 (97.1)	142 (21.8)	120 (18.4)	389 (59.8)

*MERS-CoV, Middle East respiratory syndrome coronavirus; rIFA, recombinant immunofluorescence assay with MERS-CoV spike protein; MERS-S, spike protein from MERS-CoV; A, adult; J, juvenile; CVRL, Dubai Central Veterinary Research Laboratory.
†Fluorescence signal intensity was rated as negative, +, ++, +++, and ++++.
‡Originally purchased from Saudi Arabia, Sudan, Pakistan, and Oman.

in the rIFA. Re-testing at lower dilutions of 1:20 and 1:40 confirmed absence of reactivity in these serum samples. Subcollection 1B contained serum samples from 5 animals that were born in, and had never left, a closed animal research facility in Dubai; these animals were seronegative.

A confirmatory microneutralization test was conducted at dilutions of 1:640 and 1:1,280 for all IFA-reactive serum samples. These high dilutions were chosen on the basis of our observation of high levels of neutralizing serum activity in camels (*10*). Most (59.8%, 389/651) serum samples had high neutralizing titers >1,280 (Table 2). In 18.4% (120/651) of all serum samples, neutralization titers ranged from 640 through 1,280, and 21.8% (142/651) of rIFA-positive serum samples had neutralizing titers <640.

To rule out cross-reactivity and to study additional exposure of MERS-CoV–positive camels with BCoV (*17*,*18*), all serum samples having MERS-CoV neutralizing titers >640 were tested by using a BCoV-specific microneutralization assay. At a dilution of 1:640, a total of 19.2% (23/120) of MERS-CoV–neutralizing serum samples had concomitant neutralizing activities against BCoV (Table 3). Of serum samples that had MERS-CoV neutralizing antibody titers >1,280, a total of 24.2% (94/389) had concomitant neutralizing activities against BCoV.

Fecal samples were available for 182 dromedaries in collection 1. All samples were tested by using a subfamily *Coronavirinae*–specific broad-range reverse transcription PCR (RT-PCR) and a highly sensitive RT-PCR specific for genus *Betacoronavirus* phylogenetic lineage C. Both assays were specific for the viral RNA-dependent RNA polymerase gene. Two positive fecal samples were identified by both assays. Sequencing of amplified cDNA fragments of 182 nt and 404 nt identified sequences 99% identical with BCoV strain Mebus (GenBank accession nos. KF894801 and U00735.2). To further confirm virus identity, we amplified a region within the spike protein gene (positions 24303–24702 in BCoV strain Mebus) by using RT-PCR and sequencing it. Amplicons from both animals were 97% identical at nucleotide level with BCoV strain Mebus, indicating the presence of BCoV in camels as reported (*10*). We tested all serum samples in the same way by RT-PCR and obtained uniformly negative results.

Discussion

We have shown that dromedaries from the United Arab Emirates, a country with human cases of MERS-CoV infection, have antibodies that can neutralize MERS-CoV at high rates. Antibodies were detected in serum samples obtained in 2013 and in serum samples obtained >10 years earlier, which indicated the long-standing presence of MERS-CoV or a closely related virus in dromedaries in that region. Our data add to previous studies in which our group and others have reported wide antibody prevalence in camels in various regions, including Oman, Egypt, and the Canary Islands (*10*,*11*). A 10% lower seroprevalence in collection 2, which contained young racing camels, suggests that animals might be infected as juveniles. However, because only limited data were made available by owners, a definite statement awaits confirmation.

The absence of antibodies in a control cohort from Germany might be explained by the fact that these animals belonged to a different camelid species (*C. bactrianus* vs. *C. dromedarius*). However, because MERS-CoV has a highly conserved receptor structure, we did not assign high priority to the hypothesis that the closely related camel species *C. bactrianus*, should be less susceptible than *C. dromedarius* camels to MERS-CoV (*29*,*30*). Differences in antibody prevalence rates might reflect a restricted geographic distribution of the virus, which corresponds to our previous finding of a relatively lower prevalence of antibodies against MERS CoV in camels from the Canary Islands, which have been isolated from their point of origin in Africa for many years (*10*). Therefore, MERS-CoV–like viruses in camelids might be spreading across a region covering at least the eastern Arabian Peninsula, including

Oman, the United Arab Emirates, Egypt, and Morocco from where some of the antibody-positive camels described by Reusken et al. originated (*10*).

The high rates of antibody prevalence in contemporary serum samples and samples from 2003 suggest that the virus has spread in camelids for some time. However, recognition of camelids as the bona fide reservoir for MERS-CoV has to await sequencing of camelid-associated MERS-related CoV. In this context, only animals infected with conspecific viruses can be regarded as reservoirs for a given virus. Although neutralization assays can provide evidence of infection with a virus belonging to the same serotype, no systematic studies have defined whether serotypes correlate with CoVs species. Nevertheless, for several CoV clades, serotypes defined by neutralization assay will not include >1 viral species. Members of the species *Betacoronavirus 1*, including CoV-OC43 and BCoV, show cross-neutralization with each other, but the closely related sister species (human CoV-HKU1) does not show cross-neutralization (*31*).

Feline CoV (FCoV) comprises 2 subserotypes that show limited cross-reactivity but are considered 1 virus species. Transmissible gastroenteritis virus of swine shows more efficient cross-neutralization with 1 of these FCoV subserotypes than the other and is classified as 1 species with FCoV even though it is carried by a different host (*32*). Human CoVs 229E and NL63, which form 2 closely related sister taxa, do not show cross-neutralization and concordantly form 2 different species by genetic criteria (*33*). Therefore, our finding of high neutralizing antibody titers in camelids is suggestive (but not evidentiary) of the presence of viruses conspecific with MERS-CoV in camelids. Final confirmation will depend on the identification of virus sequences in camelids, which should expectably be closely related to human-specific MERS-CoV sequences.

Camels probably acquired MERS-CoV at some unknown time. Potential sources include bats of the family Vespertilionidae, in which a virus with a close phylogenetic relationship with MERS-CoV has been detected (*9*). This virus, which is carried by vespertilionid bats of the genus *Neoromicia*, has been confirmed to be conspecific with MERS-CoV. Lineage C betacoronaviruses in other bat taxa have also been proposed to be related to MERS-CoV (*34*,*35*). However, although these viruses cluster phylogenetically with MERS-CoV, they are not conspecific with MERS-CoV on the basis of sequence distance criteria, such as that were proposed by Drexler et al. (*36*).

In vespertilionid bats, including those in the genus *Neoromicia*, virus conspecific with MERS-CoV differs from human MERS-CoV, even if formally a member of the same species. The observed degree of sequence divergence between this virus and MERS-CoV makes any direct and recent transmission from bats to humans seem unlikely. Nevertheless, it cannot be excluded from available data that the virus source population in bats has not been detected. For example, a recent investigation of *Rhinolophus* bats in China identified viruses with close relationships to the bona fide ancestor of SARS-CoV, and viruses described in many studies yielded only conspecific yet less related viruses (*37*). In that study, viruses from civet cats, which are deemed to be intermediary hosts in the transition of SARS-CoV from bats to humans, were still more closely related to human SARS-CoV than even the closest bat-borne virus.

Table 3. BCoV neutralization test results for MERS-CoV–positive dromedary serum samples, United Arab Emirates, 2003 and 2013*

	No. BCoV positive/no. MERS-CoV positive (serum dilution, %)	
Collection	640–1,280	>1,280
1A	15/74 (20.3)	14/79 (17.7)
1B	0	0
2	0/3 (0.0)	14/42 (33.3)
3	2/13 (15.4)	52/182 (28.6)
4	6/30 (20.0)	14/86 (16.3)
Total	23/120 (19.2)	94/389 (24.2)

*BCoV, bovine coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus.

If camelids should function as intermediary hosts in a similar manner, we should expect a virus in camelids that has a closer phylogenetic relationship with any bat-borne CoV and thus should be easily detectable with available RT-PCRs. Larger studies to confirm the presence of MERS-CoV in camelids should receive high priority so as to define the animal reservoir of MERS-CoV and possibly control it by such measures as vaccination or control of animal movement. However, before implementation of any control measures, whether camelids are a continuous source of infection for humans needs to be firmly established.

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Mr Meyer is a doctoral student at the Bonn Institute of Virology, Bonn, Germany. His research interests are spike protein–mediated entry of bat-borne coronaviruses into cells and advancement of specific serologic tests for antibodies against coronaviruses.

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Address for correspondence: Christian Drosten, Institute of Virology, University of Bonn Medical Centre, Sigmund Freud Strasse 25, 53105 Bonn, Germany; email: drosten@virology-bonn.de

Novel Betacoronavirus in Dromedaries of the Middle East, 2013

Patrick C.Y. Woo,[1] Susanna K.P. Lau,[1] Ulrich Wernery, Emily Y.M. Wong, Alan K.L. Tsang, Bobby Johnson, Cyril C.Y. Yip, Candy C.Y. Lau, Saritha Sivakumar, Jian-Piao Cai, Rachel Y.Y. Fan, Kwok-Hung Chan, Ringu Mareena, and Kwok-Yung Yuen

In 2013, a novel betacoronavirus was identified in fecal samples from dromedaries in Dubai, United Arab Emirates. Antibodies against the recombinant nucleocapsid protein of the virus, which we named dromedary camel coronavirus (DcCoV) UAE-HKU23, were detected in 52% of 59 dromedary serum samples tested. In an analysis of 3 complete DcCoV UAE-HKU23 genomes, we identified the virus as a betacoronavirus in lineage A1. The DcCoV UAE-HKU23 genome has G+C contents; a general preference for G/C in the third position of codons; a cleavage site for spike protein; and a membrane protein of similar length to that of other betacoronavirus A1 members, to which DcCoV UAE-HKU23 is phylogenetically closely related. Along with this coronavirus, viruses of at least 8 other families have been found to infect camels. Because camels have a close association with humans, continuous surveillance should be conducted to understand the potential for virus emergence in camels and for virus transmission to humans.

The 2003 epidemic of severe acute respiratory syndrome (SARS) boosted interest in the discovery of new coronaviruses (CoVs) (*1*–*3*). In 2004, a novel human CoV (HCoV), named HCoV-NL63, was reported (*4*), and the discovery of another novel HCoV, HCoV-HKU1, was described and further characterized in 2005 (5,*6*) and 2006 (*7*). SARS-CoV–like viruses have also been reported in Chinese horseshoe bats in Hong Kong, China, and other horseshoe bats in China (*8*,*9*). The discovery in Chinese horseshoe bats in Yunnan, China, of a new SARS-CoV–like virus that uses ACE2 as receptor has furthered interest in discovering animal origins of human infections (*10*). We have discovered 20 other animal CoVs that include 2 novel betacoronavirus lineages and a novel genus, *Deltacoronavirus* (*11*–*20*). From our studies, bats and birds were shown to be the gene sources for fueling the evolution and dissemination of alphacoronaviruses and betacoronaviruses and of gammacoronaviruses and deltacoronaviruses, respectively (*18*).

In 2012, a novel CoV, Middle East respiratory syndrome CoV (MERS-CoV) emerged as a cause of severe respiratory infections associated with high rates of death among humans; the virus is closely related to tylonycteris bat CoV HKU4 and pipistrellus bat CoV HKU5 (Pi-Bat CoV HKU5) (*21*–*23*). It has also been shown that dromedaries in the Middle East possess MERS-CoV neutralizing antibodies (*24*). To further knowledge of the evolution and dissemination of CoVs, we conducted a molecular epidemiology study of fecal samples obtained from dromedaries in Dubai, United Arab Emirates.

Materials and Methods

Samples

Dromedary fecal and serum samples used in the study were leftover specimens that had been submitted for pathogen screening (feces) or preventive health screening (serum) to Central Veterinary Research Laboratory (Dubai, United Arab Emirates) during January–July 2013. The fecal and serum samples were not obtained from the same animals. None of the dromedaries tested were known to have had contact with bats or horses.

We tested a total of 293 fecal samples: 232 from teenage and adult dromedaries (*Camelus dromedarius*) (≥1

Author affiliations: The University of Hong Kong, Hong Kong, China (P.C.Y. Woo, S.K.P. Lau, E.Y.M. Wong, A.K.L. Tsang, C.C.Y. Yip, C.C.Y. Lau, J.-P. Cai, R.Y.Y. Fan, K.H. Chan, K.-Y. Yuen); and Central Veterinary Research Laboratory, Dubai, United Arab Emirates (U. Wernery, B. Johnson, S. Sivakumar, R. Mareena)

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[1]These authors contributed equally to this article.

year of age) and 61 from dromedary calves (<1 year of age). Among the 293 samples, 6 were collected in January, 209 in February, 5 in March, 39 in April, 16 in May, 7 in June, and 11 in July 2013.

We tested a total of 59 serum samples: 55 from teenage and 4 from adult dromedaries. The serum samples were collected from female dairy farm or racing dromedaries. The dairy dromedaries were purchased from various countries (e.g., Saudi Arabia, Oman, Sudan, and Pakistan); the number of dairy dromedaries from each country and their lengths of stay in Dubai were not known. The racing dromedaries were from Dubai Emirate.

RNA Extraction, Reverse Transcription PCR, and DNA Sequencing

Viral RNA extraction was conducted as described (*20*,*23*). Initial CoV screening was performed by amplifying a 440-bp fragment of CoV RNA-dependent RNA polymerase (RdRp) gene by using conserved primers (5′-GGTTGGGACTATCCTAAGTGTGA-3′ and 5′-ACCATCATCNGANARDATCATNA-3′ and degenerative bases N, R, and D, representing A/C/T/G, A/G, and A/G/T, respectively. After the novel CoV was detected in samples, subsequent screening was performed by using specific primers 5′-ACTATGACTGGCAGAATGTT-3′ and 5′-TAATAAGGCGACGTAACATA-3′. To amplify a 126-bp fragment of RdRp gene, reverse transcription PCR (RT-PCR) and DNA sequencing were performed as described (*20*,*23*).

Virus Culture

The fecal samples from 3 dromedaries tested positive for CoV. These samples were cultured in HRT-18G, Vero E6, Caco-2, and LLC-MK2 cell lines.

Complete Genome Sequencing and Analysis

Three complete genomes of DcCoV UAE-HKU23 (265F, 362F, and 368F) were amplified and sequenced as described (*5*,*12*). RNA extracted from fecal specimens was used as template, and a database of CoV genes and genomes (CoVDB, http://covdb.microbiology.hku.hk) (*25*) was used for sequence retrieval. Sequences were assembled and edited to produce final sequences.

We used EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) to compare the nucleotide sequences of the genomes and the deduced amino acid sequences with those for other CoVs. A neighbor-joining phylogenetic tree with 1,000 bootstraps was constructed by using the Jones-Taylor-Thornton substitution model; gamma distribution among sites was conducted in MEGA5 (*26*).

Real-time Quantitative RT-PCR

According to our protocol (*19*,*20*), all samples positive for DcCoV UAE-HKU23 by RT-PCR were subjected to quantitative RT-PCR (qRT-PCR).The assay was performed, as described (*27*), using a real-time 1-step qRT-PCR with DcCoV UAE-HKU23 primers 5′-ATAGCGGCTACACGTGGTGTT-3′ and 5′-TCCCAGCCGCCATAAAACT-3′ and probe 5′-(FAM) CTGTTGTTATAGGCACCACT (BHQ1)-3′. To generate calibration curves, we prepared a series of 6 $\log_{10}$ dilutions equivalent to 10^1–10^6 copies per reaction mixture and ran them in parallel with the test samples.

Cloning and Purification

The nucleocapsid proteins of SARS-CoV (betacoronavirus lineage B), Pi-Bat CoV HKU5 (betacoronavirus lineage C), and rousettus bat coronavirus (Ro-BatCov HKU9; betacoronavirus lineage D) were obtained as described (*2*,*16*). To produce a plasmid for DcCoV UAE-HKU23 nucleocapsid protein purification, primers 5′-CATGCCATGGGCATGTCTTTTACTCCTGGTAAGC-3′ and 5′-CCGCTCGAGTATTTCTGAGGTGTTTTCAG-3′ were used to amplify the gene encoding the nucleocapsid protein of DcCoV UAE-HKU23. The sequence encoding aa 1–672 of the nucleocapsid protein was amplified and cloned into the *Nco*I and *Xho*I sites of expression vector pET-28b(+) (Merck KGaA, Darmstadt, Germany). Recombinant nucleocapsid protein was expressed and purified by using the Ni^{2+}-loaded HiTrap Chelating System (GE Healthcare Life Sciences, Little Chalfont, UK) according to the manufacturer's instructions. The nucleocapsid protein of MERS-CoV was cloned and purified by using the method described above with primers 5′-GGAATTCCATATGATGGCATCCCCTGCTGCACCTC-3′ and 5′-ATAAGAATGCGGCCGCATCAGTGTTAACATCAATCATT-3′.

Western Blot Analysis

Western blot analysis was performed as described (*5*) with 1.5 μg of purified $(His)_6$-tagged recombinant nucleocapsid protein of DcCoV UAE-HKU23 and 1:2,000, 1:4,000, and 1:8,000 dilutions of dromedary serum samples. Antigen–antibody interaction was detected by using 1:4,000 diluted horseradish peroxidase–conjugated Goat Anti-Llama IgG (Life Technologies, Carlsbad, CA, USA) and the ECL fluorescence system (GE Healthcare Life Sciences).

To determine the possibility of cross-reactivity between antibodies against the nucleocapsid protein of DcCoV UAE-HKU23 and that of other betacoronavirus lineages, we tested 3 serum samples that were positive for antibody against the nucleocapsid protein of DcCoV UAE-HKU23. We used 1.5 μg of purified $(His)_6$-tagged recombinant nucleocapsid protein of 3 betacoronaviruses (SARS-CoV [lineage B], Pi-BatCoV HKU5 [lineage C], and Ro-BatCoV HKU9 [lineage D]) and 1:2,000 dilutions of serum samples. To determine the presence of antibodies against the nucleocapsid protein

of MERS-CoV, we tested the serum samples by using 1.5 μg of purified $(His)_6$-tagged recombinant nucleocapsid protein of MERS-CoV and 1:2,000 dilutions of serum samples. Antigen–antibody interaction was detected as described above.

Indirect Immunofluorescence

Anti–MERS-CoV antibody detection by indirect immunofluorescence was performed as described (*28*) with minor modifications. In brief, Vero cells infected with MERS-CoV were prepared as described (*28*). Camel serum samples were screened at a dilution of 1:160 on infected and noninfected control cells. Antigen–antibody interaction was detected by using fluorescein isothiocyanate–conjugated Goat Anti-Llama IgG (Life Technologies). Serum samples positive at a screening dilution of 1:160 were further titrated with serial 2-fold dilutions. The indirect immunofluorescence antibody titer was the highest dilution giving a positive result.

Neutralization Antibody Test

The neutralization antibody test was performed as described (*28*). In brief, starting with a serum dilution of 1:10, we prepared serial 2-fold dilutions of serum in 96-well microtiter plates. For each serum dilution, 0.05 mL of the dilution was mixed with 0.05 mL of 200 MERS-CoV 50% tissue culture infectious doses and incubated at 37°C for 1.5 h in a CO_2 incubator. Then 0.1 mL of virus–serum mixture was inoculated in duplicate wells of 96-well microtiter plates with preformed monolayers of Vero cells and further incubated at 37°C for 3–4 days. Cytopathic effects were observed by using an inverted microscope on days 3 and 4 after inoculation. The neutralizing antibody titer was determined as the highest dilution of serum that completely suppressed the cytopathic effects in at least half of the infected wells.

Estimation of Substitution Rates and Divergence Dates

The number of synonymous substitutions per synonymous site, *Ks*, and the number of nonsynonymous substitutions per nonsynonymous site, *Ka*, for each coding region between each pair of strains were calculated by using the Nei-Gojobori method (Jukes-Cantor) in MEGA5 (*26*). Divergence time was calculated on the basis of RdRp gene sequence data by using a Bayesian Markov Chain Monte Carlo approach as implemented in BEAST version 1.8.0 (http://beast.bio.ed.ac.uk), as described (*15*,*19*,*29*,*30*). Bayesian skyline under a relaxed-clock model with an uncorrelated exponential distribution was adopted for making inferences because Bayes factor analysis for the RdRp gene indicated that this model fitted the data better than other models tested.

Results

Identification of CoV in Dromedaries

Of the 293 fecal samples tested, 14 (4.8%) were RT-PCR positive for the CoV RdRp gene; 1 (0.4%) of the samples was from an adult dromedary and 13 (21.3%) were from calves (Table 1). Of the 14 positive samples, 11 were collected in April and 3 were collected in May. Ten of the 14 samples were submitted to the Central Veterinary Research Laboratory for routine checking, and the other 4 were collected because the dromedaries had diarrhea. Sequencing results indicated a 126-nt sequence identical to that of equine CoV, a betacoronavirus 1 species in lineage A (betacoronavirus A1).

Virus Culture and Virus Load

Attempts to stably passage DcCoV UAE-HKU23 in cell cultures were unsuccessful; no cytopathic effect or viral replication was detected. Real-time qRT-PCR showed that the amounts of DcCoV UAE-HKU23 RNA ranged from 5.7×10^4 copies/mL to 9.8×10^7 copies/mL (median 8.4×10^5) in the 14 fecal samples positive for DcCoV UAE-HKU23 (Table 1).

Genome Organization and Coding Potential

The 3 complete genomes of DcCoV UAE-HKU23 (GenBank accession nos. KF906249–KF906251) were 31,036 bases and had a G+C content of 37% (Table 2). The genome organization is similar to that of other betacoronavirus lineage A CoVs (Figure 1). Additional open-reading frames (ORFs) coding for nonstructural proteins (NSPs) NS2 and NS5 are found. DcCoV UAE-HKU23 and other CoVs in betacoronavirus lineage A possess the same putative transcription regulatory sequence motif, 5′-UCUAAAC-3′, at the 3′ end of the leader sequence and preceding most ORFs (Table 3; online Technical

Table 1. Epidemiologic data for dromedaries in the Middle East that were positive for a novel betacoronavirus, DcCoV UAE-HKU23, 2013*

Age category	Reason for sample testing	Virus load
Calf	Routine check	3.3×10^6
Calf	Routine check	1.1×10^6
Calf	Routine check	1.5×10^5
Calf	Routine check	3.4×10^5
Calf	Routine check	3.2×10^6
Calf	Routine check	6.5×10^5
Calf	Routine check	9.5×10^5
Calf	Routine check	5.7×10^4
Calf	Routine check	2.1×10^5
Calf	Routine check	4.4×10^5
Adult	Diarrhea	7.3×10^5
Calf	Diarrhea	9.8×10^7
Calf	Diarrhea	4.4×10^7
Calf	Diarrhea	1.0×10^7

*DcCoV, dromedary camel coronavirus; calf, <1 year of age.

Table 2. Comparison of representative coronaviruses with a novel betacoronavirus, DcCoV UAE-HKU23, discovered in dromedaries in the Middle East, 2013*

Coronavirus (genome)	Genome features		% Pairwise amino acid identity with DcCoV UAE-HKU23 (265F)				
	Size, bases	G+C content	3CL^{pro}	RdRp	Helicase	S protein	N protein
Alphacoronavirus							
HCoV-229E	27,317	0.38	44.4	55.8	57.9	26.9	25.1
HCoV-NL63	27,553	0.34	42.8	56.0	57.5	26.4	24.1
Betacoronavirus							
Lineage A							
BCoV	31,028	0.37	99.7	99.9	99.5	94.1	98.4
SACoV	30,995	0.37	99.7	99.8	99.3	94.1	98.9
DcCoV UAE-HKU23 (362F)	31,036	0.37	99.7	100	100	99.8	100
DcCoV UAE-HKU23 (368F)	31,036	0.37	99.7	100	100	99.8	100
Lineage B							
SARS-CoV	29,751	0.41	48.4	66.3	68.0	30.4	34.4
Lineage C							
MERS-CoV	30,119	0.41	53.3	68.2	67.9	31.8	34.7
Lineage D							
Ro-BatCoV HKU9	29,114	0.41	48.4	66.5	67.5	29.2	32.5
Gammacoronavirus							
Infectious bronchitis virus	27,608	0.38	42.1	60.6	60.2	24.8	28.0
BdCoV HKU22	31,750	0.39	45.3	60.0	58.2	25.7	28.9
Deltacoronavirus							
BuCoV HKU11	26,476	0.39	38.5	51.2	47.5	26.4	23.8
PorCoV HKU15	25,421	0.43	38.8	51.8	48.6	26.0	23.1

*The representative coronaviruses were those with complete genomes sequences available. DcCoV, dromedary camel coronavirus; 3CL^{pro}, chymotrypsin-like protease; RdRp, RNA-dependent RNA polymerase; S protein, spike protein; N protein, nucleocapsid protein; HCoV, human CoV; BCoV, bovine CoV; SACoV, sable antelope CoV; SARS-CoV, severe acute respiratory syndrome CoV; MERS-CoV, Middle East respiratory syndrome CoV; Ro-BatCoV, rousettus bat CoV; BdCoV, bottlenose dolphin CoV; BuCoV, bulbul CoV; PorCoV, porcine CoV.

Appendix Table 1, wwwnc.cdc.gov/EID/article/20/4/13-1769-Techapp1.pdf) (*19*,*30*).

The characteristics of putative NSPs in ORF1ab of DcCoV UAE-HKU23 are shown in online Technical Appendix Table 2. The ORF1ab polyprotein shared 70.7%–99.3% aa identity with polyproteins of other betacoronavirus lineage A CoVs. The predicted putative cleavage sites were conserved between DcCoV UAE-HKU23 and other members of betacoronavirus A1 of the *Betacoronavirus* genus. The lengths of NSPs 1–3, 13, and 15 in DcCoV UAE-HKU23 differed from those in equine CoV, porcine hemagglutinating encephalomyelitis virus, and/or HCoV-OC43 as a result of deletions/insertions.

The amino acid sequence of the predicted spike protein of DcCoV UAE-HKU23 is most similar to that of bovine coronavirus (BCoV) and sable antelope CoV, with which DcCoV UAE-HKU23 has 94.1% similarity (Table 2). A comparison of the amino acid sequences of DcCoV UAE-HKU23 spike protein and BCoV spike protein showed 81 aa polymorphisms, of which 24 were seen within the region previously identified as hypervariable among the spike protein of other betacoronavirus lineage A CoVs (*31*) (Figure 2); this finding suggests that this region in DcCoV UAE-HKU23 is also subject to strong immune selection. BCoV has been found to use *N*-acetyl-9-*O* acetyl neuramic acid as a receptor for initiation of infection (*32*). Among the 5 aa that may affect S1-mediated receptor binding in BCoV (*31*), 2 aa (threonine at position 11 and glutamine at position 179) were conserved in DcCoV UAE-HKU23 (Figure 2). However, at positions 115, 118, and 173, aspartic acid, methionine, and asparagine observed in BCoV and were replaced by serine, threonine, and histidine, respectively, in DcCoV UAE-HKU23. A recent report identified 4 aa acids that were critical sugar-binding residues in the spike protein of BCoV (tyrosine, glutamic acid, tryptophan, and histidine at positions 162, 182, 184, and 185, respectively) (*32*); all 4 aa were also present in spike protein of DcCoV UAE-HKU23 (Figure 2). Another study identified 7 aa substitutions in the spike protein of BCoV that differed between virulent and avirulent, cell culture-adapted strains (*31*); 5 of the 7 aa from virulent strains were also conserved in DcCoV UAE-HKU23, and amino acid substitutions were observed in the other 2 aa (threonine→valine at position 40 and aspartic acid→asparagine at position 470). It has also been reported that an amino acid change at position 531 of the spike protein of BCoV discriminated between enteric (aspartic acid/asparagine) and respiratory (glycine) strains (*33*). At this position, a threonine was conserved in all 3 genomes of DcCoV UAE-HKU23.

NS5 of DcCoV UAE-HKU23 shares 83.5%–98.2% aa identity with the corresponding NSPs of betacoronavirus A1 members. In murine hepatitis virus, translation of the envelope protein is cap-independent, through an internal ribosomal entry site (*19*,*30*). However, a preceding transcription regulatory sequence, 5′-UCCAAAC-3′, can be identified upstream of the envelope protein in DcCoV UAE-HKU23, as in other betacoronavirus A1 members

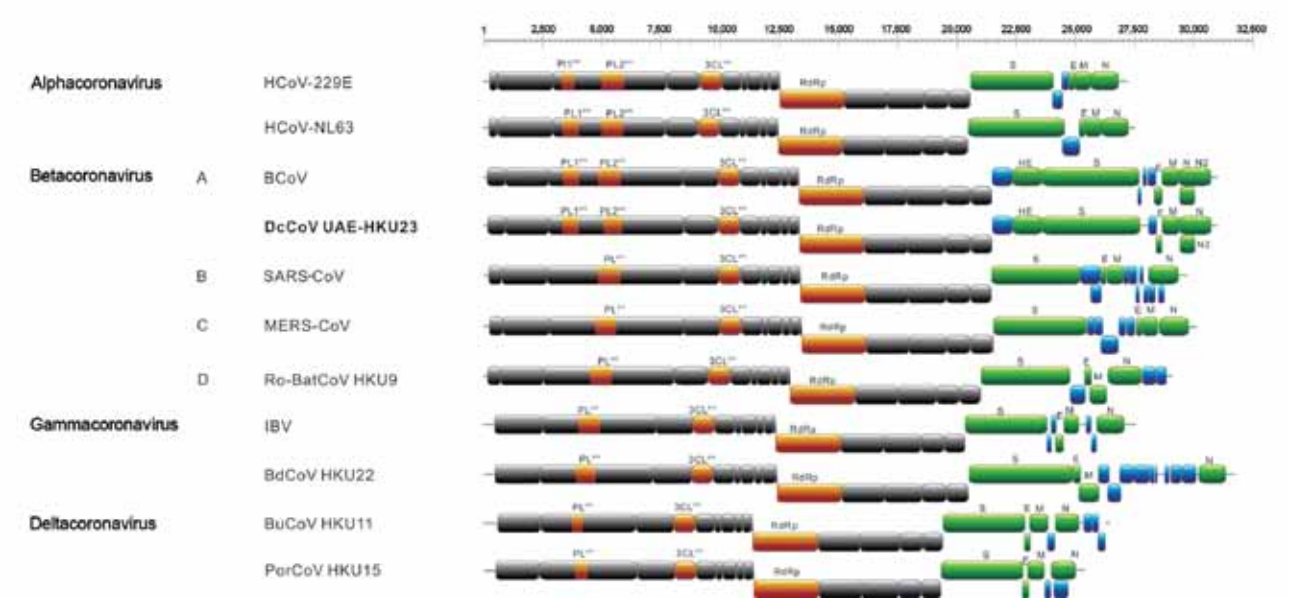

Figure 1. Genome organizations of a novel betacoronavirus, in boldface, discovered in dromedaries in the Middle East in 2013, and representative coronaviruses from each coronavirus genus (labeled on left). Numbers at top represent genome position. A, B, C, and D represent betacoronavirus lineages. Papain-like proteases ($PL1^{pro}$, $PL2^{pro}$, and PL^{pro}), chymotrypsin-like protease ($3CL^{pro}$), and RNA-dependent RNA polymerase (RdRp) are represented by orange boxes. Hemagglutinin esterase (HE), spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins are represented by green boxes. Putative accessory proteins are represented by blue boxes. HCoV, human coronavirus; BCoV, bovine CoV; DcCoV, dromedary camel CoV; SARS-CoV, severe acute respiratory syndrome CoV; MERS-CoV, Middle East respiratory syndrome CoV; Ro-BatCoV, rousettus bat CoV; IBV, infectious bronchitis virus; BdCoV, bottlenose dolphin CoV; BuCoV, bulbul CoV; PorCoV, porcine CoV.

(Table 3; online Technical Appendix Table 1) (*19*,*30*). Downstream to nucleocapsid protein, the 3′-untranslated region contains a predicted bulged stem-loop structure of 68 nt (nt position 30747–30814) that is conserved in betacoronaviruses (*34*). Overlapping with the bulged stem-loop structure by 5 nt, is a conserved pseudoknot structure (nt position 30810–30863) that is important for CoV replication.

Phylogenetic Analyses

Phylogenetic trees constructed by using the amino acid sequences of ORF1b polyprotein, spike protein, and

Table 3. Putative transcription regulatory sequence of betacoronavirus A1 members*

	Putative transcription regulatory sequence (distance, in bases, to AUG)†							
ORF	DcCoV UAE-HKU23	BCoV	CRCoV	SACoV	GiCoV	PHEV	HCoV-OC43	ECoV
1ab	UCUAAAC (140)	UCUAAAC (140)	UCUAAAC (140)	UCUAAAC (140)	UCUAAAC (140)	UCUAAAC (140)	UCUAAAC (140)	UCUAAAC (140)
NS2	UCUAAAC (7)	UCUAAAC (7)	UCUAAAC (7)	UCUAAAC (7)	UCUAAAC (7)	UCUAAAC (1)	UCUAAAC (7)	UCUAAAA (12)
HE	ACUAAAC (9)	ACUAAAC (9)	ACUAAAC (9)	ACUAAAC (9)	ACUAAAC (9)	ACUAAAC (9)	AUUAAAC (9)	UCUAAAC (9)
S	UCUAAAC (0)	UCUAAAC (0)	UCUAAAC (0)	UCUAAAC (0)	UCUAAAC (0)	UCUAAAC (0)	UCUAAAC (0)	UCUAAAC (0)
NS5	GGUGAAC (51)	GGU**AG**AC (**50**)	GGU**AG**AC (51)	GGU**AG**AC (**50**)	GGU**AG**AC (**50**)	**UUAAGCA** (**32**)	**UCUAGCA** (**20/32**)	**UAUACUUUAUAA** (**41**)
E	UCCAAAC (123)	UCCAAAC (123)	UCCAAAC (123)	UCCAAAC (123)	UCCAAAC (123)	UCCAAAC (123)	UCCAAAC (123)	UCCAAAC (123)
M	UCCAAAC (3)	UCCAAAC (3)	UCCAAAC (3)	UCCAAAC (3)	UCCAAAC (3)	UCCAAAC (3)	UCCAAAC (3)	UCCAAAC (3)
N	UCUAAAC (7)	UCUAAAC (7)	UCUAAAC (7)	UCUAAAC (7)	UCUAAAC (7)	UCUAAAC (7)	UCUAAAU (7)	UCUAAAC (7)

*ORF, open reading frame; DcCoV, dromedary camel coronavirus; BCoV, bovine CoV; CRCoV, canine respiratory CoV; SACoV, sable antelopeCoV; GiCoV, giraffe CoV; PHEV, porcine hemagglutinating encephalomyelitis virus; HCoV, human CoV; ECoV, equine CoV; NS, nonstructural protein; HE, hemagglutinin esterase; S, spike protein; E, envelope protein; M, membrane protein; N, nucleocapsid protein.
†Boldface indicates bases and distances to AUG that are different from those for DcCoV UAE-HKU23.

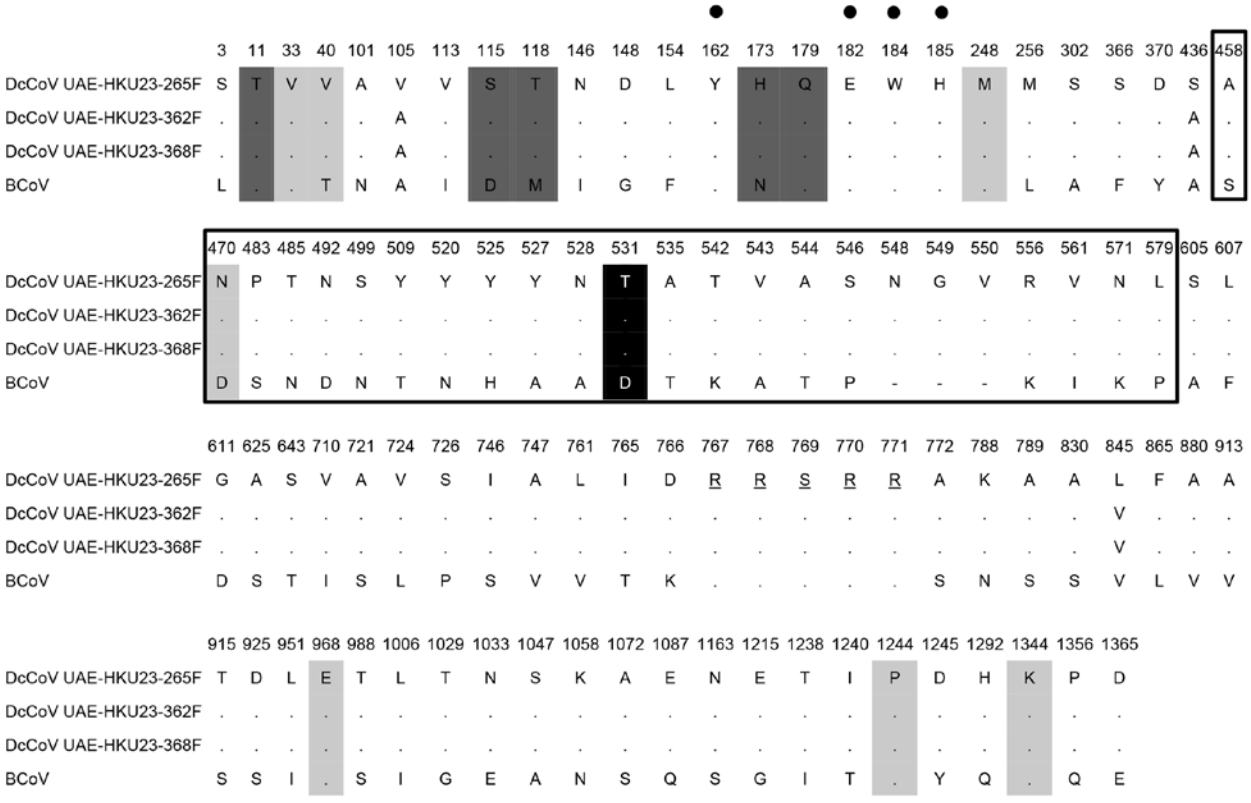

Figure 2. Amino acid comparison of the spike protein of a novel betacoronavirus, dromedary camel coronavirus (DcCoV) UAE-HKU23, discovered in dromedaries in the Middle East in 2013, with that of bovine coronavirus (BCoV; GenBank accession no. AF391541). Amino acid substitution sites, key amino acids for virulence and receptor binding in BCoV, and cleavage sites are shown. Amino acid positions are given with reference to DcCoV UAE-HKU23. Conserved amino acids, compared with those of DcCoV UAE-HKU23 (strain 265F), are represented by dots. Amino acids of putative cleavage sites are underlined. Amino acids within the S1 hypervariable region of BCoV are marked with open boxes. Amino acid sites central to virulence in BCoV are highlighted in light gray. Amino acid sites shown to affect S1-mediated receptor binding in BCoV are highlighted in dark gray. The 4 critical sugar-binding residues are indicated by black dots. The amino acid site that discriminated between enteric and respiratory BCoV strains is highlighted in black.

nucleocapsid protein of DcCoV UAE-HKU23 and other CoVs are shown in Figures 3–5. The pairwise amino acid identities of chymotrypsin-like protease ($3CL^{pro}$), RdRp, helicase, spike protein, and nucleocapsid protein are shown in Table 2. In all 3 phylogenetic trees, DcCoV UAE-HKU23 clustered with other members of betacoronavirus A1, including BCoV, sable antelope CoV, equine CoV, HCoV-OC43, giraffe CoV, porcine hemagglutinating encephalomyelitis virus, canine respiratory CoV, and rabbit CoV (RbCoV) HKU14 (Figures 3–5).

Antibody Detection

Nucleocapsid protein of DcCoV UAE-HKU23 was purified. Prominent immunoreactive bands were visible for 31 (52%) of 59 dromedary serum samples; 25 of the 31 samples had titers of 2,000, three had titers of 4,000, and 3 had titers of 8,000 (Figure 6). Serum samples for all 4 adult dromedaries were positive for DcCoV UAE-HKU23 antibodies, and 27 (49%) of the 55 samples for teenage dromedaries were positive. Band sizes were ≈50 kDa, consistent with the expected size of 50.4 kDa for the full-length $(His)_6$-tagged recombinant nucleocapsid protein. Only very faint bands were observed when the 3 serum samples positive for DcCoV UAE-HKU23 antibodies were incubated with nucleocapsid proteins of SARS-CoV, Pi-BatCoV HKU5, or Ro-BatCoV HKU9, indicating minimal cross-reactivity. This finding concurs with our previous observation that minimal cross-reactivity occurs between CoVs in different lineages in betacoronavirus (*16*). For MERS-CoV antibody testing, results were positive for 57 (97%) of the 59 samples by Western blot analysis, for all 59 samples by indirect immunofluorescence, and for 58 (98%) of the 59 samples by neutralization antibody test (Table 4).

Estimation of Substitution Rates and Divergence Dates

The *Ka*, *Ks*, and *Ka*/*Ks* of the various coding regions in DcCoV UAE-HKU23 are shown in Table 5.

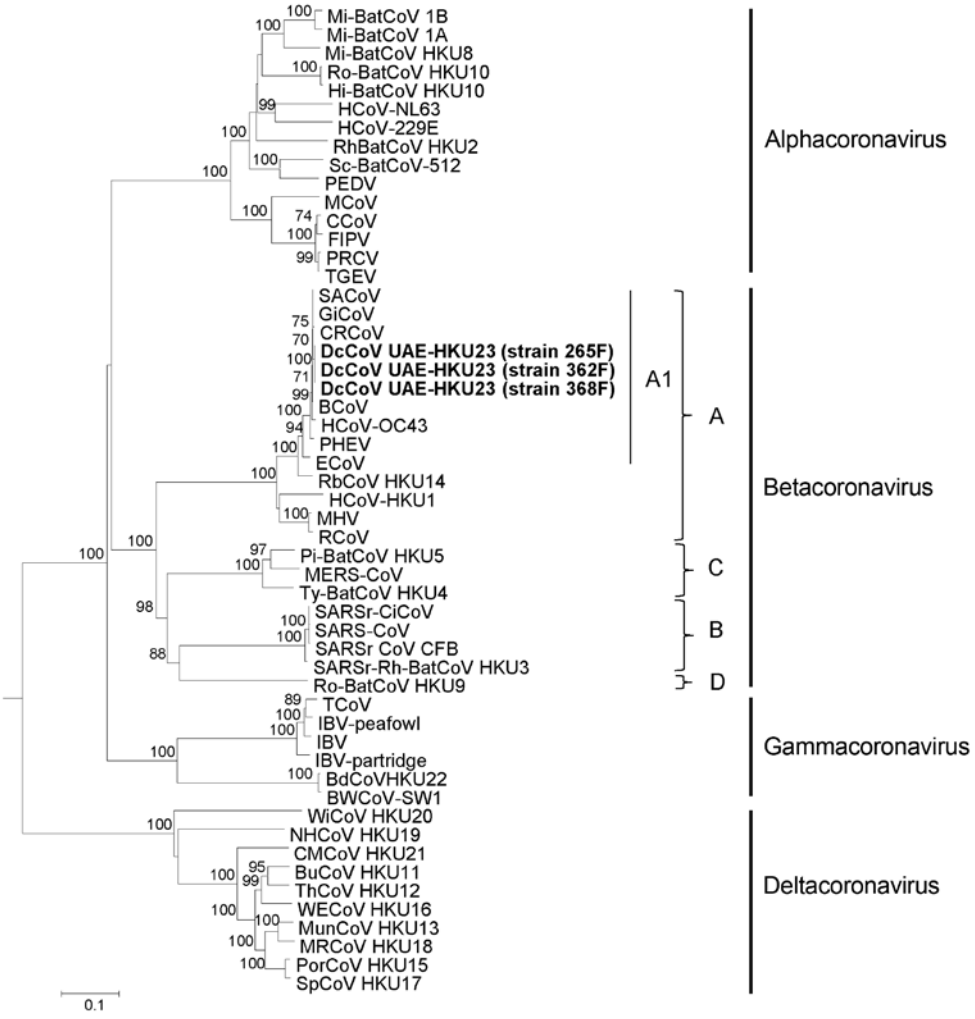

Figure 3. Phylogenetic analysis of open reading frame (ORF) 1b polyprotein of dromedary camel coronavirus (DcCoV) UAE-HKU23 from dromedaries of the Middle East, 2013. The tree was constructed by the neighbor-joining method, using Jones-Taylor-Thornton substitution model with gamma distributed rate variation and bootstrap values calculated from 1,000 trees. Bootstrap values of <70% are not shown. A total of 2,725 aa positions in ORF1b polyprotein were included in the analysis. The tree was rooted to Breda virus (GenBank accession no. AY_427798). Betacoronavirus lineages A1 and A–D are indicated on the right. Boldface indicates the 3 strains of DcCoV UAE-HKU23 characterized in this study. Virus definitions and GenBank accession numbers (in parentheses) follow: Mi-BatCoV 1B, miniopterus bat CoV 1B (NC_010436); Mi-BatCoV 1A (NC_010437); Mi-BatCoV HKU8 (NC_010438); Ro-BatCoV HKU10, rousettus bat CoV HKU10 (JQ989270); Hi-BatCoV HKU10, hipposideros bat CoV HKU10 (JQ989266); HCoV-NL63, human CoV NL63 (NC_005831); HCoV-229E (NC_002645); RhBatCoV HKU2, rhinolophus bat CoV HKU2 (EF203064); Sc-BatCoV-512, scotophilus bat CoV 512 (NC_009657); PEDV, porcine epidemic diarrhea virus (NC_003436); MCoV, mink CoV (HM245925); CCoV, canine CoV (GQ477367); FIPV, feline infectious peritonitis virus (AY994055); PRCV, porcine respiratory CoV; TGEV, transmissible gastroenteritis virus; SACoV, sable antelope CoV; GiCoV, giraffe CoV (EF424622); CRCoV, canine respiratory CoV (JX860640); BCoV, bovine CoV (NC_003045); HCoV-OC43, human CoV OC43 (NC_005147); PHEV, porcine hemagglutinating encephalomyelitis virus (NC_007732); ECoV, equine CoV (NC_010327); RbCoV HKU14, rabbit CoV HKU14 (JN874559); HCoV-HKU1, human CoV HKU1 (NC_006577); MHV, murine hepatitis virus (NC_001846); RCoV, rat CoV (NC_012936); Pi-BatCoV HKU5, pipistrellus bat CoV HKU5 (NC_009020); MERS-CoV, Middle East respiratory syndrome CoV (JX869059); Ty-BatCoV HKU4, tylonycteris bat CoV HKU4 (NC_009019); SARSr-CiCoV, SARS-related palm civet CoV (AY304488); SARS-CoV, severe acute respiratory syndrome–associated human CoV (NC_004718); SARSrCoV CFB, SARS-related Chinese ferret badger CoV (AY545919); SARS-related rhinolophus bat CoV HKU3 (DQ022305); Ro-BatCoV HKU9, rousettus bat CoV HKU9 (NC_009021); TCoV, turkey CoV (NC_010800); IBV-peafowl, peafowl CoV (AY641576); IBV, infectious bronchitis virus (NC_001451); IBV-partridge, partridge CoV (AY646283); BdCoV HKU22, bottlenose dolphin CoV HKU22 (KF793824); BWCoV-SW1, Beluga whale CoV SW1 (NC_010646); WiCoV HKU20, wigeon CoV HKU20 (JQ065048); NHCoV HKU19, night-heron CoV HKU19 (JQ065047); CMCoV HKU21, common-moorhen CoV HKU21 (JQ065049); BuCoV HKU11, bulbul CoV HKU11 (FJ376619); ThCoV HKU12, thrush CoV HKU12 (FJ376621); WECoV HKU16, white-eye CoV HKU16 (JQ065044); MunCoV HKU13, munia CoV HKU13 (FJ376622); MRCoV HKU18, magpie–robin CoV HKU18 (JQ065046); PorCoV HKU15, porcine CoV HKU15 (JQ065042); SpCoV HKU17, sparrow CoV HKU17 (JQ065045). Numbers at nodes represent bootstrap values. Scale bar indicates the estimated number of substitutions per 10 aa.

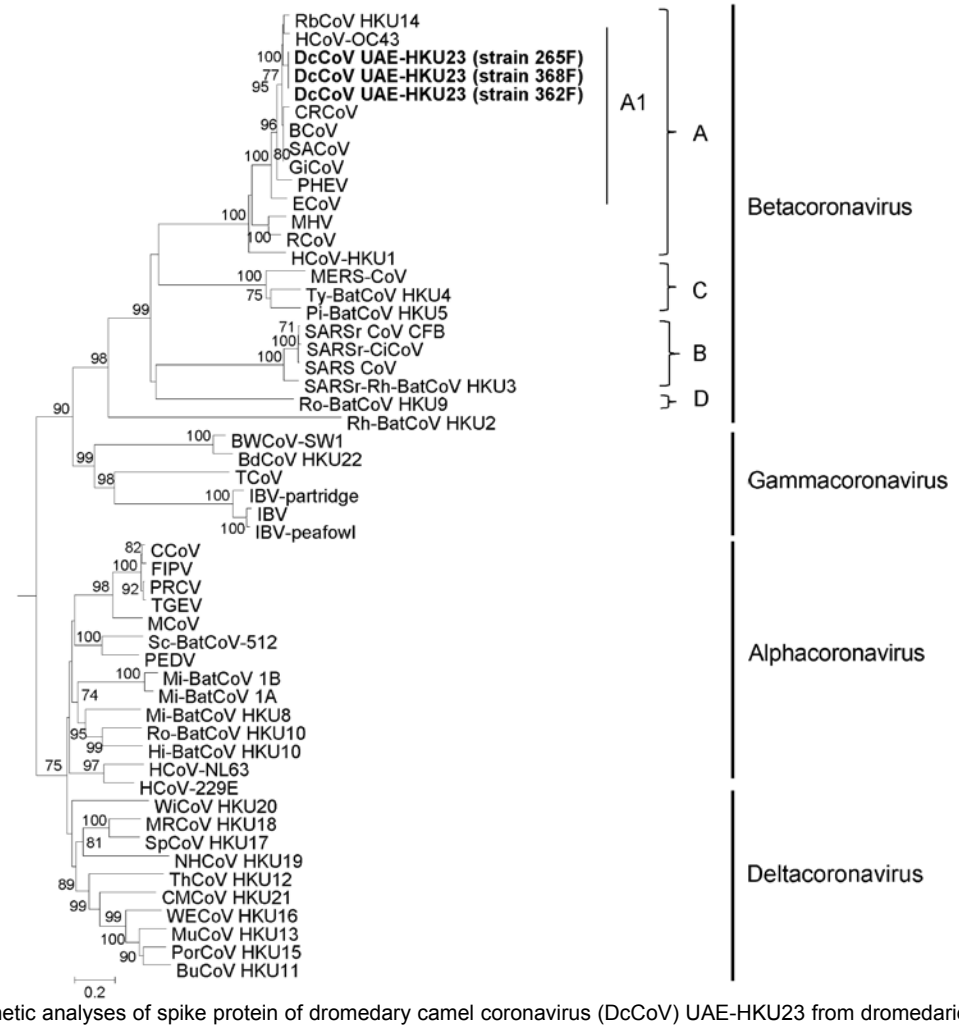

Figure 4. Phylogenetic analyses of spike protein of dromedary camel coronavirus (DcCoV) UAE-HKU23 from dromedaries of the Middle East, 2013. The tree was constructed by the neighbor-joining method, using Jones-Taylor-Thornton substitution model with gamma distributed rate variation and bootstrap values calculated from 1,000 trees. Bootstrap values of <70% are not shown. A total of 1,366 aa positions in spike protein were included in the analysis. The tree was rooted to Breda virus (GenBank accession no. AY_427798). Betacoronavirus lineages A1 and A–D are indicated on the right. Boldface indicates the 3 strains of DcCoV UAE-HKU23 characterized in this study. Virus definitions and GenBank accession numbers (in parentheses) follow: RbCoV HKU14, rabbit CoV HKU14 (JN874559); HCoV-OC43, human CoV OC43 (NC_005147); CRCoV, canine respiratory CoV (JX860640); BCoV, bovine CoV (NC_003045); SACoV, sable antelope CoV (EF424621); GiCoV, giraffe CoV (EF424622); PHEV, porcine hemagglutinating encephalomyelitis virus (NC_007732); ECoV, equine CoV (NC_010327); MHV, murine hepatitis virus (NC_001846); RCoV, rat CoV (NC_012936); HCoV-HKU1, human CoV HKU1 (NC_006577); MERS-CoV, Middle East respiratory syndrome CoV (JX869059); Ty-BatCoV HKU4, tylonycteris bat CoV HKU4 (NC_009019); Pi-BatCoV HKU5, pipistrellus bat CoV HKU5 (NC_009020); SARSrCoV CFB, SARS-related Chinese ferret badger CoV (AY545919); SARSr-CiCoV, SARS-related palm civet CoV (AY304488); SARS-CoV, severe acute respiratory syndrome–associated human CoV (NC_004718); SARSr-Rh-BatCoV HKU3, SARS-related rhinolophus bat CoV HKU3 (DQ022305); Ro-BatCoV HKU9, rousettus bat CoV HKU9 (NC_009021); RhBatCoV HKU2, rhinolophus bat CoV HKU2 (EF203064); BWCoV-SW1, Beluga whale CoV SW1 (NC_010646); BdCoV HKU22, bottlenose dolphin CoV HKU22 (KF793824); TCoV, turkey CoV (NC_010800); IBV-partridge, partridge CoV (AY646283); IBV, infectious bronchitis virus (NC_001451); IBV-peafowl, peafowl CoV (AY641576); CCoV, canine CoV (GQ477367); FIPV, feline infectious peritonitis virus (AY994055); PRCV, porcine respiratory CoV (DQ811787); TGEV, transmissible gastroenteritis virus (DQ811789); MCoV, mink CoV (HM245925); Sc-BatCoV-512, scotophilus bat CoV 512 (NC_009657); PEDV, porcine epidemic diarrhea virus (NC_003436); Mi-BatCoV 1B, miniopterus bat CoV 1B (NC_010436); Mi-BatCoV 1A, miniopterus bat CoV 1A (NC_010437); Mi-BatCoV HKU8, miniopterus bat CoV HKU8 (NC_010438); Ro-BatCoV HKU10, rousettus bat CoV HKU10 (JQ989270); Hi-BatCoV HKU10, hipposideros bat CoV HKU10 (JQ989266); HCoV-NL63, human CoV NL63 (NC_005831); HCoV-229E, human CoV 229E (NC_002645); WiCoV HKU20, wigeon CoV HKU20 (JQ065048); MRCoV HKU18, magpie–robin CoV HKU18 (JQ065046); SpCoV HKU17, sparrow CoV HKU17 (JQ065045); NHCoV HKU19, night-heron CoV HKU19 (JQ065047); ThCoV HKU12, thrush CoV HKU12 (FJ376621); CMCoV HKU21, common-moorhen CoV HKU21 (JQ065049); WECoV HKU16, white-eye CoV HKU16 (JQ065044); MunCoV HKU13, munia CoV HKU13 (FJ376622); PorCoV HKU15, porcine CoV HKU15 (JQ065042); BuCoV HKU11, bulbul CoV HKU11 (FJ376619). Numbers at nodes represent bootstrap values. Scale bar indicates the estimated number of substitutions per 5 aa.

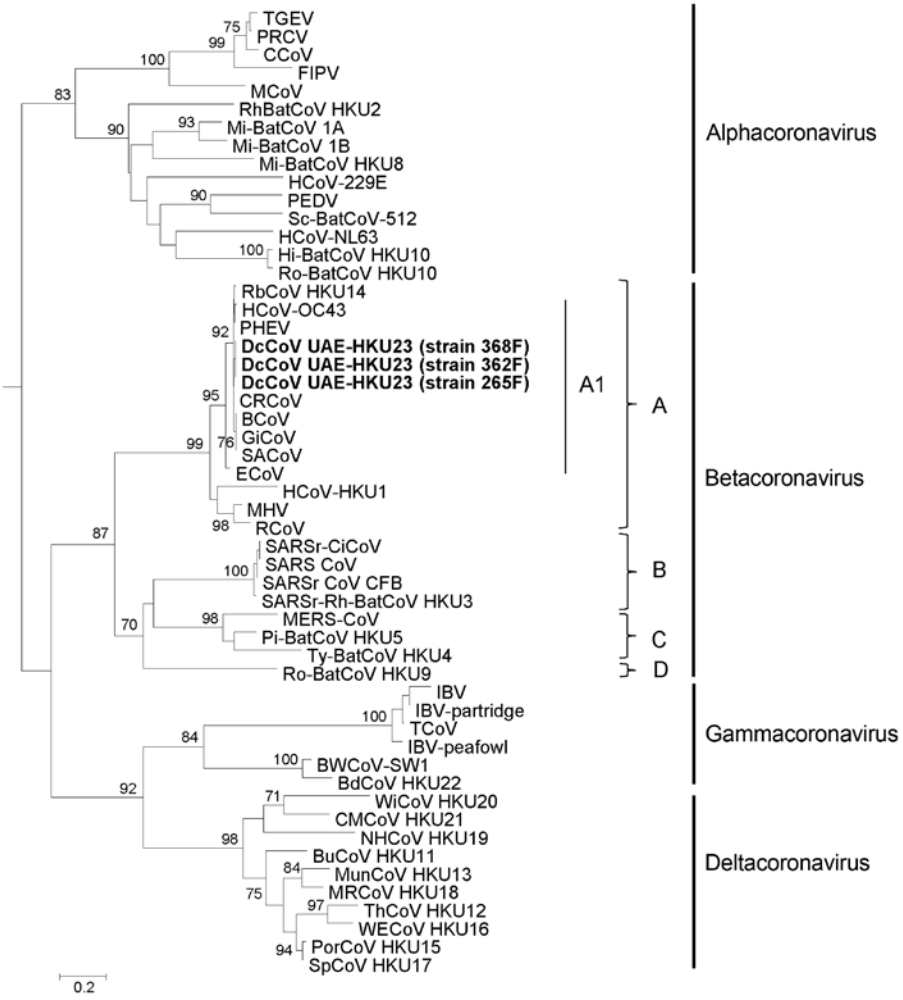

Figure 5. Phylogenetic analyses of the nucleocapsid protein of a novel coronavirus (CoV), dromedary camel CoV (DcCoV) UAE-HKU23, discovered in dromedaries of the Middle East, 2013. The tree was constructed by the neighbor-joining method, using Jones-Taylor-Thornton substitution model with gamma distributed rate variation and bootstrap values calculated from 1,000 trees. Bootstrap values of <70% are not shown. A total of 448 aa positions were included in the analysis. The tree was rooted to Breda virus (GenBank accession no. AY_427798). Betacoronavirus lineages A1 and A–D are indicated on the right. Boldface indicates the 3 strains of DcCoV UAE-HKU23 characterized in this study. Virus definitions and GenBank accession numbers (in parentheses) follow: TGEV, transmissible gastroenteritis virus (DQ811789); PRCV, porcine respiratory CoV (DQ811787); CCoV, canine CoV (GQ477367); FIPV, feline infectious peritonitis virus (AY994055); MCoV, mink CoV (HM245925); RhBatCoV HKU2, rhinolophus bat CoV HKU2 (EF203064); Mi-BatCoV 1A, miniopterus bat CoV 1A (NC_010437); Mi-BatCoV 1B, miniopterus bat CoV 1B (NC_010436); Mi-BatCoV HKU8, miniopterus bat CoV HKU8 (NC_010438); HCoV-229E, human CoV 229E (NC_002645); PEDV, porcine epidemic diarrhea virus (NC_003436); Sc-BatCoV-512, scotophilus bat CoV 512 (NC_009657); HCoV-NL63, human CoV NL63 (NC_005831); Hi-BatCoV HKU10, hipposideros bat CoV HKU10 (JQ989266); Ro-BatCoV HKU10, rousettus bat CoV HKU10 (JQ989270); RbCoV HKU14, rabbit CoV HKU14 (JN874559); HCoV-OC43, human CoV OC43 (NC_005147); PHEV, porcine hemagglutinating encephalomyelitis virus (NC_007732); CRCoV, canine respiratory CoV (JX860640); BCoV, bovine CoV (NC_003045); GiCoV, giraffe CoV (EF424622); SACoV, sable antelope CoV (EF424621); ECoV, equine CoV (NC_010327); HCoV-HKU1, human CoV HKU1 (NC_006577); MHV, murine hepatitis virus (NC_001846); RCoV, rat CoV (NC_012936); SARSr-CiCoV, SARS-related palm civet CoV (AY304488); SARS-CoV, severe acute respiratory syndrome–associated human CoV (NC_004718); SARSrCoV CFB, SARS-related Chinese ferret badger CoV (AY545919); SARSr-Rh-BatCoV HKU3, SARS-related rhinolophus bat CoV HKU3 (DQ022305); MERS-CoV, Middle East respiratory syndrome CoV (JX869059); Pi-BatCoV HKU5, pipistrellus bat CoV HKU5 (NC_009020); Ty-BatCoV HKU4, tylonycteris bat CoV HKU4 (NC_009019); Ro-BatCoV HKU9, rousettus bat CoV HKU9 (NC_009021); IBV, infectious bronchitis virus (NC_001451); IBV-partridge, partridge CoV (AY646283); TCoV, turkey CoV (NC_010800); IBV-peafowl, peafowl CoV (AY641576); BWCoV-SW1, Beluga whale CoV SW1 (NC_010646); BdCoV HKU22, bottlenose dolphin CoV HKU22 (KF793824); WiCoV HKU20, wigeon CoV HKU20 (JQ065048); CMCoV HKU21, common-moorhen CoV HKU21 (JQ065049); NHCoV HKU19, night-heron CoV HKU19 (JQ065047); BuCoV HKU11, bulbul CoV HKU11 (FJ376619); MunCoV HKU13, munia CoV HKU13 (FJ376622); MRCoV HKU18, magpie–robin CoV HKU18 (JQ065046); ThCoV HKU12, thrush CoV HKU12 (FJ376621); WECoV HKU16, white-eye CoV HKU16 (JQ065044); PorCoV HKU15, porcine CoV HKU15 (JQ065042); SpCoV HKU17, sparrow CoV HKU17 (JQ065045). Numbers at nodes represent bootstrap values. Scale bar indicates the estimated number of substitutions per 5 aa.

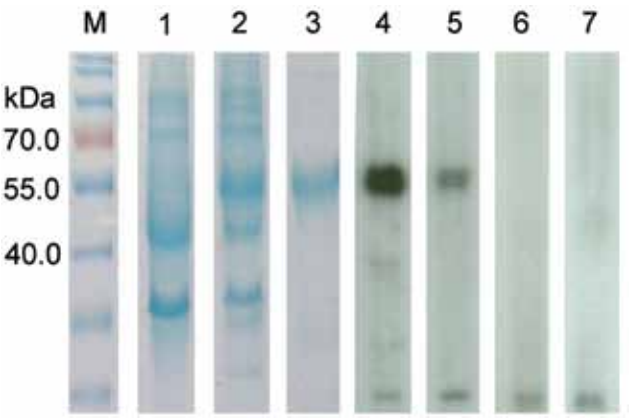

Figure 6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis of a novel coronavirus, dromedary camel coronavirus UAE-HKU23, discovered in dromedaries of the Middle East, 2013. Nucleocapsid protein was expressed in *Escherichia coli*. M, protein molecular-mass marker; kDa, kilodaltons. Lanes: 1, non-induced crude *E. coli* cell lysate; 2, induced crude *E. coli* cell lysate of DcCoV UAE-HKU23 nucleocapsid protein; 3, purified recombinant DcCoV UAE-HKU23 nucleocapsid protein; 4, dromedary camel serum sample strongly positive for antibody against nucleocapsid protein; 5, dromedary camel serum sample moderately positive for antibody against nucleocapsid protein; 6 and 7: dromedary camel serum sample negative for antibody against nucleocapsid protein.

The *Ka*/*Ks* of all the coding regions in DcCoV UAE-HKU23 was ≤0.5.

By using the uncorrelated relaxed clock model on RdRp gene sequences, we estimated the date of divergence between DcCoV UAE-HKU23 and BCoV to be ≈46 years ago. We estimated that the 3 strains of DcCoV UAE-HKU23 diverged from their most recent common ancestor in March 2010 (the 95% highest posterior density interval, August 2006–September 2012) (online Technical Appendix Figure 1).

Discussion

We discovered a novel CoV, but no MERS-CoV, in dromedaries from the Middle East. Dromedaries are 1 of 2 surviving camel species. Dromedaries (*C. dromedarius*; 1-humped camels) inhabit the Middle East and northern and northeastern Africa; Bactrian camels (*C. bactrianus*, 2-humped camels) inhabit Central Asia. Among the 20 million camels on earth, 90% are dromedaries. In 2012, there were ≈360,000 dromedaries in the United Arab Emirates.

In this study, we discovered a novel CoV, DcCoV UAE-HKU23, from 4.8% of 293 fecal samples collected from dromedaries in Dubai. The positive samples were not collected from the same farm or stable. Moreover, there was >0.2% nt difference among the 3 complete genomes sequenced, indicating that the positive samples were not collected from a clonal outbreak. In our study, 21.3% of dromedary calves, but only 0.4% of adult dromedaries, were RT-PCR positive for DcCoV UAE-HKU23; this finding indicates that dromedary calves are probably more susceptible than adult dromedaries to infection with DcCoV UAE-HKU23. Furthermore, DcCoV UAE-HKU23 is probably stably evolving in dromedaries because the *Ka*/*Ks* of all the coding regions in the genome were ≤0.5. In this study, 4 of the 12 positive samples were collected from dromedaries with diarrhea. A previous report also described the presence of a betacoronavirus in the fecal sample of a dromedary calf with diarrhea (*35*). This finding raises the question of the pathologic significance of DcCoV UAE-HKU23 for camelids and warrants further animal studies.

Our serologic data showed little cross-reactivity between DcCoV UAE-HKU23 and SARS-CoV, Pi-BatCoV HKU5, and Ro-BatCoV HKU9. This finding is in line with findings from our previous studies of Ro-BatCoV HKU9, which also showed minimal serologic cross-reactivity among the 4 lineages of betacoronaviruses (*16*). These results suggest that there should be minimal cross-reactivity between DcCoV UAE-HKU23 and MERS-CoV, which belong to 2 different CoV lineages. Because we showed an extremely high prevalence of MERS-CoV antibodies in the serum samples by Western blot analysis, indirect immunofluorescence, and neutralization antibody testing, concurring with findings in a previous study (*24*), we would also expect a similar high prevalence of DcCoV UAE-HKU23 antibodies if there was major serologic cross-reactivity between MERS-CoV and DcCoV UAE-HKU23. However, our serologic data only revealed the presence of DcCoV UAE-HKU23 antibodies in 52% of the serum samples, indicating that no correlation exists between seropositivity to DcCoV UAE-HKU23 and seropositivity to MERS-CoV. Furthermore, we found no correlation between seropositivity to DcCoV UAE-HKU23 and MERS-CoV antibody titers.

In this study, correlation between DcCoV UAE-HKU23 RT-PCR positivity and seropositivity also

Table 4. Detection of antibodies to MERS-CoV in dromedaries in the Middle East, 2013*

Test, antibody titer	No. (%) positive samples
Indirect immunofluorescence	
160	2 (3.4)
320	12 (20.3)
640	16 (27.1)
1280	11 (18.6)
2560	14 (23.7)
5120	4 (6.8)
Neutralization antibody test	
<10	1 (1.7)
10	7 (11.9)
20	14 (23.7)
40	28 (47.5)
80	8 (13.6)
160	1 (1.7)

*MERS-CoV, Middle East respiratory syndrome coronavirus.

Table 5. Estimates of nonsynonymous and synonymous substitution rates in the genomes of a novel betacoronavirus, DcCoV UAE-HKU23, discovered in dromedaries of the Middle East, 2013*

Gene	*Ka*	*Ks*	*Ka*/*Ks*
NSP1	0	0.004	0
NSP2	0	0.002	0
NSP3	0.001	0.006	0.167
NSP4	0.001	0.007	0.143
NSP5	0.001	0.003	0.333
NSP6	0.001	0.003	0.333
NSP7	0	0	–
NSP8	0	0.016	0
NSP9	0	0	–
NSP10	0	0	–
NSP11	0	0	–
NSP12	0	0.002	0
NSP13	0	0.002	0
NSP14	0.001	0	–
NSP15	0.001	0	–
NSP16	0	0	–
NS2	0.001	0.008	0.125
HE	0.001	0.002	0.5
Spike	0.001	0.004	0.25
NS5	0	0.009	0
Envelope	0	0	–
Membrane	0	0	–
Nucleocapsid	0	0	–
N2	0	0	–

DcCoV, dromedary camel coronavirus; *Ka*, nonsynonymous site; *Ks*, synonymous site; NSP, nonstructural protein; NS, nonstructural; HE, hemagglutinin esterase; N, nucleocapsid.

cannot be ascertained because the fecal samples and serum samples were collected from different dromedaries. Because MERS-CoV was not present in dromedaries in the present study, an intensive search in dromedaries and other animals in other locations in the Middle East would be helpful in the search for the animal source of MERS-CoV.

DcCoV UAE-HKU23 is a member of betacoronavirus A1 (Figure 7). Comparison of the amino acid identities of the 7 conserved replicase domains for species demarcation (i.e., ADP-ribose 1″-phosphatase, NSP5 [3CL^{pro}], NSP12 [RdRp], NSP13 [helicase], NSP14 [ExoN], NSP15 [NendoU], and NSP16 [O-MT]) (*36*) between DcCoV UAE-HKU23 and other CoVs of betacoronavirus A1 revealed that in all 7 domains, the amino acid sequences of DcCoV UAE-HKU23 and other betacoronavirus A1 members shared >90% identity. This finding indicates that DcCoV UAE-HKU23 should be a member of betacoronavirus A1.

Furthermore, the genome characteristics of DcCoV UAE-HKU23 showed features similar to those of other betacoronavirus A1 members. The genomes of all betacoronavirus A1 members have G+C contents of 0.37 and the genome of RbCoV HKU14, a recently discovered CoV closely related to betacoronavirus A1 (*19*), has a G+C content of 0.38. This G+C content differs substantially from those of other CoV species of lineage A betacoronaviruses, which have G+C contents of 0.32 (HCoV-HKU1), 0.41 (rat CoV) and 0.42 (murine hepatitis virus) (Table 2). The difference in the genome characteristics between A1 and non-A1 members of betacoronavirus is also reflected by their codon usage bias, in which the general preference of using G/C in the third position of the codons decreases from murine hepatitis virus and rat CoV to betacoronavirus A1 members and RbCoV HKU14 to HCoV-HKU1 (online Technical Appendix Figure 2). The cleavage site for spike protein of betacoronavirus A1 members is RRS/QRR, whereas those of HCoV-HKU1, RbCoV HKU14, and murine hepatitis virus are RRKRR, LRSRR, and RAR/H/DR/S, respectively. The length of membrane genes for betacoronavirus A1 members and RbCoV HKU14 is 693

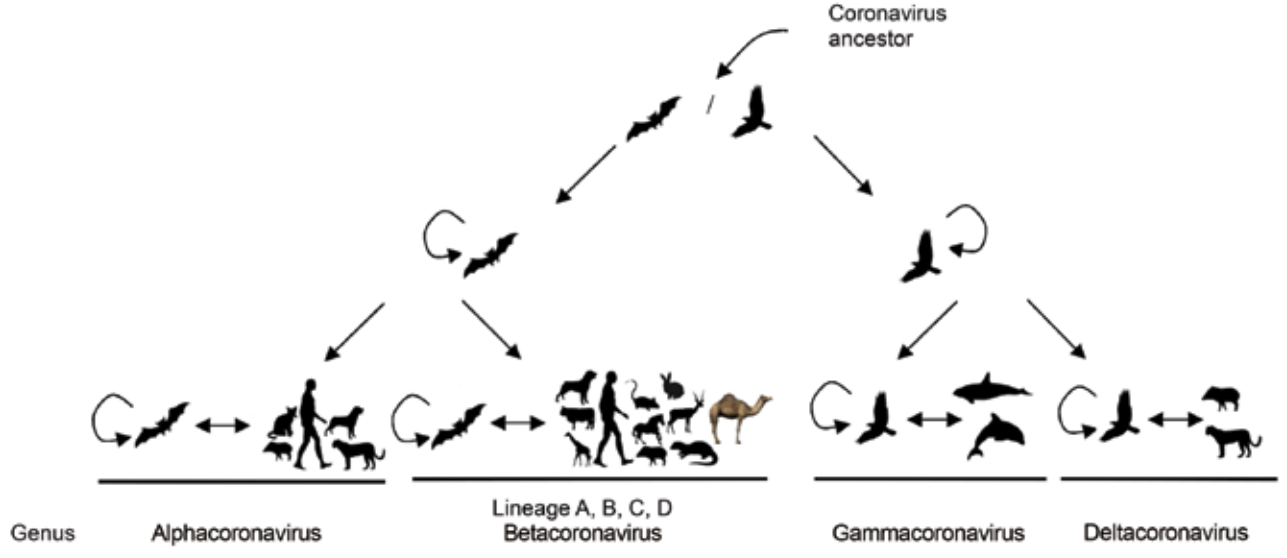

Figure 7. The evolution of coronaviruses from their ancestors in bat and bird hosts to new virus species that infect other animals.

bases, whereas the lengths for HCoV-HKU1 and murine hepatitis virus are 672 and 687 bases, respectively.

DcCoV UAE-HKU23 is phylogenetically closely related to other betacoronavirus A1 members, which in turn are closely related to other CoVs of betacoronavirus lineage A. Despite their close relationships, no recombination was detected between DcCoV UAE-HKU23 and other betacoronavirus A1 members by bootscan analysis (data not shown). These CoVs of betacoronavirus A1 may be using different receptors in their corresponding hosts because their spike protein is one of the proteins that show the largest difference among different CoVs. Most of the differences among the spike proteins in different betacoronavirus A1 members were also observed in the N terminal half of their spike protein, where the receptor binding domains should be located.

Camels are one of the most unique mammals on earth. In particular, they have shown perfect adaptation to desert life, which presents temperature extremes and a scarce supply of food and water. In the past, camels were used for transportation of humans and goods and for military uses. Moreover, for humans, they provide a good source of meat, milk, and wool. Camels are also important recreational animals in the Middle East and are used for camel racing. Having been associated with humans for at least 5,000 years, camels usually pose little physical danger to humans. However, infectious pathogens, such as brucellosis, can occasionally be transmitted from camels to humans. Apart from the present novel CoV, viruses of at least 8 taxonomic families (i.e., *Paramyxoviridae*, *Flaviviridae*, *Herpesviridae*, *Papillomaviridae*, *Picornaviridae*, *Poxviridae*, *Reoviridae*, and *Rhabdoviridae*) have been found to infect camels (*37*–*39*). Because camels are closely associated with humans, continuous surveillance of viruses in this hardy group of animals is needed to understand the potential for virus emergence and transmission to humans.

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Dr Woo is a professor and head of the Department of Microbiology at The University of Hong Kong. His research focuses on novel microbe discovery and microbial genomics.

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Address for correspondence: Patrick C.Y. Woo, State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology, University of Hong Kong, University Pathology Bldg, Queen Mary Hospital, Hong Kong, China; email: pcywoo@hkucc.hku.hk

Rotavirus Surveillance in Urban and Rural Areas of Niger, April 2010–March 2012

Anne-Laure Page, Viviane Jusot, Abdoul-Aziz Mamaty, Lagare Adamou, Jérôme Kaplon, Pierre Pothier, Ali Djibo, Mahamane L. Manzo, Brahima Toure, Céline Langendorf, Jean-Marc Collard, and Rebecca F. Grais

Knowledge of rotavirus epidemiology is necessary to make informed decisions about vaccine introduction and to evaluate vaccine impact. During April 2010–March 2012, rotavirus surveillance was conducted among 9,745 children <5 years of age in 14 hospitals/health centers in Niger, where rotavirus vaccine has not been introduced. Study participants had acute watery diarrhea and moderate to severe dehydration, and 20% of the children were enrolled in a nutrition program. Of the 9,745 children, 30.6% were rotavirus positive. Genotyping of a subset of positive samples showed a variety of genotypes during the first year, although G2P[4] predominated. G12 genotypes, including G12P[8], which has emerged as a predominant strain in western Africa, represented >80% of isolates during the second year. Hospitalization and death rates and severe dehydration among rotavirus case-patients did not differ during the 2 years. The emergence of G12P[8] warrants close attention to the characteristics of associated epidemics and possible prevention measures.

As the leading cause of severe gastroenteritis in children, rotavirus is responsible for ≈450,000 deaths each year among children <5 years of age, mainly in low-income countries (*1*,*2*). Two rotavirus vaccines that have been prequalified by the World Health Organization, Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium) and Rotateq (Merck, Whitehouse Station, NJ, USA), have been introduced widely in high- and middle-income countries, where their effect on rotavirus-related hospital admissions and deaths has been demonstrated (*3*). These vaccines are being introduced in several low-income countries in Africa and Asia, where their efficacy is lower (*4–6*) and impact data are limited (*7*).

Four rotavirus genotypes were historically recognized as predominant: G1P[8], G2P[4], G3P[8], and G4P[8]. These genotypes represented 88% of all rotavirus strains worldwide, and genotype G1P[8] has been responsible for >70% of the rotavirus infections in North America, Europe, and Australia (*8*). Since 2000, the prevalence of G1 strains has been declining, and other genotypes, such as G9 and G12, have emerged (*9*). In Africa, G12 strains were first detected in southern Africa, mostly in association with P[6] (*10–12*), and G12P[8] recently emerged as a predominant strain in western Africa (*13*,*14*) and some regions of Spain (*15*), Argentina (*16*), and the United States (*17*).

Because questions remain about vaccine efficacy and impact in low-resource settings, countries planning to introduce rotavirus vaccine must have knowledge of rotavirus epidemiology and circulating genotypes to evaluate the potential effect of vaccine programs. Niger is one of 34 countries approved by GAVI Alliance for financial support, but rotavirus vaccine has not yet been introduced in the country. We present rotavirus surveillance data for children <5 years of age in 14 hospitals and health centers in urban and rural Niger.

Patients and Methods

Study Sites

Gastroenteritis surveillance was conducted in 2 urban areas (Niamey, the capital city of Niger, and Maradi, the administrative center of the Maradi region) and in 3 rural districts (Madarounfa, Aguié, and Guidan Roumdji)

Author affiliations: Epicentre, Paris, France (A.-L. Page, C. Langendorf, R.F. Grais); Epicentre, Niamey, Niger (V. Jusot, A.-A. Mamaty, B. Toure); Centre de Recherche Médicale et Sanitaire (CERMES), Niamey (L. Adamou, J.-M. Collard); University Hospital of Dijon, Dijon, France (J. Kaplon, P. Pothier); Ministry of Health, Niamey (A. Djibo, M.L. Manzo); and Niamey University, Niamey (A. Djibo)

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in Maradi region, which is located ≈500 km from Niamey. Results of a 2009 survey of health-seeking behavior showed that hospital-based surveillance would capture <10% of severe diarrhea cases (*18*); thus, to capture more cases, we included hospitals and health centers in the study. Surveillance was implemented in several stages, beginning in December 2009 at 3 health centers in Madarounfa and at the regional hospital in Maradi. In 2010, a total of 10 other sites were added to the surveillance: in January, 3 health centers in Aguié were included; in February, 4 health centers in Guidan Roumdji were included; and in April, the 3 main hospitals in Niamey were included. After 1 year of surveillance, the number of sites was reduced for organizational and budgetary reasons. The analysis presented here is restricted to a 24-month period, April 2010–March 2012.

Study Population

Children 0–59 months of age were included in the study if they sought medical care at a study site, had watery diarrhea and signs of moderate or severe dehydration, and their parents or legal guardians accepted participation in the study. Children in Maradi region with reported bloody diarrhea were included during the second year, but they were excluded from this analysis.

Watery diarrhea was defined as ≥3 loose or liquid stools per day. Moderate and severe dehydration were defined on the basis of the child's general state and on thirst and ability to drink, sunken eyes, and skin-pinch assessment, according to the Integrated Management of Childhood Illness guidelines (*19*). The Vesikari score was calculated at the medical consultation or hospital admission (*20*). Vesikiari scores ≥11 were considered as severe.

Data Collection

A standardized questionnaire was used to obtain sociodemographic data, clinical signs and symptoms at the first medical visit, and outcomes. Enrolment of the child in a nutrition program was also recorded. If the child was transferred to another health facility, outcome information was obtained at the transfer site. If a child died at a health facility, the reported cause(s) of death, as assessed by the clinicians in charge, was recorded.

Specimen Collection and Rotavirus Assay

Fecal specimens were collected into sterile plastic containers or by using a rectal swab. We performed the Vikia Rota-Adeno rapid test (bioMérieux, Marcy l'Etoile, France) on site, following the manufacturer's recommendations.

Rotavirus-positive specimens and a sample of negative specimens were stored in a cool box and transported to the central laboratories in Maradi (every other day) and Niamey (every day), where they were aliquoted and stored at –20°C. At the central laboratories, the rapid test was repeated on ≈10% of the samples; good concordance was found with the original test results (κ 0.83). When discordant results were reported, training on use and interpretation of the rapid test was reinforced at the concerned study site(s).

Genotyping

For the first year of surveillance, a sample size of 420 specimens for genotyping was calculated to estimate the most frequent genotype expected at a 50% level with ±5% precision. For each 3-month period during the first year (April 1, 2010–March 31, 2011), a stratified random selection was used to select 70 and 35 specimens from Maradi region and Niamey, respectively, from the list of rotavirus-positive samples; selection was made regardless of the health facility of origin. A descriptive sample of 150 specimens (100 from the Maradi region, 50 from Niamey) was used to follow genotype evolution during the second year of surveillance (April 1, 2011–March 31, 2012). These samples were randomly selected separately for Niamey and Maradi at the end of the second year.

Rotavirus genotyping was conducted at CERMES (Niamey), according to the EuroRotaNet method (www.eurorota.net/docs.php), with the exception of the G12 primer. For the specimens that could not be genotyped, VP6 gene amplification was performed (*21*); the VP4 and/or VP7 first-round reverse transcription PCR (RT-PCR) products were sequenced directly at the National Reference Center for Enteric Viruses (Dijon, France) by using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit on a 3130XL DNA Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For the specific amplification of rotavirus G12 strains, a new set of primers (forward primer: G12Fcnr, 5′-GTTGTCGTCATACTGCCAT-3′, nt 169–187; reverse primer: G12Rcnr 5′-ATGAATTTTGGTACTGTATT-3′, nt 471–490) was designed on the basis of the VP7 coding sequences of G12 strains from Niger and other countries.

Statistical Analysis

We used EpiData version 3.1 (EpiData, Odense, Denmark) for double data entry and Stata version 12.1 (College Station, TX, USA) for data analysis. We performed a weighted analysis by month and region of study inclusion to extrapolate the results of genotyping of a random subset of rotavirus-positive fecal samples to the population of rotavirus-positive patients.

Ethical Considerations

Ethical approval was granted by the National Ethics Committee of Niger (reference no. 02/2009/CCNE) and the Comité de Protection des Personnes, Ile de France XI, Saint-Germain en Laye, France. Written informed consent

was obtained from each participant's parent or legal guardian; study participation was voluntary.

Results

Characteristics of the Study Population

In total, 12,355 children with diarrhea and dehydration sought care at the study sites during April 2010–March 2012, of whom 2,038 (16%) were not included in the study for the following reasons: seeking care outside of the study hours, 1,183 (9.6%) children; reporting bloody diarrhea, 837 (6.8%) children; and refusing study participation, 18 (0.1%) children. We further excluded 270 children with bloody diarrhea, 35 children with who were not tested for rotavirus, 127 children with a delay of >3 days between hospital admission and testing, 134 children who sought care ≥14 days after onset of diarrhea, and 4 children without clinical signs of dehydration. Thus, we included 9,747 children in the analysis. Although sociodemographic characteristics for the children were similar between districts, other characteristics (e.g., degree of dehydration, receipt of intravenous treatment, and percentage hospitalized) differed because of differences in the level of care between study sites (i.e., hospital vs. health centers) (Table 1).

Proportion of Rotavirus-associated Gastroenteritis

Overall, 2,982 (30.6%) children were positive for rotavirus. The percentage of positive study participants varied substantially across sites, especially during the first year (Table 2). Overall and after stratifying by type of site (hospital vs. health center) and for children included in a nutrition program, the percentage of rotavirus-positive cases was lower among hospitalized patients, those with severe dehydration, and those included in a nutrition program (Table 3).

The percentage of rotavirus-positive specimens was lower among children from whom rectal swab samples (24.4%, 725/2,974) rather than stools samples (33.3%, 2,253/6,765) were obtained ($p<0.001$). This variation could not explain the difference in the percentage of positive cases by site and by clinical characteristics because these differences remained when the analysis was restricted to patients from whom stool samples (not rectal swab samples) were obtained (Tables 2, 3).

Children <1 year of age represented 67.4% of all diarrhea cases and 79.1% of rotavirus-positive cases (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/20/4/13-1328-Techapp1.pdf). More specifically, children <6 months of age accounted for 16.9% of all rotavirus-positive cases, and those 6–11 months of age accounted for 62.2%.

Rotavirus infections occurred year-round, and there was a consistent peak in October–November in Maradi region and in November–December in Niamey (online Technical Appendix Figure 2). The 2011 peaks in March and April in Guidan Roumdji and Madarounfa, respectively, were not observed in 2010, and only a small increase was seen in the first trimester of 2012.

Clinical Signs and Severity

The percentage of children with vomiting was consistently higher among rotavirus-infected than noninfected children, but the percentage with severe dehydration was lower (Table 4). The median Vesikari score was almost 1 point higher in children with rotavirus (Table 4).

Table 1. Sociodemographic and clinical and treatment characteristics of children in a rotavirus surveillance study in Niamey and Maradi region, Niger, April 2010–March 2012*

		Children, by location				
			Maradi region, district			
Characteristic	Total, N = 9,747	Niamey, n = 1,196	Maradi, n = 962	Madarounfa, n = 2,965	Aguie, n = 748	Guidan Roumdji, n = 3,876
Sex						
F	4,329 (44.4)	481 (40.2)	442 (46.0)	1,401 (47.3)	309 (41.3)	1,696 (43.8)
M	5,416 (55.6)	715 (59.8)	518 (54.0)	1,564(52.8)	439 (58.7)	2,180 (56.2)
Age, mo, median (IQR)	9 (7–12)	9 (6–12)	9 (6–14)	9 (7–12)	8 (6–11)	10 (7–13)
Type of sample collected						
Stool	6,765 (69.5)	678 (56.7)	749 (77.9)	2,224 (75.1)	665 (88.9)	2,449 (63.3)
Rectal swab	2,974 (30.5)	518 (43.3)	213 (22.1)	739 (24.9)	83 (11.1)	1,421 (36.7)
Clinical signs/symptoms						
Severe dehydration	1,976 (20.3)	724 (60.5)	382 (39.8)	158 (5.3)	175 (23.4)	537 (13.9)
Fever	2,360 (24.2)	410 (34.3)	290 (30.2)	593 (20.0)	251 (33.6)	816 (21.1)
Vomiting	6,499 (66.8)	818 (68.6)	353 (36.7)	2,146 (72.5)	545 (72.9)	2,637 (68.3)
Severe Vesikari score rating	7,156 (73.5)	963 (80.6)	631 (65.7)	2,227 (75.2)	563 (75.5)	2,772 (71.7)
IV treatment received	1,392 (14.3)	599 (50.1)	577 (60.0)	56 (1.9)	29 (3.9)	131 (3.4)
Hospitalized	2,529 (26.0)	917 (76.7)	924 (96.2)	153 (5.2)	57 (7.6)	478 (12.3)
Enrolled in nutrition program	2,046 (21.0)	396 (33.2)	539 (56.0)	314 (11.0)	93 (12.4)	704 (18.2)
Died	255 (2.6)	119 (10.0)	48 (5.0)	50 (1.7)	16 (2.1)	22 (0.6)

*Data are no. (%) unless otherwise indicated. IQR, interquartile range; IV, intravenous.

Table 2. Number of rotavirus-positive study participants identified during a 2-year surveillance study in Niamey and Maradi region, Niger, April 2010–March 2012*

Variable	April 2010–March 2011		April 2011–March 2012	
	No. positive/no. total (%)	95% CI	No. positive/no. total (%)	95% CI
Study site				
All areas	1,714/5,845 (29.3)	28.2–30.5	1,268/3,902 (32.5)	31.0–34.0
Niamey†	180/796 (22.6)	19.7–25.5	148/478 (32.3)	27.7–36.8
Maradi region, district				
Maradi	95/484 (19.6)	16.1–23.2	105/478 (22.0)	18.2–25.7
Madarounfa	611/1,711 (35.7)	33.4–38.0	478/1,254 (38.1)	35.4–40.8
Aguie	252/748 (33.7)	30.3–37.1	NA	NA
Guidan Roumdji	576/2,106 (27.4)	25.5–29.3	556/1,770 (31.4)	29.2–33.6
Patients with stool samples tested	1,312/4,074 (32.2)	30.8–33.6	941/2,691 (34.9)	33.2–36.8
Niamey†	110/409 (26.9)	22.6–31.2	100/269 (37.1)	31.4–42.9
Maradi region, district				
Maradi	80/349 (22.9)	18.5–27.3	95/400 (23.8)	19.6–27.9
Madarounfa	485/1,293 (37.5)	34.9–40.2	364/931 (39.1)	36.0–42.2
Aguie	231/665 (34.7)	31.1–38.4	NA	NA
Guidan Roumdji	406/1,358 (29.9)	27.5–32.3	382/1,091 (35.0)	32.2–37.9

*NA, not applicable because surveillance was interrupted in all study sites in the district of Aguie at the end of the first year.
†Surveillance was interrupted in 1 hospital in Niamey at the end of the first year.

Of the 9,747 study participants, 255 (2.6%) died in health facilities; 34 (13.3%; 95% CI 9.1%–17.5%) of those who died were rotavirus positive (Table 4). The most frequently cited causes of death among all patients who died were diarrhea or dehydration (124 [48.6%] patients; 95% CI 42.4%–54.8%) and malnutrition (109 [42.7%] patients; 95% CI 36.6%–48.9%). Of the 124 study participants with diarrhea and/or dehydration cited as a cause of death, 23 (18.9%) were positive for rotavirus.

Rotavirus Genotypes

A total of 570 fecal samples that were positive for rotavirus by the rapid test were randomly selected for genotyping. Two of these specimens were lost, 3 were excluded because the delay between patient hospital admission and specimen collection was >3 days, and 2 were excluded because the patients had bloody diarrhea. Of the remaining 563 samples, 58 (10.3%) were rotavirus negative by RT-PCR and 56 were excluded because they were collected before April 2010; the latter group of samples comprised 15 G2P[6], 14 G1P[8], 12 G2P[4], 3 G12P[8], 10 mixed infection, and 2 partially typed samples.

A variety of G and P combinations were detected during the study, but G12P[8] and G2P[4] were the most prevalent rotavirus strains (Table 5). The seasonal distribution of these 2 genotypes extrapolated to all rotavirus-positive

Table 3. Number and percentage of rotavirus-positive study participants in a surveillance study in urban and rural areas of Niger, April 2010–March 2012

Variable	Urban and rural, N = 9,747		Urban, n = 2,158*		Rural, n = 7,589†	
	No. positive/ no. total (%)	95% CI	No. positive/ no. total (%)	95% CI	No. positive/ no. total (%)	95% CI
All patients	2,982 (30.6)	29.7–31.5	509 (23.6)	21.8–25.4	2,473 (32.6)	31.5–33.6
Hospitalization status						
Hospitalized	628/2,529 (24.8)	23.1–26.5	428/1,841 (23.2)	21.3–25.2	200/688 (29.1)	25.7–32.5
Not hospitalized	2,353/7,217 (32.6)	31.5–33.7	80/316 (25.3)	20.5–30.1	2,273/6,901 (32.9)	31.8–34.0
Dehydration status						
Severe	491/1,976 (24.8)	22.9–26.8	230/1,106 (20.8)	18.4–23.2	261/870 (30.0)	27.0–33.0
Moderate	2,490/7,770 (32.0)	31.0–33.1	278/1,051 (26.5)	23.8–29.1	2,212/6,719 (32.9)	31.8–34.0
Nutrition program status						
Enrolled	415/2,046 (20.3)	18.5–22.0	149/935 (15.9)	13.6–18.3	266/1,111 (23.9)	21.4–26.5
Not enrolled	2,564/7,687 (33.4)	32.3–34.4	359/1,221 (29.4)	26.8–32.0	2,205/6,466 (34.1)	32.9–35.3
Patients with stool sample tested	2,253/6,765 (33.3)	32.2–34.4	385/1,427 (27.0)	24.7–29.3	1,868/5,338 (35.0)	33.7–36.3
Hospitalization status						
Hospitalized	456/1,669 (27.3)	25.2–29.5	336/1,274 (26.4)	24.0–28.8	120/395 (30.4)	25.8–34.9
Not hospitalized	1,796/5,095 (35.3)	33.9–36.6	48/152 (31.6)	24.2–39.0	1,748/4,943 (35.3)	34.0–36.7
Dehydration status						
Severe	356/1,269 (28.1)	25.6–30.5	171/713 (24.0)	20.8–27.1	185/556 (33.3)	29.4–37.2
Moderate	1,896/5,495 (34.5)	33.2–35.8	213/713 (29.9)	26.5–33.2	1,683/4,782 (35.2)	33.8–36.5
Nutrition program status						
Enrolled	265/1,235 (21.5)	19.2–23.7	111/613 (18.1)	15.1–21.2	154/622 (24.8)	21.4–28.2
Not enrolled	1,985/5,521 (36.0)	34.7–37.2	273/813 (33.6)	30.3–21.2	1,712/4,708 (36.4)	35.0–37.7

*Hospitals in Niamey and Maradi.
†Health centers in Madarounfa, Guidan Roumdji, and Aguie districts.

Table 4. Age and clinical characteristics of study participants in a 2-year rotavirus surveillance study in urban and rural areas of Niger, April 2010–March 2012*

	All participants, April 2010–March 2012, N = 9,747			RV-positive participants, n = 2,982		
Patient variable	RV negative	RV positive	p value	First year†	Second year‡	p value
Age, mo, mean (± SD)	11.8 (6.9)	8.8 (4.4)	0.0001	9.1 (4.2)	8.4 (3.9)	0.0001
No. stools in 24-h, mean (± SD)	6.1 (1.7)	6.4 (1.8)	0.0001	6.4 (1.9)	6.4 (1.7)	0.76
Vomiting present, % (95% CI)	60.0 (58.9–61.2)	82.3 (80.9–83.7)	<0.001	81.8 (80.0–83.6)	83.0 (81.0–85.1)	0.37
No. vomiting episodes in 24-h, mean (± SD)	3.8 (1.9)	4.5 (2.8)	0.0001	4.6 (2.2)	4.4 (3.5)	0.012
Severe dehydration present, % (95% CI)	22.0 (21.0–22.9)	16.5 (15.1–17.8)	<0.001	17.2 (15.4–18.9)	15.5 (13.6–17.5)	0.23
Fever present, % (95% CI)	24.4 (23.4–25.5)	23.7 (22.1–25.2)	0.2	25.9 (23.9–28.0)	20.6 (18.4–22.8)	0.001
Vesikari score, mean (± SD)	12.1 (2.7)	12.9 (2.2)	0.0001	13.0 (2.2)	12.9 (2.1)	0.057
Hospitalized, % (95% CI)	28.3 (27.2–29.3)	20.8 (19.4–22.2)	<0.001	22.9 (20.9–24.9)	18.5 (16.4–20.7)	0.003
Died, % (95% CI)	3.3 (2.8–3.7)	1.1 (0.8–1.5)	<0.001	1.3 (0.8–1.9)	0.9 (0.4–1.4)	0.2

*RV, rotavirus.
†April 2010–March 2011.
‡April 2011–March 2012.
Vesikari score ≥11 indicates severe diarrhea.

patients showed a clear shift to predominance of G12P[8] during the second year (Figure).

Comparison of First- and Second-Year Characteristics

Because rotavirus diarrhea was due to a variety of genotypes during the first year and primarily to G12 genotype during the second year, we compared characteristics of rotavirus cases for the 2 years to detect differences that could be linked to the G12 genotype. Although the percentage of rotavirus infections among study participants was substantially higher during the second year, the total number of rotavirus cases, and thus incidence of infection, was not higher in districts where the number of surveillance sites did not change during the 2 years (Table 2). Rotavirus-infected patients during the second year were significantly younger than those during the first year, but clinical characteristics for patients were similar during the 2 years, except for a lower percentage of patients with fever and a lower percentage of patient hospitalizations during the second year (Table 4).

Discussion

The results of this large study on the epidemiology of rotavirus in urban and rural settings in Niger confirm the high morbidity rate for rotavirus in this area, particularly among children <1 year of age and during the dry and cool season. This 2-year study captured the emergence of G12P[8] strains, which are emerging in several areas of the world (*9*,*13*–*16*).

The emergence of G12P[8] warrants close attention to the characteristics of associated epidemics and possible

Table 5. Weighted analysis of rotavirus genotypes identified during a 2-year surveillance study in Niamey and Maradi region, Niger, April 2010–March 2012

	April 2010–March 2011				April 2011–March 2012			
Genotype and	Niamey, n = 121		Maradi region, n = 194		Niamey, n = 49		Maradi region, n = 85	
G- and P-type	No. (%)	95% CI	No. (%)	95% CI	No. (%)	95% CI	No. (%)	95% CI
Genotype								
G1P[8]	6 (4.8)	2.1–10.5	3 (1.8)	0.6–5.7	1 (1.8)	0.2–12.4	4 (3.8)	1.3–10.6
G2P[4]	30 (25.1)	18.0–33.9	137 (60.5)	51.6–68.8	0	–	0	–
G2P[6]	9 (6.2)	3.2–11.6	5 (3.7)	9.0–1.6	0	0	2 (2.7)	0.6–10.3
G6P[6]	12 (9.4)	5.4–16.1	0	–	1 (1.3)	0.2–9.2	0	–
G9P[8]	8 (5.9)	2.9–11.6	7 (5.9)	2.8–11.9	0	–	1 (1.3)	0.2–8.9
G12P[8]	36 (31.4)	23.4–40.5	19 (10.9)	6.6–17.5	43 (89.2)	76.3–95.5	56 (64.1)	52.4–74.4
Others*	15 (13.2)	8.0–21.0	13 (10.0)	5.4–17.8	2 (2.7)	0.6–11.4	9 (12.3)	6.1–23.3
Mixed	5 (4.0)	1.6–9.6	10 (7.1)	3.5–13.7	2 (5.1)	1.2–19.3	13 (15.8)	9.1–26.1
G-type†								
G1	10 (8.1)	4.3–14.7	4 (2.7)	1.0–7.1	2 (4.8)	1.1–18.7	4 (3.7)	1.3–10.6
G2	51 (41.9)	33.2–51.1	148 (66.5)	57.5–74.4	0	–	6 (7.9)	3.5–16.7
G3	3 (2.7)	0.8–8.5	17 (16.0)	10.0–24.7	0	–	5 (6.0)	2.4–14.3
G6	13 (10.3)	6.0–17.2	1 (0.1)	0.0–1.0	2 (3.0)	0.7–12.3	0	–
G9	10 (7.2)	3.8–13.1	13 (11.1)	6.4–18.5	1 (2.1)	0.3–14.2	9 (10.0)	5.0–18.7
G12	36 (31.4)	23.4–40.5	20 (11.1)	6.8–17.6	46 (95.2)	85.3–98.5	71 (82.8)	72.3–89.9
P-type†								
P[4]	32 (27.0)	19.7–35.9	145 (67.8)	59.1–75.4	0	–	0	–
P[6]	27 (20.1)	13.9–28.0	14 (9.7)	5.6–16.2	2 (2.2)	0.5–9.0	13 (17.6)	10.1–28.9
P[8]	63 (53.7)	44.6–62.6	38 (23.6)	16.9–31.9	47 (97.8)	91.0–99.5	72 (83.7)	72.9–90.7

*Including G1P[6] (2), G2P[8] (9), G3P[4] (5), G3P[6] (7), G3P[8] (2), G6P[8] (3), and G12P[6] (7).
†Mixed infections counted in each G- or P-type found, potentially leading to a total number greater than N.

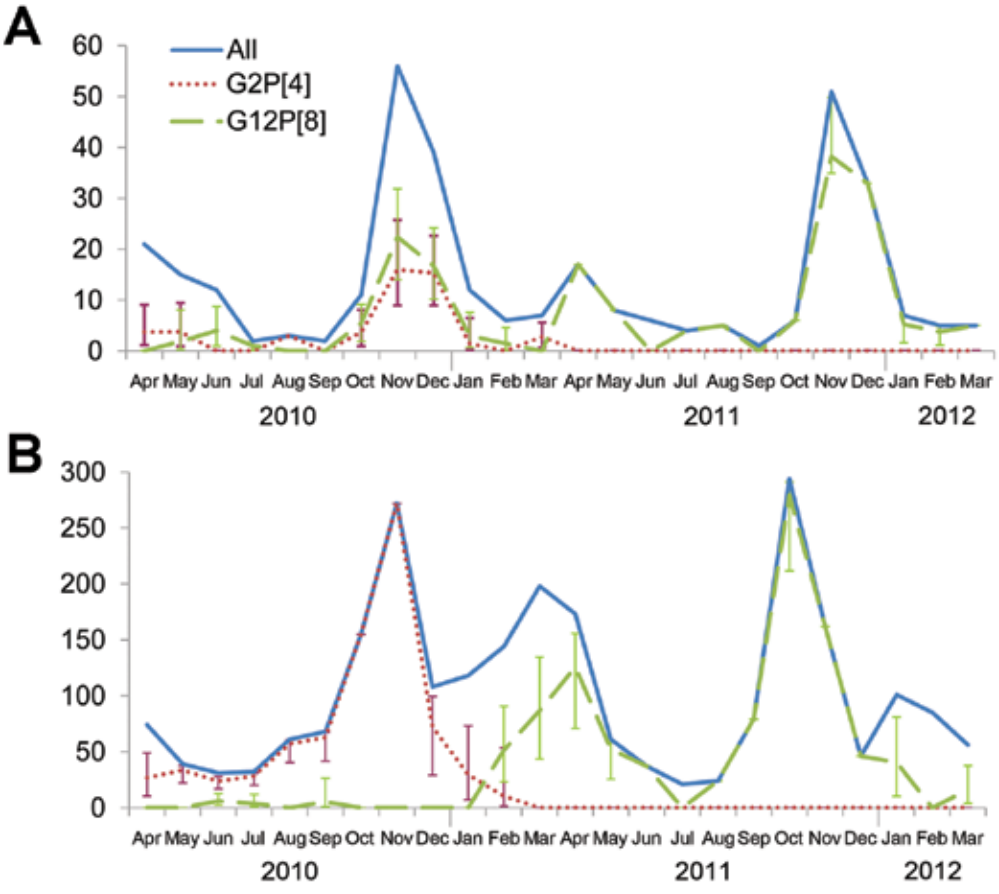

Figure. Number of rotavirus cases and extrapolated number of the 2 most frequent genotypes, G2P[4] and G12P[8], identified each month during a 2-year surveillance study in urban and rural areas of Niger, April 2010–March 2012. A) Cases in Niamey, the capital of Niger. B) Cases in Maradi region. Vertical bars indicate CIs.

prevention measures. Although only available for Rotarix vaccine, the first data on the efficacy of vaccine against rotavirus G12 strains in the clinical trials in Africa suggest that the vaccine provides heterotypic protection, although confidence intervals are wide (*22*). The lower mean age of rotavirus-infected children during the G12P[8] season, compared with the mean age during the previous year, emphasizes the need for early vaccination. A lower protection provided by maternal antibodies acquired transplacentally or through breast-feeding might partly explain the lower mean age of infection during the second year. Other characteristics of the rotavirus cases during the second year of the study suggested that G12P[8]-associated diarrhea might be clinically slightly less severe than diarrhea caused by other strains. Only 1 previous study compared characteristics of a G12 epidemic with characteristics of previous epidemics in which G1 or G9 strains were dominant, and no difference in mean age and hospitalization rates was found (*15*).

In the context of our study, rotavirus infection was not associated with severity criteria, except for the Vesikari score. In particular, severe dehydration, hospitalizations, and deaths were less frequent among children with than without rotavirus. One factor that might explain this result is the high prevalence of acute malnutrition; this factor was associated with a higher proportion of study participants who were hospitalized or who had severe dehydration but with a lower proportion of study participants with rotavirus infection. The lower percentage of rotavirus cases among children who were included in nutrition programs does not imply a lower incidence of rotavirus among these children. A study in South Africa reported that the percentage of rotavirus cases among HIV-infected children with diarrhea was lower than the percentage among non–HIV-infected children with diarrhea; however, when the overall incidence of acute gastroenteritis was taken into account, the incidence of rotavirus in HIV-infected children was 2.3-fold higher than in non–HIV-infected children (*23*). More precise data on the incidence and natural history of rotavirus infection and on vaccine efficacy in children with severe acute malnutrition are needed. Diarrhea-associated mortality data are also needed because the direct extrapolation of the percentage of rotavirus-associated diarrhea cases to diarrhea-associated deaths may not be relevant, particularly in contexts with high levels of malnutrition (*24*).

Our study has several limitations. First, malnutrition status was monitored only through the study participant inclusion in a nutrition program. In addition, children with

severe acute malnutrition can show signs and symptoms similar to those of dehydration. However, in this study, the duration of diarrhea, the number of watery stools, and the Vesikari score were similar in children with or without malnutrition (data not shown), suggesting that dehydration was truly associated with diarrhea. Second, the rapid test used in this study is not ideal, as suggested by the fact that 10.3% of the rotavirus-positive stool samples selected for genotyping were negative by RT-PCR. The rapid test was chosen for practical reasons and for its good performance in initial evaluations (*25*,*26*), and the test had similar sensitivity but lower specificity than the Premier Rotaclone immunoassay (Meridian Bioscience, Inc., Cincinnati, Ohio, USA) in a post hoc evaluation (A.-L. Page, unpub. data). The lower test specificity might have led to a slightly higher estimate than if an immunoassay had been used. The last limitation is that the sensitivity of rotavirus detection was lower among patients for whom rectal swab samples rather than stool samples were used for testing; the difference could reflect the lower sensitivity of rectal swab sample testing. This lower sensitivity might have led to underestimating the percentage of rotavirus cases by ≈3% overall and by a slightly higher percent in Niamey and Guidan Roumdji, where the proportion of patients tested by using rectal swab samples was higher than in other study areas.

Rotavirus is a major cause of diarrhea with dehydration in Niger, but it is not associated with severity criteria (e.g., hospitalization, severe dehydration, and death), probably because of the large number of children with malnutrition. The emergence of G12P[8] in Niger and other areas of Africa indicates the complex natural evolution of rotavirus strains, even in the absence of any external pressure (e.g., vaccination), and the need for continuous surveillance. Better data on the efficacy and effect of existing and new rotavirus vaccines against currently circulating strains in countries with a high prevalence of malnutrition are crucially needed before the nationwide introduction of vaccine in these countries.

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Dr Page is a biologist and epidemiologist at Epicentre/Médecins Sans Frontières, Paris, France. Her primary research interests are the etiology and diagnosis of infectious diseases.

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Address for correspondence: Anne-Laure Page, Epicentre, 8 Rue Saint-Sabin, 75011 Paris, France; email: anne-laure.page@epicentre.msf.org

Large Outbreak of *Cryptosporidium hominis* Infection Transmitted through the Public Water Supply, Sweden

Micael Widerström, Caroline Schönning, Mikael Lilja, Marianne Lebbad, Thomas Ljung, Görel Allestam, Martin Ferm, Britta Björkholm, Anette Hansen, Jari Hiltula, Jonas Långmark, Margareta Löfdahl, Maria Omberg, Christina Reuterwall, Eva Samuelsson, Katarina Widgren, Anders Wallensten, and Johan Lindh

In November 2010, ≈27,000 (≈45%) inhabitants of Östersund, Sweden, were affected by a waterborne outbreak of cryptosporidiosis. The outbreak was characterized by a rapid onset and high attack rate, especially among young and middle-aged persons. Young age, number of infected family members, amount of water consumed daily, and gluten intolerance were identified as risk factors for acquiring cryptosporidiosis. Also, chronic intestinal disease and young age were significantly associated with prolonged diarrhea. Identification of *Cryptosporidium hominis* subtype IbA10G2 in human and environmental samples and consistently low numbers of oocysts in drinking water confirmed insufficient reduction of parasites by the municipal water treatment plant. The current outbreak shows that use of inadequate microbial barriers at water treatment plants can have serious consequences for public health. This risk can be minimized by optimizing control of raw water quality and employing multiple barriers that remove or inactivate all groups of pathogens.

Protozoan parasites of the genus *Cryptosporidium* can cause gastrointestinal illness in humans and animals (*1*). Twenty-six species and >60 genotypes have been identified (*2*). *C. parvum* and *C. hominis* are the most prevalent species that infect humans (*1*,*3*). Cryptosporidiosis is transmitted mainly by the fecal–oral route, usually through oocyst-contaminated water or food or by direct contact with an infected person or animal (*2*). Infectivity is dose dependent and certain subtypes are apparently more virulent, requiring only a few oocysts to establish infection (*1*,*4*). In healthy persons, gastrointestinal symptoms usually resolve spontaneously within 1–2 weeks, although asymptomatic carriage can occur (*2*). Nonetheless, in immunocompromised patients, severe life-threatening watery diarrhea can develop (*2*). Information is limited regarding the long-term effects of *Cryptosporidium* infection (*3*,*5*,*6*).

The global incidence of cryptosporidiosis is largely unknown, although the disease was recently identified as one of the major causes of moderate to severe diarrhea in children <5 years of age in low-income countries (*7*). In Sweden, cryptosporidiosis has been a notifiable disease since 2004, and ≈150 cases (≈1.7/100,000 population/year) were reported annually until 2009. However, cryptosporidiosis is probably underreported, mainly because sampling from patients with gastrointestinal symptoms and requests for diagnostic tests are insufficient (*3*,*8*).

Because of some inherent characteristics of the pathogen, *Cryptosporidium* infection has critical public health implications for drinking water and recreational waters. The oocysts are excreted in large numbers in feces, can survive for months in the environment (*5*), and are resistant to the concentrations of chlorine commonly used to treat drinking water (*9*). The first reported outbreak of waterborne human cryptosporidiosis occurred in the United States in 1984 (*10*), and since then, numerous outbreaks involving up to hundreds of persons have been identified in several parts of the world (*11*,*12*). However, only a few very large outbreaks have been documented (*13*–*15*); the most extensive occurred in 1993 in Milwaukee, Wisconsin, USA, in which ≈400,000 persons were infected with *Cryptosporidium* oocysts by drinking water from a

Author affiliations: Umeå University, Umeå, Sweden (M. Widerström, M. Lilja, M. Ferm, C. Reuterwall, E. Samuelsson); Jämtland County Council, Östersund, Sweden (M. Widerström, M. Omberg); Public Health Agency of Sweden, Solna, Sweden (C. Schönning, M. Lebbad, G. Allestam, B. Björkholm, A. Hansen, J. Långmark, M. Löfdahl, K. Widgren, A. Wallensten, J. Lindh); Mid Sweden University, Östersund (T. Ljung); Östersund Municipality, Östersund (J. Hitula); and Karolinska Institutet, Stockholm (J. Lindh)

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water treatment plant (WTP) (*14*). *Cryptosporidium* spp. are the predominant protozoan parasites causing waterborne outbreaks worldwide (*11*). In 2012, an increase in *Cryptosporidium* infections, particularly by *C. hominis* IbA10G2, was reported in Europe (*16*).

In Sweden, only 1 drinking water outbreak involving *Cryptosporidium* has been recognized (Y. Andersson, pers. comm.), and a *C. parvum* outbreak associated with fecal contamination of a public swimming pool occurred in 2002 and affected ≈1,000 persons (*17*). A study of *Cryptosporidium* species and subtypes isolated from samples from 194 patients in Sweden during 2006–2008 identified 111 *C. parvum* infections and 65 *C. hominis* infections. Most patients with *C. hominis* infection had been infected abroad, and only 3 were considered to have sporadic domestic infections (*3*). A recent investigation of *Cryptosporidium* in raw water from 7 large WTPs in Sweden (not including the WTP of interest in the present study) identified 23 (11.5%) of 200 positive samples containing 1–30 oocysts/10 L, although neither species nor subtypes were analyzed (*18*).

The city of Östersund is located in central Sweden and has a population of ≈60,000. The major WTP in Östersund (WTP-Ö) draws surface water from nearby Lake Storsjön and supplies drinking water to ≈51,000 of the city's inhabitants. At the time of the onset of the outbreak reported here, the purification process at WTP-Ö included preozonation, flocculation, and sedimentation, followed by rapid sand filtering and chloramination. WTP-Ö is situated 4 km upstream from the major wastewater treatment plant (WWTP-Ö) to ensure that the drinking water intake will not be affected by the wastewater outlet (Figure 1).

In late November 2010, the County Medical Office in Östersund received reports from several employers that 10%–20% of employees had gastroenteritis. The office advised that patients with acute gastroenteritis be tested for bacterial, viral, and protozoan pathogens. Among 20 patients from whom samples were obtained, 14 cases of cryptosporidiosis were detected on November 26. The local health advice line received numerous calls from persons with gastroenteritis, most of whom lived within the municipality (*19*). These facts indicated that the outbreak could be traced to the drinking water, and thus a boil-water advisory was issued for the municipality on November 26. This study describes the outbreak investigation and outlines the extent of the outbreak, clinical characteristics of persons infected, and risk factors for acquiring cryptosporidiosis.

Methods

Epidemiologic Investigation

Electronic Survey

To estimate the extent of the outbreak, the municipality published a questionnaire on its website during November 27–December 13, 2010. Persons in Östersund who

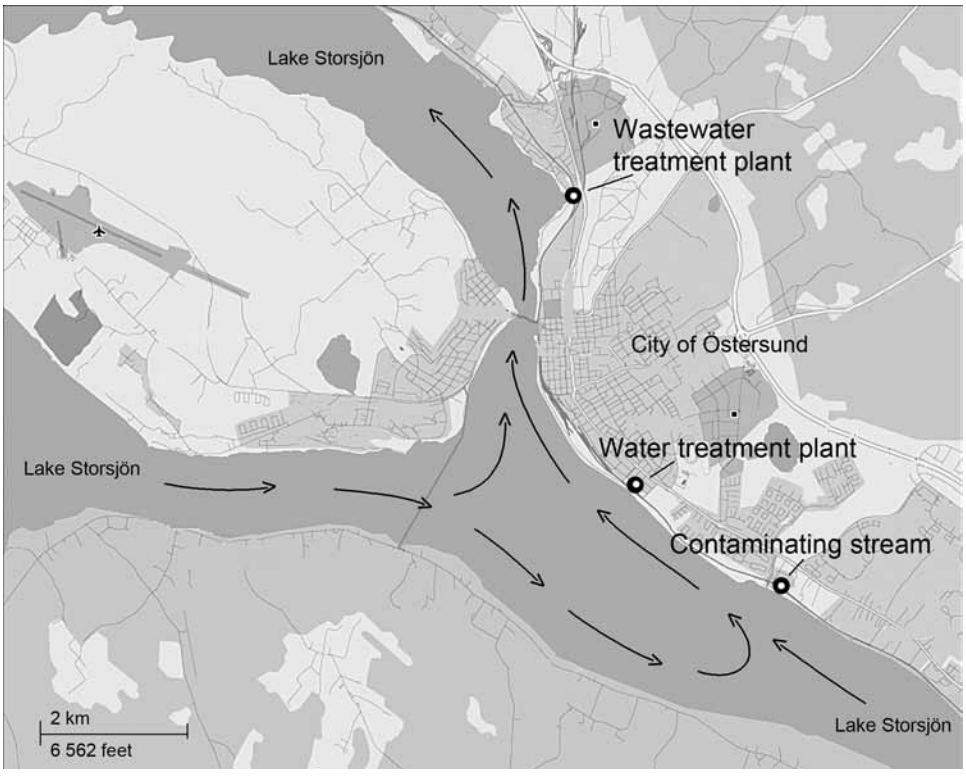

Figure 1. Map of Lake Storsjön, showing water currents (arrows) and locations of wastewater treatment plant, water treatment plant, and contaminating stream during *Cryptosporidium* infection outbreak, Östersund, Sweden, 2010–2011.

had gastrointestinal symptoms were encouraged to provide information about day of onset, home address, and recent food intake.

Written Questionnaire

Two months after the outbreak began, we conducted a retrospective cohort study, which included a random sample of 1,524 persons living in Östersund, to assess the extent of the outbreak, clinical characteristics of infected persons, and risk factors for acquiring cryptosporidiosis.

We estimated the proportion infected among the population of Östersund with a 3% margin of error (95% CI) by assuming a 50% attack rate and a 70% response rate when calculating the sample size. The patient questionnaire contained items on demographic characteristics, onset and occurrence of possible symptoms of cryptosporidiosis, water consumption, underlying diseases, and whether the WTP-Ö supplied water to the person's workplace. Residential WTP supply was ascertained through population registers. Parents or guardians were asked to respond for children <15 years of age. A case-patient was defined as a person who lived in Östersund in mid-January 2011 and had had ≥3 episodes of diarrhea daily and/or watery diarrhea with onset after November 1, 2010, and before January 31, 2011. The study was approved by the Research Ethics Committee of the Faculty of Medicine, Umeå University, Umeå, Sweden.

Microbiological Investigation

Human Samples

From November 1, 2010, through January 31, 2011, fecal samples from inhabitants of Östersund who had acute gastroenteritis were tested for various pathogens. *Cryptosporidium* oocysts were analyzed by standard concentration techniques and modified Ziehl-Neelsen staining (*20*); enteric bacterial pathogens by standard methods; noroviruses and sapoviruses by PCR; and *Entamoeba* spp. and *Giardia duodenalis* by conventional light microscopy.

Environmental Samples

During the outbreak, 163 samples of drinking water, raw water, and wastewater were collected to trace the source and monitor the presence of oocysts. Most water samples were collected at or near WTP-Ö and at WWTP-Ö. However, as the outbreak spread to nearby regions, sampling was also conducted at 14 other WTPs and 6 additional WWTPs. The municipality identified 4 different streams with high counts of *Escherichia coli* that may have contaminated the raw water, and samples from those streams were analyzed for *Cryptosporidium*. Also, as part of a then-ongoing national survey regarding presence of parasites in wastewater, 7 preoutbreak samples were collected at WWTP-Ö. The methods used are described in the online Technical Appendix (wwwnc.cdc.gov/EID/article/20/4/12-1415-Techapp1.pdf).

Molecular Analysis/Typing

In a subset of fecal samples, *Cryptosporidium* species were determined by PCR–restriction fragment-length polymorphism analysis of the 18S rRNA gene (*21*). Species were further characterized by sequence analysis of the 60-kDa glycoprotein (*gp60*) gene (*22*).

Oocysts in wastewater and stream water samples were isolated from the contaminating debris by immunomagnetic separation (IMS), and DNA was extracted (online Technical Appendix). DNA was also extracted from oocysts that had been obtained from 1 raw water sample and 1 drinking water sample by use of Envirochek filters (Pall Life Science, Ann Arbor, MI, USA) followed by IMS. Microscope slides containing 1–13 oocysts from 4 raw water samples and 4 drinking water samples were sent to the Cryptosporidium Reference Unit, Swansea, United Kingdom (online Technical Appendix), where molecular analyses were performed.

Statistical Analysis

We conducted statistical analyses to test associations between risk factors and duration of diarrhea after controlling for age, sex, and residence in the area served by WTP-Ö. Student *t* test was used to analyze differences in attack rate and relapse rate. Relationships between risk factors and clinical cryptosporidiosis as the outcome variable were investigated by logistic regression. For dichotomous predictors, odds ratios were used to measure associations between clinical cryptosporidiosis and risk factors. Because of overdispersion in the data, negative binomial regression was applied to model the duration of infection in accordance with the case definition. Age and number of glasses of water consumed per day were evaluated as continuous variables. All statistical analyses were performed by using SPSS software version 19 (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered significant.

Results

Epidemiologic Investigation

Electronic Survey

Gastrointestinal symptoms were reported by 10,653 persons over a period of 2.5 weeks, confirming the large outbreak in the city and contamination of the drinking water (Figure 2). The number of cases of gastrointestinal illness increased from mid-November and peaked on November 29, three days after the boil-water advisory was issued. Thereafter, the number of new cases reported per day rapidly declined.

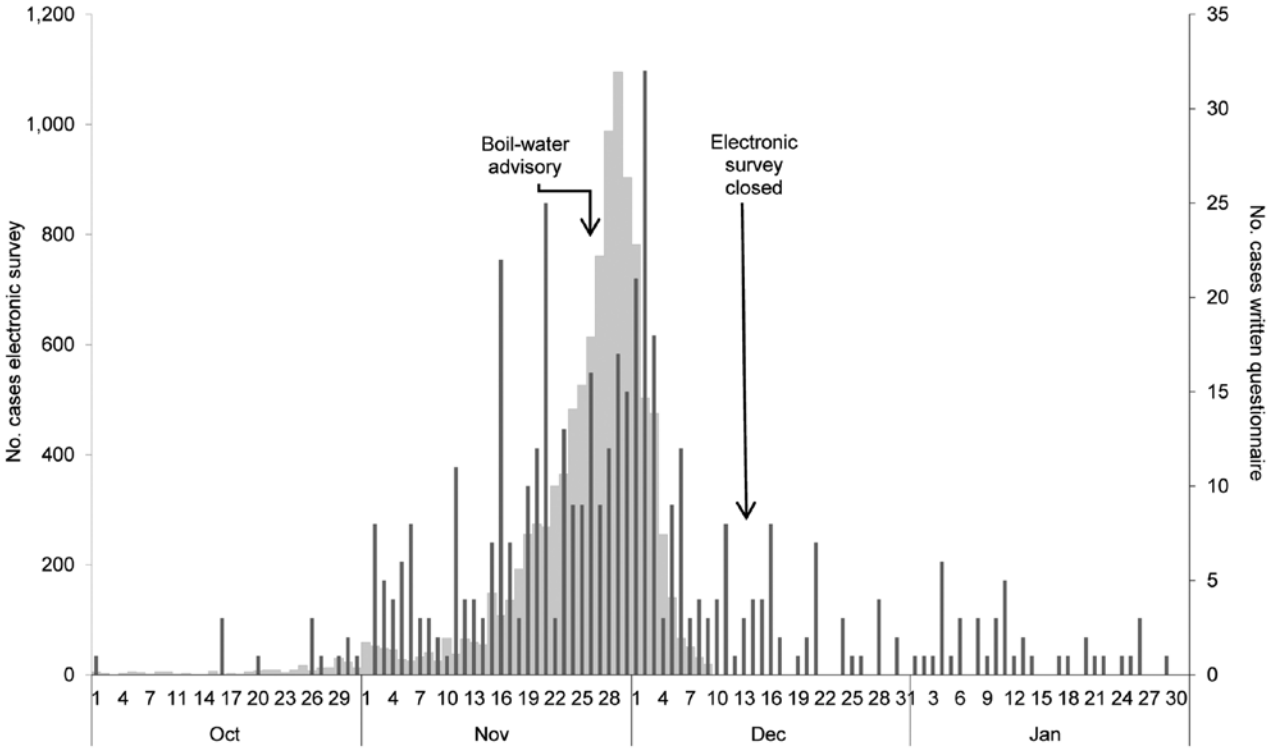

Figure 2. Epidemiologic curve of data from the electronic survey (10,653 participants; light gray) and written questionnaire (434 participants; dark gray) showing number of patients with suspected cases by date of onset of illness during *Cryptosporidium* infection outbreak, Östersund, Sweden, 2010–2011.

Written Questionnaire

Questionnaires were distributed by mail to 1,524 addressees; 10 persons had moved, and 6 were unable to respond. Of the remaining 1,508, a total of 1,044 (69.2%) responded: 481 men (46.1%) and 563 women (53.9%) (median age 44 years [range 0–98 years]) (Table 1). The response rate was highest for women 60–69 years of age (90.0%) and lowest for men 20–29 years (43.8%), and 45.2% (95% CI 42.1%–48.3%) of all the responders met the case definition criteria. When the rate of 45.2% was applied to the total population of Östersund (59,500), results indicated that ≈27,000 (95% CI 25,049–28,738) inhabitants contracted clinical cryptosporidiosis during the survey period. The attack rate decreased with age ($p<0.0001$; Table 1, Figure 3), was highest (58.0%) for persons 20–29 years of age and lowest (26.1%) for persons >69 years of age (Table 1), and was similar for men and women. The attack rate was 52.2% for respondents who lived and worked in areas served by the WTP-Ö but only 12.8% for inhabitants of Östersund who neither lived nor worked in areas served by that plant ($p<0.0001$; data not shown). The most common symptoms among case-patients were episodes of diarrhea ≥3 times daily (89.0%), watery diarrhea (84.3%), abdominal cramps (78.8%), fatigue (73.1%), nausea (63.9%), and headache (57.1%) (Table 2). Diarrhea lasted a median of 4 days (range 1–51 days). Duration of diarrhea decreased significantly with age ($p<0.0001$; Table 3, Figure 3), as did the incidence of

Table 1. Distribution of survey respondents and attack rate in *Cryptosporidium* infection outbreak, Östersund, Sweden, 2010–2011

	No. respondents (%)			Attack rate, %			
Age group, y	All	Female	Male	All	Women	Men	p value
0–9	115 (67.3)	58 (67.4)	57 (67.1)	50.9	42.6	58.9	0.09
10–19	117 (66.5)	58 (61.1)	59 (72.8)	47.2	55.6	38.5	0.08
20–29	103 (48.8)	57 (53.8)	46 (43.8)	58.0	58.2	57.8	0.97
30–39	110 (55.8)	58 (60.4)	52 (51.5)	52.8	51.9	53.8	0.84
40–49	150 (66.7)	71 (70.3)	79 (63.7)	55.0	52.9	57.0	0.62
50–59	145 (79.2)	85 (84.2)	60 (73.2)	42.1	45.1	37.9	0.40
60–69	148 (89.2)	81 (90.0)	67 (88.2)	35.3	41.3	27.6	0.10
>69	156 (87.2)	95 (88.8)	61 (84.7)	26.1	24.4	28.8	0.57
Total	1,044 (69.2)	563 (72.0)	481 (66.3)	45.2	45.1	45.4	0.94

Table 2. Clinical characteristics of surveyed case-patients and non–case-patients in *Cryptosporidium* infection outbreak, Östersund, Sweden, 2010-2011

Symptom	No. positive answers/total no. respondents (%)*		
	All respondents, N = 972†	Case-patients, n = 434	Non–case-patients, n = 538
Diarrhea, ≥3 stools/d	382/967 (39.5)	382/429 (89.0)	0/538 (0)
Watery diarrhea	343/945 (36.3)	343/407 (84.3)	0/538 (0)
Abdominal cramps	382/952 (40.1)	328/416 (78.8)	54/536 (10.1)
Fatigue	342/921 (37.1)	302/413 (73.1)	40/508 (7.9)
Nausea	301/931 (32.3)	253/396 (63.9)	48/535 (9.0)
Headache	267/920 (29.0)	232/406 (57.1)	35/514 (6.8)
Fever ≥38.0°C	128/909 (14.1)	121/393 (30.8)	7/516 (1.4)
Muscle or joint aches	95/875 (10.9)	80/366 (21.9)	15/509 (2.9)
Vomiting	89/894 (10.0)	76/357 (21.3)	13/537 (2.4)
Eye pain	81/877 (9.2)	67/367 (18.3)	14/510 (2.7)
Bloody diarrhea	16/883 (1.8)	15/345 (4.3)	1/538 (0.2)

*Respondents who answered yes (case-patients) compared with those who answered no (non–case-patients) about whether they had experienced ≥3 episodes of diarrhea daily and/or watery diarrhea with onset after November 1, 2010.
†Results on the basis of answers from 972 of 1,044 respondents.

fever, headache, nausea, vomiting, and fatigue (data not shown). Recurrence of diarrhea after ≥2 days of normal stools (defined as a relapse) was reported in 49.1% of the cases, and >1 relapse occurred significantly more often among women than men (p = 0.016; Table 4). Higher consumption of water and gluten intolerance were significant risks for *Cryptosporidium* infection (Table 3). Chronic intestinal disease (defined as inflammatory bowel disease [IBD], lactose intolerance, or gluten intolerance) and young age were significantly associated with more days with diarrhea (Table 3).

Microbiological Investigation

Human Samples

A total of 186 laboratory-confirmed cases of cryptosporidiosis related to the outbreak were reported to the national surveillance system: 149 in Jämtland County and 37 in other counties. Genotyping identified *C. hominis* subtype IbA10G2 in 37 samples. A representative sequence has been deposited into GenBank under accession no. KF574041. Analyses showed that the 149 *Cryptosporidium*-positive samples from Jämtland County were negative for other gastrointestinal pathogens.

Environmental Samples

Cryptosporidium oocysts were found in drinking water and raw water samples collected at the WTP-Ö on November 27 and in all samples of WTP-Ö drinking water, water from the distribution network, and raw water from Lake Storsjön over the next 2 months (Table 5). The highest number of oocysts in drinking water (1.4 presumptive oocysts/10 L) was detected on December 12, 2010 (online Technical Appendix Figure 1. During the outbreak, the average oocyst density in drinking water was 0.32/10 L in WTP-Ö samples and 0.20/10 L in samples from the distribution network. Densities in raw water samples were generally higher: 0.2–3.1 oocysts/10 L. In WWTP-Ö wastewater, the pre-outbreak low density (≤200 oocysts/10 L), had increased to 1,800/10 L on November 16, was highest at 270,000/10 L on November 29, and then gradually declined to preoutbreak levels from December 31 onward (online Technical Appendix Figure 2).

Oocysts were detected in 4 of 22 raw water samples from other municipalities near Lake Storsjön but in only 1 drinking water sample from a WTP (online Technical Appendix Table). All samples of untreated wastewater, most samples of treated wastewater (11/18), and samples from recipient water bodies (6/9) contained oocysts. Two of the 4 investigated streams connected to Lake Storsjön

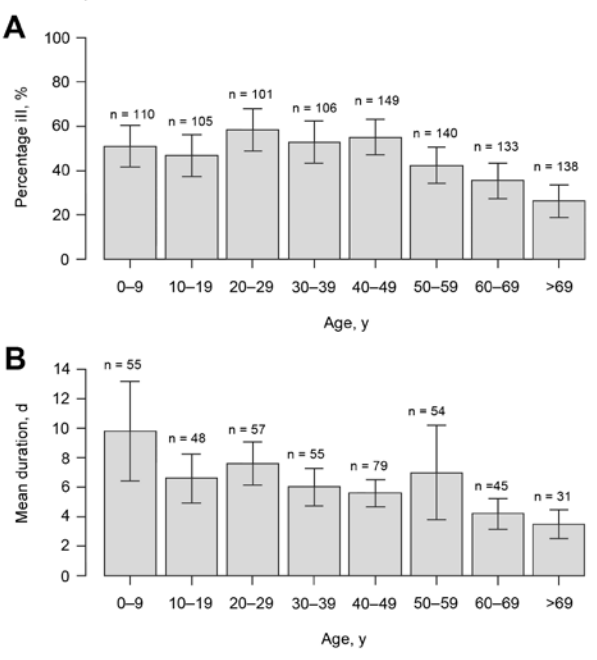

Figure 3. Percentage of ill persons (A) and mean duration of symptoms fulfilling the case definition (B), stratified by age group during *Cryptosporidium* infection outbreak, Östersund, Sweden, 2010–2011 . Error bars represent ±1 SE.

Table 3. Risk factors for cryptosporidiosis and duration of infection in *Cryptosporidium* infection outbreak, Östersund, Sweden, 2010 –2011*

Risk factor	Infection†		Duration,‡ p value
	Adjusted OR (95% CI)	p value	
Age, continuous	0.99 (0.98–0.99)	<0.0001	<0.0001
Chronic intestinal disease§	1.86 (0.95–2.63)	0.08	<0.01
Chronic underlying disease#	1.15 (0.73–1.8)	0.55	0.59
Gluten intolerance	4.06 (1.24–13.29)	0.02	0.05
Lactose intolerance	1.40 (0.79–2.46)	0.25	<0.01
No. additional family members with cryptosporidiosis	1.99 (1.70–2.33)	<0.0001	NA
No. glasses of water consumed daily	1.07 (1.03–1.11)	<0.0001	0.07
No. persons in household	0.98 (0.87–1.07)	0.54	NA
Peptic ulcer or medication	1.26 (0.72–2.22)	0.42	0.43
Smoking	1.01 (0.58–1.75)	0.98	0.40

*OR, odds ratio, adjusted for age, sex, and residence in the water treatment plant area; NA, not applicable.
†Participants with watery diarrhea and/or ≥3 episodes of diarrhea daily were defined as having cryptosporidiosis.
‡Duration (i.e., time fulfilling the case definition).
§Defined as inflammatory bowel disease, lactose intolerance, or gluten intolerance.
#Defined as cancer, rheumatic disease, cardiac failure, asthma, chronic obstructive pulmonary disease, or diabetes.

contained oocysts (Table 5). The stream closest to WTP-Ö (Figure 1) had densities of 1,300 and 5,000 oocysts/10 L on November 30 and December 2, respectively; this finding could be explained by wastewater leaking from an apartment building into the storm water system, which was repaired on December 3.

Isolated DNA from 1 concentrate of raw water, separated from other particulate matter by IMS, was successfully amplified at the 18S rRNA gene locus, and *C. hominis* was determined by restriction fragment length polymorphism and sequence analysis. Subtyping was not possible because amplification of the *gp60* gene failed. Also, despite repeated attempts, we were unable to amplify any DNA sequences from oocysts detected in raw water and drinking water by microscopy and removed from microscope slides.

C. hominis IbA10G2 was identified in 2 samples from the stream closest to WTP-Ö, in 5 from untreated wastewater at WWTP-Ö, and in 4 from other WWTPs in Jämtland County. No other *Cryptosporidium* species or subtypes were detected in any of the analyzed samples.

Discussion

We describe a confirmed outbreak of *Cryptosporidium* infection affecting at least 27,000 inhabitants of Östersund, Sweden, which represents the largest known outbreak in Europe and the second largest worldwide after the Milwaukee outbreak. The etiologic agent was detected in drinking water, repeatedly over >2 months. Although *Cryptosporidium* spp. are occasionally found in untreated surface water, to our knowledge, this is the first time this pathogen has been detected in drinking water in Sweden.

Three factors facilitated detection of the outbreak. First, before the outbreak was recognized, alert staff at the county laboratory suspected oocysts in wet smears of unstained, concentrated fecal specimens and subsequently confirmed the presence of *Cryptosporidium* spp. by modified Ziehl-Neelsen staining, even though this analysis had not been specifically requested. Second, data from the local health advice line indicated that most persons with gastroenteritis resided within the city limits, which proved to be crucial for the decision to issue a boil-water advisory. Third, the electronic survey was a valuable tool for daily monitoring of the epidemic curve and evaluating the effect of the boil-water advisory. Previous research has demonstrated the benefits of event-based surveillance data and website questionnaires in early detection and monitoring of an outbreak (*23*,*24*).

The distribution of symptoms among case-patients with cryptosporidiosis in this study is comparable to observations from other studies (*6*,*17*,*25*), except regarding muscle or joint aches, which were reported less frequently in Östersund. Moreover, the median duration of diarrhea, the level of attack rates in different age groups, and recurrence rate of diarrhea correspond to findings in other outbreaks (*6*,*14*).

We identified young age, amount of water consumed, and number of infected family members as risk factors, which agrees with results from other studies (*26*,*27*). Also, gluten intolerance remained a risk factor after we controlled for age, sex, and residence in the WTP area, but this analysis was based on information from only 17 persons and hence should be interpreted with caution. The mechanism by which gluten intolerance might constitute a risk factor for cryptosporidiosis is unknown. Duration of diarrhea was significantly associated with young age and chronic intestinal disease. Exacerbation of IBD in cryptosporidiosis patients has been documented (*28*), and *Cryptosporidium*-induced loss of intestinal barrier function has been suggested to mimic changes seen in IBD (*29*). Additional studies are needed to clarify any long-term effects of *Cryptosporidium* infection and are being undertaken in relation to the current outbreak.

Molecular typing identified *C. hominis* IbA10G2 in both human and environmental samples. This early identification of non–livestock-associated *Cryptosporidium*

Table 4. Distribution of respondents and relapse of diarrhea among surveyed case-patients in the *Cryptosporidium* infection outbreak, Östersund, Sweden, 2010–2011

Age group, y	All relapses, %	1 Relapse, %			>1 Relapse, %		
		Female	Male	p value	Women	Men	p value
0–9	68.5	50.0	43.8	0.66	22.7	21.9	0.94
10–19	48.9	20.7	50.0	0.04	20.7	10.0	0.30
20–29	40.4	22.6	19.2	0.76	22.6	15.4	0.50
30–39	47.3	25.9	32.1	0.63	29.6	7.1	0.03
40–49	51.3	27.8	36.4	0.42	25.0	13.6	0.21
50–59	47.4	22.2	23.8	0.89	25.0	23.8	0.92
60–69	47.8	22.6	20.0	0.85	29.0	20.0	0.52
>69	35.3	15.0	35.7	0.20	15.0	7.1	0.50
Total	49.1	25.4	33.5	0.07	24.1	15.0	0.016

isolates facilitated the outbreak investigation by indicating that the cause was contamination of surface water by human sewage rather than contamination from an animal source (*4*,*30*). *C. hominis* IbA10G2 is reported to be highly virulent; is excreted in high numbers in feces (*1*,*31*,*32*); and is the most commonly identified subtype in waterborne cryptosporidiosis outbreaks, including that in Milwaukee (*3*,*30*,*33*,*34*). These characteristics, along with occurrence of the outbreak in a population that may have been particularly susceptible because of limited previous exposure, contributed to the high attack rate (*35*,*36*).

Although the infectious dose for *Cryptosporidium* infection is low, the oocyst densities in the Östersund drinking water (maximum ≈1/10 L) cannot readily explain the high attack rate, even if the low recovery rate is taken into account. Densities may have been higher at the onset of the outbreak because of a surge of oocysts in the inlet before sampling, and secondary household transmission could have contributed to some of the cases. However, similar low numbers of oocysts have been detected in drinking water samples in other outbreaks (*26*,*37*). The level of recovery efficiency of the methods used in the outbreak required analysis of at least 100 L of water to identify the low level of *Cryptosporidium* contamination, which agrees with findings reported by other investigators (*26*).

Recovery studies were not performed during the acute phase of the Östersund outbreak, which underscores the uncertainty of extrapolating the numbers of oocysts detected in raw and drinking water to the actual density of oocysts (*38*). Moreover, no reliable assays to test viability and infectivity of oocysts are available (*1*). Other limitations of the present study include potential response bias in the electronic survey and the mailed questionnaire (*39*). Moreover, we could not assess the contribution of secondary transmission to the attack rate or ascertain the number of subclinical cases by serologic testing.

Several possible factors could explain *Cryptosporidium* contamination of the drinking water. In the routine bacteriologic analysis performed weekly at WTP-Ö, *E. coli* densities were ≈10 times greater than the average level on 3 occasions a few weeks before the outbreak (H. Dahlsten, pers. comm.), which implies abnormally high fecal contamination of the source water. Furthermore, *Cryptosporidium* oocysts were detected repeatedly in both raw and drinking water for months after the outbreak peaked, which illustrates the environmental persistence of oocysts and/or continuing contamination. Survival of the oocysts in Lake Storsjön was probably prolonged because the outbreak occurred in winter when the lake was covered with ice. The municipality of Östersund made

Table 5. Presence of *Cryptosporidium* oocysts in environmental samples collected in Östersund, Sweden, November 27, 2010– March 22, 2011*

Sample type	No. samples	No. positive samples	Analyzed volume, L	Presumptive no. oocysts, min–max/10 L	Confirmed no. oocysts, min–max/10 L	Time span for positive samples
Raw water†	18	10	100	0.2–3.1	0.1–0.7	2010 Nov 27–2011 Feb 9
Drinking water, WTP-Ö†	7	7	800–1,500	0.047–1.4	0.02–1.3	2010 Nov 27–2011 Jan 20
Drinking water, distribution network	9	9	800–1,400	0.063–0.36	0.05–0.05	2010 Nov 29–2011 Jan 31
Wastewater, untreated†	21	13	0.05‡	200–270,000	§–160,000	2010 Nov 29–2011 Feb 17
Wastewater, treated	15	14	0.25–0.3‡	30–21,000	30–10,000	2010 Dec 1–2011 Jan 24
Recipient (Lake Storsjön)	14	8	9–10	2–21	1–18	2010 Nov 29–2011 Mar 22
Connected streams	8	5	2–10	1,300–5,000	950–3,500	2010 Nov 30–Dec 14
Other¶	10	2	10–17	1–3	1–3	2010 Nov 30–2011 Jan 17
Total	102	68		0.047–270,000	0.02–160,000	2010 Nov 27–2011 Mar 22

*Min, minimum; max, maximum; WTP-Ö, water treatment plant–Östersund.
†Details are available in Technical Appendix Figures 1 and 2, wwwnc.cdc.gov/EID/article/20/4/12-1415-Techapp1.pdf.
‡These samples consisted of 30-mL aliquots from every 50–60 m^3 of wastewater produced over 24 h.
§Not possible to determine the lowest density by microscopy because of substantial background material in the concentrated water sample.
¶Samples from sources, such as swimming pools, water used to flush the distribution network, and sediment from fire hydrants.

considerable efforts to trace the sources of *Cryptosporidium* contamination, and tentatively identified 2 streams, 1 of which was located closer to (upstream of) the raw water intake (Figure 1) and had higher densities of oocysts. However, we could not establish whether the initial input of oocysts to Lake Storsjön and the raw water intake had actually come from these streams, or whether it resulted from the outbreak itself. Perhaps these 2 streams contributed to a transmission cycle in which infectious persons were shedding oocysts into leaking wastewater that reached the raw water intake. Because only *C. hominis* IbA10G2 was identified in environmental samples, we suggest that the outbreak was caused by a single common source of contamination, although this hypothesis could not be definitively demonstrated.

Failure of the WTP-Ö and onset of the outbreak has several plausible explations. To our knowledge, no posttreatment contamination or extensive failures in the treatment processes occurred, and routine tests of the drinking water showed no increased levels of fecal indicator bacteria. The WTP-Ö had 2 microbiological barriers (ozonation and chloramination) as recommended by the drinking water regulations in Sweden for surface waterworks, but these barriers were simply inadequate to remove or inactivate the *Cryptosporidium* oocysts in the raw water. The long-term solution to reduce infective parasites in Östersund was to install a UV water disinfection system, which was done after the outbreak in December 2010. In addition, pipes were repeatedly flushed, and and further sampling was conducted to verify that no potentially viable oocysts remained in the distribution network.

Previous research has suggested that analysis of *Cryptosporidium* in wastewater can aid in early detection of an outbreak (*40*). In Östersund, the number of *Cryptosporidium* oocysts in influent wastewater increased slightly 10 days before the boil-water advisory (1,800 oocysts/10 L), which indeed implies that monitoring the level of oocysts in influent wastewater might facilitate early detection of an ongoing outbreak, although the cost of such an approach would render it impractical.

Six months after the outbreak in Östersund, another waterborne outbreak of *C. hominis* IbA10G2 infection occurred in the city of Skellefteå, 450 km northeast of Östersund, possibly because persons from that city had visited Östersund during the outbreak there and had subsequently spread *Cryptosporidium* oocysts on their return to Skellefteå. In Sweden, recommendations to prevent outbreaks of parasites include identifying and limiting sources of contamination of raw water in combination with sampling (100-L volumes). The awareness of parasites as a probable cause of waterborne outbreaks has increased tremendously in this country since these outbreaks, and many WTPs have evaluated the efficiency of their current barriers, for example, by quantitative microbial risk assessment.

This study has documented the largest outbreak of waterborne cryptosporidiosis in Europe, affecting ≈27,000 persons. *C. hominis* subtype 1bA10G2 was identified in clinical samples and in wastewater. Low levels of oocysts were repeatedly detected in drinking water for >2 months. Our results emphasize the value of assessing microbial risks in raw water and using multiple barriers in WTPs to substantially reduce or inactivate all groups of microorganisms, including parasites such as *Cryptosporidium* spp.

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Dr Widerström is the county medical officer at the Department of Communicable Diseases Control and Prevention, County Council of Jämtland, Sweden, and senior infectious disease consultant at the Department of Infectious Diseases, Östersund Hospital. His primary research interests include epidemiology of communicable diseases, especially healthcare-associated staphylococcal infections.

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Address for correspondence: Micael Widerström, Department of Clinical Microbiology—Clinical Bacteriology, Umeå University, Umeå 90185, Sweden; email: micael.widerstrom@jll.se

Efficiency of Points of Dispensing for Influenza A(H1N1)pdm09 Vaccination, Los Angeles County, California, USA, 2009

Shubhayu Saha, Brandon Dean, Steven Teutsch, Rebekah H. Borse, Martin I. Meltzer, DeeAnn Bagwell, Alonzo Plough, and Jonathan Fielding

During October 23–December 8, 2009, the Los Angeles County Department of Public Health used points of dispensing (PODs) to improve access to and increase the number of vaccinations against influenza A(H1N1)pdm09. We assessed the efficiency of these units and access to vaccines among ethnic groups. An average of 251 persons per hour (SE 65) were vaccinated at the PODs; a 10% increase in use of live-attenuated monovalent vaccines reduced that rate by 23 persons per hour (SE 7). Vaccination rates were highest for Asians (257/10,000 persons), followed by Hispanics (114/10,000), whites (75/100,000), and African Americans (37/10,000). Average distance traveled to a POD was highest for whites (6.6 miles; SD 6.5) and lowest for Hispanics (4.7 miles; SD ±5.3). Placing PODs in areas of high population density could be an effective strategy to reach large numbers of persons for mass vaccination, but additional PODs may be needed to improve coverage for specific populations.

Mass vaccination outside clinical settings (e.g., in pharmacies, workplaces, businesses, schools, and religious institutions) has been used to safely and efficiently provide a high volume of influenza vaccinations (*1*) and expand access to the vaccine (*2*,*3*). Success for such operations depends on the rapid dispensation of vaccines, the number of vaccines administered, and the communities reached. In Los Angeles County, California, USA, the 2009 influenza A(H1N1) pandemic was considered widespread by September 20, 2009 (*4*,*5*). Distributing the influenza A(H1N1)pdm09 (pH1N1) vaccine through points of dispensing (PODs) was the principal prevention strategy of the Los Angeles County Department of Public Health (LACDPH). PODs are vaccination clinics that operate at designated locations throughout the community for the temporary, large-scale dispensing of vaccines to persons at risk during a public health emergency. During October 23–December 8, 2009, the LACDPH distributed the pH1N1 vaccine through 60 POD locations in Los Angeles County. Clinical and nonclinical staff (including LACDPH-trained volunteers) registered patients and facilitated the vaccination process. The PODs were placed throughout the county to reach diverse, high-risk populations who would be less likely to receive the vaccine otherwise (*6*). We reviewed data from this effort to determine how future mass vaccination campaigns can improve the efficiency of vaccination at PODs and provide equitable access to PODs among demographic groups considered especially vulnerable to the vaccine-preventable outcome.

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (S. Saha, R.H. Borse, M.I. Meltzer); and Los Angeles County Department of Public Health, Los Angeles, California, USA (B. Dean, S. Teutsch, D. Bagwell, A. Plough, J. Fielding)

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Methods

We combined data collected at the PODs with census tract–level demographic information for Los Angeles County. We used a combination of multivariate regression analysis and geospatial methods to determine what factors affected the rate of vaccination (throughput) in the PODs; if the distance to PODs was similar for the 4 major ethnic groups living in Los Angeles County (white, African American, Hispanic, and Asian); how proximity to PODs affected visit patterns across these ethnic groups; and how the rate of POD visits varied by the underlying ethnic concentration and income status among the census tracts in Los Angeles County.

To examine throughput, we used data from each of the 101 POD events (some of the 60 locations had POD events on ≥1 day). Trained personnel completed patient

registration forms that contained information about age, sex, ethnicity, address, existing medical conditions, pregnancy, and the type of pH1N1 vaccine received (i.e., live-attenuated monovalent vaccine [LAMV] administered nasally or monovalent inactivated vaccine injected from a multidose vial or as a single-dose unit). The number of hours staff worked was recorded, and each staff member was classified as clinical or nonclinical. We analyzed vaccine throughput (the average number of doses of vaccine administered per hour per POD event [dependent variable]) as a linear function of the following: vaccine mix (percentage of LAMV administered at each POD event); clinical staff time (percentage of hours worked by clinical staff/POD event); queue length (average number of persons waiting in line at each POD event); PODs same day (the number of PODs that were in operation in separate locations on the same day); high-risk patients (percentage of patients who were ≤10 years of age, were pregnant, or reported high-risk medical conditions and vaccine-related contraindications); previous influenza vaccination clinic held at site (previous seasonal influenza vaccination held at the same POD location); and vaccine shortage (POD operating on a day during the reported shortage of vaccine supply, which ended November 21).

We found the vaccine throughput data to be normally distributed by using the Shapiro-Wilk statistic (*7*), providing justification for using least squares regression (*8*). Because some locations had POD events on multiple days, we tested for systematic patterns of vaccination throughput by using White test for heteroscedasticity. Such patterns existed, and we corrected by estimating robust standard errors in which we used PODs as the clustering variable (*8*). A Ramsey test confirmed that no combination of higher order terms of the explanatory variables would fit the data better (*9*). Because estimates of the average number of persons waiting outside the POD were skewed, we log transformed the data measuring queue length. We used Stata version 10 (StataCorp, College Station, Texas, USA) for the regression analysis.

The datasets used for the spatial analyses were population-weighted geometric center of each census tract from the 2000 census, census tract–level distribution of ethnic populations and median household income in 2009, geocoded addresses of patients obtained from patient registration forms, and geocoded POD locations. We used Centrus software (Stamford, CT, USA) for geocoding and ARCGIS version 9.2 software (ESRI, Redlands, CA, USA) for spatial analyses. For each patient, we geocoded the residence address, identified the census tract containing the geocoded address, and calculated the Euclidean (straight-line) distance between the geocoded address and each of the POD locations. We also calculated the Euclidean distance between each population-weighted census tract geographic center and each of the POD locations. We used census tract–level demographic data to classify each census tract into ethnic population quartiles (white, African American, Hispanic, and Asian). Each census tract was also assigned to an income quartile on the basis of the tract's median household income. Thus, each of the 2,052 census tracts in Los Angeles County was classified into 4 ethnic quartiles and 1 income quartile.

We first assessed the average distance to PODs for members belonging to the 4 ethnic groups. For each census tract, we identified the POD closest to the tract geographic center and assumed that all persons living in the census tract traveled the same distance to reach that closest POD. We then used the number of persons in each ethnic group in each census tract as weights to calculate a weighted average distance to the nearest POD for each ethnic group. For sensitivity analysis, we repeated the process to calculate the average distance to the second and third closest POD.

We then assessed how proximity to PODs affected visit patterns to PODs across ethnic groups. We calculated the percentage of patients in the 4 ethnic groups who visited any of the 3 PODs closest to the census tract where they lived by using the census tract in which the geocoded address of a patient fell and geocoded address of the POD that each patient visited. For each ethnic group, we then calculated the percentage of patients who visited any of the 3 PODs closest to them.

To ensure that certain subpopulations (e.g., low income, ethnic minorities) have adequate access to PODs, the units are often located in areas with a high density of those subpopulations to achieve high rates of vaccination. Thus, we assessed how frequency and rates of POD visits varied by the distribution of ethnic population and median household income. We used the geocoded patient address data to identify the number of patients in each ethnic group residing in each census tract. For each census tract, we then calculated the number of patients by each ethnic group. Using this count as the numerator and the total population for each ethnic group as the denominator, we calculated an ethnicity-specific POD visit rate for each census tract. We summarized these numbers across census tracts by ethnic population and income quartile to calculate count and average visit rates by ethnic groups.

Results

Descriptive statistics of the variables used in the regression analysis of vaccine throughput during the 101 POD events are shown in Table 1. A total of 179,688 vaccine doses were administered at the PODs; the average number of doses administered per hour per POD was 239 (range 40–427). LAMV constituted an average of 29% of the vaccines administered at a POD (range 0%–62%). Clinical staff contributed an average of 56% (range

Table 1. Patient characteristics and POD data from 101 influenza vaccine events, Los Angeles County, California, USA, October 23–December 8, 2009*

Variable	Mean	SD	Min	Max
Patient characteristic, % patients				
Age 0–10 y	22	7	0	41
Pregnancy	4	3	0	25
Contraindications	3	7	0	42
Preexisting medical conditions	23	7	0	36
POD data				
Throughput	239	87	40	427
Vaccine mix	29	13	0	62
Clinical staff time	56	13	12	100
Queue length	247	277	7	1,614
PODs same day	5.7	3.7	1	13
Proportion of POD locations with influenza vaccine clinics before 2009	0.14	0.08	0	1
Proportion of POD events during vaccine shortage ending on 2009 Nov 21	0.81	0.15	0	1

*POD, point of distribution; throughput, average vaccine doses administered per hour per POD event; vaccine mix, percentage of live-attenuated monovalent vaccine; clinical staff time, percentage of clinical staff hours; queue length, average number of patients in queue outside PODs per hour per event; PODs same day, number of POD events held on the same day.

12%–100%) of total staff time, which included hours worked by nonclinical staff and volunteers. Approximately 6 (range 1–13) POD events were held per day, but delays in vaccine availability caused variation over time. pH1N1 vaccines were in short supply until November 21, 2009, and ≈81% of the PODs were in operation before that date. Approximately 14% of POD locations had previously been used for seasonal influenza vaccinations. An average of 247 persons (range 7 1,614) every hour were estimated to be in line outside a POD waiting to be vaccinated. Of all patients, 22% were 0 10 years of age, 4% were pregnant, and 23% reported medical conditions that influenced the type of vaccine they received.

The baseline rate of vaccination was ≈251 patients per hour (SE 65; p<0.01) (Table 2). A 10% increase in LAMV in the vaccine mix was associated with a 23.2% decrease in throughput (SE 0.7; p<0.01) (Table 2). A similar increase in the percentage of clinical staff hours was associated with an 11% decrease in persons vaccinated per hour (SE 0.61; p = 0.06). A 10% increase in the average number of persons waiting in line outside a POD was associated with an increase in throughput of 3 patients per hour (SE 6.88; p<0.01). Operation of other PODs on the same day at another location, percentage of patients who were 0–10 years of age, and percentage of patients who reported contraindications and preexisting medical conditions did not significantly affect vaccine throughput.

The largest ethnic group in Los Angeles County is Hispanic (47% of the population); a similar percentage (44%) for this group was found among those came to PODs for vaccination (Table 3). The rate of vaccination for the total population across all PODs was highest for Asians (257/10,000 persons), followed by Hispanics (114/10,000 persons), whites (75/10,000 persons), and African Americans (37/10,000 persons).

Of persons who received the 179,688 vaccine doses administered in the PODs, 157,176 were residents of Los Angeles County. A total of 125,849 addresses provided at the POD registration could be geocoded; spatial analyses were restricted to this sample. On the basis of the calculated

Table 2. Factors affecting average number of patients vaccinated per hour (throughput) per influenza vaccine POD event, Los Angeles County, California, USA, October 23–December 8, 2009*†‡

Variable†	No. persons/h (95% CI)	SE‡	p value
Baseline	251.19 (124.43 to 377.95)	64.68	0.00
Patient demographic			
Age 0–9 y	–2.54 (–5.87 to 0.79)	1.70	0.14
Pregnancy	4.55 (–0.78 to 9.88)	2.72	0.09
Contraindications	–2.53 (–6.14 to 1.09)	1.85	0.17
Preexisting medical conditions	1.38 (–1.69 to 4.44)	1.56	0.38
POD data			
Vaccine mix	–2.32 (–3.69 to –0.96)	0.70	0.00
Clinical staff time	–1.14 (–2.33 to 0.06)	0.61	0.06
Queue length	32.05 (18.57 to 45.53)	6.88	0.00
PODs same day	–3.21 (–8.06 to 1.63)	2.47	0.19
Influenza clinic in same location before 2009	37.32 (–13.78 to 88.43)	26.07	0.15
Period of vaccine shortage	–17.56 (–64.43 to 29.31)	23.92	0.46

*N = 101. Adjusted R^2 = 0.57; Wald χ^2 = 144.6 (Prob > χ^2 = 0.00). POD, point of distribution; throughput, average vaccine doses administered per hour per POD event; vaccine mix, percentage of live-attenuated monovalent vaccine (LAMV); clinical staff time, percentage of clinical staff hours; queue length, average number of patients in queue outside PODs per hour per event; PODs same day, number of POD events held on the same day.
†The analysis estimates the effect of these variables on the baseline number of persons vaccinated per hour per individual POD. For example, 1% increase in LAMV reduced throughput by 2.32 persons vaccinated per hour.
‡Robust clustered SEs with POD as clustering variable.

Table 3. Residence, rate of vaccination, and distance traveled for persons visiting PODs for influenza vaccination, Los Angeles County, California, USA, October 23–December 8, 2009*

Variable	White	African American	Hispanic	Asian
% Residents of Los Angeles†	30	9	47	13
Total % patients in PODs,‡ n = 125,849	19	3	44	28
Number vaccinated per 10,000 population	75	37	114	257
Average distance traveled, miles (SD)§	6.6 (6.5)	5.6 (6.3)	4.7 (5.3)	6.3 (6.2)
Median distance traveled, miles§	4.7	3.8	2.9	4.5

*PODs, points of distribution.
†2009 tract-level demographic data from the Los Angeles Department of Public Health.
‡Information from questionnaire used at POD before vaccination.
§Euclidean distance from residence to POD based on geocoded addresses of vaccines.

Euclidean distance between geocoded residence and PODs, the average distance traveled to a POD was highest for whites (6.6 miles; SD 6.5) and lowest for Hispanics (4.7 miles; SD 5.3). The average distance to the closest POD was relatively the same across ethnic groups: 2.7 miles for whites, 2.2 miles for Asians, and 2.0 miles for African Americans and Hispanics (Table 4). The average distances to the second and third closest PODs were lowest for African Americans and highest for whites. Hispanics were most likely to visit the POD closest to the geographic center of the census tract where they lived (44%), followed by whites (39%), African Americans (35%), and Asians (31%) (Table 4). By adding the percentages of patients who visited the 3 closest PODs, we found that >50% of patients in each ethnic group attended 1 of the 3 PODs closest to where they lived (Table 4).

Of patients who attended a POD, 84% resided in census tracts in the third and fourth quartiles for population density of the associated ethnic group. This finding indicates that placing PODs in closer proximity to high population density centers could increase the total number of persons vaccinated. However, these patterns are less apparent when rates of POD visits are examined. For example, the rate of POD visits for Asians (267/10,000), Hispanics (132/10,000), and African Americans (83/10,000) were highest from the census tracts in the bottom of their respective population quartiles (Table 5). Differences between the rates across the population quartiles did not vary by ethnic group, except for African Americans, for whom the average rate for the bottom 2 quartiles was more than double that of the average rate for the top 2 quartiles (Table 5).

POD visit patterns differed across ethnicities based on median household income in census tracts. A total of 55% of African American and 68% of Hispanic POD attendees came from census tracts in the lowest 2 income quartiles. This percentage was much smaller for whites (18%) and Asians (36%) (Table 6). However, the rate of POD visits uniformly increased from the lowest income quartile to the highest income quartile (Table 6). The difference in the rate across the income quartiles was most apparent among African Americans, from 28/10,000 for the lowest to 71/10,000 for the highest income quartile (Table 6).

Discussion

We retrospectively evaluated pH1N1 vaccine distribution through PODs in Los Angeles County to identify factors associated with throughput of patients and vaccine coverage for different racial/ethnic groups. We found that a higher proportion of LAMV among vaccines administered and higher proportion of clinical staff among all personnel were associated with a reduction in throughput of vaccine. An increase in the proportion of pregnant woman among patients was associated with increased throughput; anecdotal evidence suggests the reason may be that pregnant women were provided a dedicated, priority queue at the PODs, and only 1 type of vaccine was used.

The rate of vaccination in the PODs was highest for the Asian residents of Los Angeles County, followed by Hispanics, whites, and African Americans. PODs were placed throughout Los Angeles County equally close to different racial/ethnic groups. Across all 4 ethnic groups, ≥80% patients resided in top 2 population density quartiles of their respective ethnicities. Thus, placing PODs in census tracts of high population density could be an effective strategy to reach large numbers of persons. Income quartile notably affected rates of vaccination for whites and African Americans but had very little effect for Hispanics and Asians.

Our findings add to the understanding of the association between POD-level features and vaccination rates and helps elucidate the usefulness of geographic information systems in planning improved community-level access to

Table 4. Average distance persons traveled to the 3 closest PODs for influenza vaccination, Los Angeles County, California, USA, October 23–December 8, 2009*

	Distance traveled to POD, miles (% persons visiting POD)			
POD location	White	African American	Hispanic	Asian
Closest	2.7 (39)	2.0 (35)	2.0 (44)	2.2 (31)
2nd closest	5.0 (15)	3.4 (12)	3.5 (12)	3.8 (13)
3rd closest	6.3 (8)	4.4 (8)	4.4 (9)	4.8 (10)

*Values do not equal 100% for each category because some patients visited PODs not among the 3 closest to where they live. PODs, points of distribution.

Table 5. Numbers and rates of persons receiving influenza vaccine at PODs by ethnic group and population density, Los Angeles County, October 23–December 8, 2009*

Population density quartile	No. persons (rate†)			
	White	African American	Hispanic	Asian
Bottom	646 (74)	353 (83)	2,682 (132)	1,835 (267)
Second	2,033 (58)	392 (60)	7,362 (109)	3,314 (221)
Third	6,191 (70)	572 (36)	16,385 (114)	7,053 (239)
Top	14,606 (81)	2,157 (32)	29,567 (114)	22,832 (266)

*Quartiles based on ethnic population distribution across census tracts. POD, point of distribution
†No./10,000 population for the ethnic group.

health care (*10,11*). Spatially indexed clinic-level health data aligned with commonly available census information have been used to identify patient catchment areas and assess underserved populations (*12*). Spatial statistics has been used to identify patterns in use of psychiatric outpatient care among racial and ethnic groups to inform the design of interventions to improve access to care (*13*). Whereas access to care in this study was measured on the basis of Euclidean distances between POD location and patient addresses (specific travel information to PODs for each patient was not available), actual travel route and time may be more informative, but reports in the literature do not agree on this point (*13,14*).

Our study has several limitations. Most of the information used in the analyses was recorded in paper-based questionnaires before vaccines were administered at the PODs. Use of modern technology (e.g., electronic data recorders) at times of such emergencies could possibly reduce the rate of missing and incomplete information. Our analysis further did not account for the differential effectiveness of crowd-management techniques (e.g., dedicated lines for certain persons) that could facilitate faster movement of patients through the PODs. Such information was not available for all PODs. The patterns in POD visits we found for Los Angeles County depended on the underlying spatial distribution of different ethnic populations, which also limits the generalizability of the patterns we would find for other jurisdictions. Patterns of POD visits during the 2009–10 pandemic were likely influenced by a wide variety of unmeasured factors (e.g., perceived severity, availability of vaccine relative to timing of pandemic). Although we controlled for some of these supply issues in the regression analysis, we did not have the necessary information to take these factors into account for the spatial analyses.

In a community without spatially pronounced racial and ethnic or socioeconomic population distributions, selecting POD sites on the basis of population densities may prove the most efficient strategy. Such a method may ensure the greatest possible community-wide coverage during a pandemic. However, ensuring equitable reach to all ethnic subgroups may require the use of more strategies designed to target select subpopulations, particularly for a community with marked clustering of definitive population subgroups. Similar analyses should be conducted in other locations to inform public health preparedness activities in similar future scenarios (*15*).

Making comprehensive policy recommendations for emergency public health operations is a challenge because all emergencies are local and driven by factors unique to each emergency and location (*16*). However, this vaccination campaign represents one of the largest POD-based efforts in the history of emergency public health response, and several approaches could be used to improve outcomes in future public health emergencies. Limiting the variety of available medical countermeasure products (i.e., different vaccine types, multiple antimicrobial drugs) at PODs could reduce the amount of time and resources needed to triage and match clients to a particular product. In addition, optimizing the ratio of clinical staff to nonclinical staff would maximize efficiency while ensuring a safe system. Furthermore, placing PODs close to population clusters would serve the dual objectives of wide coverage and representation of population subgroups (*17*).

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Table 6. Numbers and rates of persons receiving influenza vaccine at PODs by ethnic group and median household income, Los Angeles County, California, USA, October 23–December 8, 2009*

Income quartile	No. persons (rate†)			
	White	African American	Hispanic	Asian
Bottom	1,245 (54)	1,013 (28)	18,032 (103)	4,825 (227)
Second	3,058 (57)	909 (31)	19,878 (116)	7,866 (238)
Third	6,771 (67)	846 (46)	13,324 (124)	11,728 (270)
Top	12,402 (91)	706 (71)	4,762 (126)	10,615 (274)

*Quartiles based on median household income distribution across census tracts. POD, point of distribution
†No./10,000 population for the ethnic group.

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Dr Saha is a health scientist at the Centers for Disease Control and Prevention in Atlanta. His research interests include linking environmental exposures with health outcomes over space and time to assess the epidemiologic risk associated with those exposures and analyzing cost-effectiveness of community-level health intervention strategies.

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Address for correspondence: Shubhayu Saha, Centers for Disease Control and Prevention, 4770 Buford Hwy, Mailstop F59, Atlanta, GA 30341, USA; email: ssaha@cdc.gov

Underdiagnosis of Foodborne Hepatitis A, the Netherlands, 2008–2010[1]

Mariska Petrignani, Linda Verhoef, Harry Vennema, Rianne van Hunen, Dominique Baas, Jim E. van Steenbergen, and Marion P.G. Koopmans

Outbreaks of foodborne hepatitis A are rarely recognized as such. Detection of these infections is challenging because of the infection's long incubation period and patients' recall bias. Nevertheless, the complex food market might lead to reemergence of hepatitis A virus outside of disease-endemic areas. To assess the role of food as a source of infection, we combined routine surveillance with real-time strain sequencing in the Netherlands during 2008–2010. Virus RNA from serum of 248 (59%) of 421 reported case-patients could be sequenced. Without typing, foodborne transmission was suspected for only 4% of reported case-patients. With typing, foodborne transmission increased to being the most probable source of infection for 16%. We recommend routine implementation of an enhanced surveillance system that includes prompt forwarding and typing of hepatitis A virus RNA isolated from serum, standard use of questionnaires, data sharing, and centralized interpretation of data.

Hepatitis A virus (HAV) infection is an acute, usually self-limiting, illness; transmission is associated with suboptimal hygiene. Transmission occurs by the oral route, and infected persons can shed high amounts of infectious virus in their feces (*1*). Over recent decades, the incidence of HAV infections has been declining to a low level of transmission in high-income and middle-income countries. This epidemiologic shift results in a gradual shift in patient age and severity of first infection, from asymptomatic infections in very young children toward more severe illness in older children and adults. The World Health Organization estimates a case-fatality rate ranging from 0.1% for children <15 years of age to 2.1% for adults ≥40 years of age (*2*). As incidence of HAV decreases, the proportion of the population vulnerable to infection increases. Thus, paradoxically, hepatitis A virus could reemerge in regions where it is not endemic, affecting mostly adults. Risk for outbreaks with more severe illness becomes greater in countries where such epidemiologic transition has occurred.

In countries with low levels of HAV, the main risk comes from travel, secondary waves of transmission in households and schools, and (ongoing and sometimes epidemic) transmission among men who have sex with men (MSM) (*3–11*). However, the probable source of infection remains unknown for 20%–30% of cases, possibly because of transmission by persons with subclinical or missed primary cases, but alternatively because of food contamination. Although HAV is listed as the second most common foodborne virus (*12*), foodborne HAV infections are rarely reported, except when triggered by an unusual outbreak or event. In general, detection of a food source is difficult because the incubation period for hepatitis A is long (average 4 weeks); therefore, responses to food-consumption questionnaires, if administered, might be unreliable because of recall bias. Moreover, the food industry is a complex multinational system, and many high-risk products (shellfish, fresh or frozen fruits and vegetables) are produced in HAV-endemic countries. The common methods used for microbiological quality control

Author affiliations: Municipal Health Service Zoetermeer, Zoetermeer, the Netherlands (M. Petrignani, R. van Hunen); National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands (L. Verhoef, H. Vennema, D. Baas, J.E. van Steenbergen, M.P.G. Koopmans); Erasmus Medical Center, Rotterdam, the Netherlands (M.P.G. Koopmans); and Leiden Medical University, Leiden, the Netherlands (J.E. van Steenbergen)

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of food do not reliably predict presence or absence of virus contamination (*13*). Virus contamination of high-risk foods is not uncommon; some of these products have a long shelf life as frozen or dried products in which HAV can survive for at least 2–3 months (*14*,*15*), and these products can be marketed over a wide geographic region. For these reasons, foodborne HAV infections are difficult to recognize.

These surveillance challenges might discourage physicians from trying to signal foodborne outbreaks. Large outbreaks are detected because of their large numbers. Slow and dispersed clusters can be detected through use of molecular typing, which enables linking of cases that otherwise could not be recognized as a cluster (*16*,*17*).

We assessed the role of food as a source of HAV in the Netherlands, a country with low-level endemic circulation of HAV. To do so, we conducted a 2-year study in which we combined detailed epidemiologic investigation with real-time strain sequencing for reported case-patients.

Methods

Routine Surveillance

In the Netherlands, HAV infection is a reportable disease. Physicians and medical laboratories report cases to a municipal health service (MHS) according to national notification criteria: presence of a predefined set of clinical signs of hepatitis combined with HAV IgM in serum. MHS Consultants for Communicable Disease Control contact the patient and administer a questionnaire that collects routine demographic and epidemiologic data consisting of age, sex, country of birth, time of disease onset, related cases, travel history, homosexual contacts, and other possible modes of transmission (full questionnaire available on request to M.P.). MHS enters the suspected transmission route and other anonymized information into a national electronic registration system hosted by the National Institute for Public Health and the Environment (RIVM). All cases reported during July 1, 2008, through June 30, 2010, were included in the study.

Enhanced Surveillance

During the 2-year period, an enhanced surveillance system, which included systematic typing of viruses from patients, was deployed. All medical microbiological laboratories and MHSs in the Netherlands were asked to send serum samples from all reported patients to the Laboratory for Infectious Diseases Research, Diagnostics and Screening at RIVM. RNA was extracted from the serum and tested for HAV by reverse transcription PCR selective for the viral protein (VP) 1–2A region of the genome (*3*,*4*). HAV genotyping was conducted by sequencing of a 460-nt fragment of the VP1–2A region. Sequence data were stored in a Bionumerics database (Sint-Martens-Latem, Belgium). Sequencing results were merged with the national registration data, according to laboratory name and serum sample number. For cases lacking a unique serum sample number, notification data and sequences were linked by using combinations of variables to match records (birth year, 4-digit postal code, date of illness onset, date of diagnosis).

The reporting MHSs were contacted twice by telephone for interviews. We asked for the MHS conclusion as to the most probable modes of transmission immediately after the notification and then after results from sequencing were available. This approach was taken because public health measures for different transmission categories might differ (Table 1) and interventions could be adjusted accordingly. The initial interviews were also used to inform MHS about the study and to emphasize the need for collection of serum samples. The conclusions as to possible mode of transmission before and after inclusion of typing information were logged separately.

Because we used serum already available for diagnostic purposes, ethics approval was not needed. Patients were approached according to existing guidelines, and analyses maintained patient anonymity.

Sequence Analyses and Strain Comparisons

The Bionumerics database already contained patient data and strain sequences from previous studies conducted in the Netherlands (*3*–*6*) and all available sequences from GenBank (*19*). These data were used for background comparison if sequences covered a minimum of 300 nt of the VP1–2a region and if information was available on the most probable country of infection (for travelers) or other risk activities (*20,21*). The geographic fingerprints and other risk-group associations (e.g., Dutch MSM strains)

Table 1. Hepatitis A virus transmission categories and supplementary public health actions, the Netherlands, 2008–2010

Category	Description	Public health action*
Travel-associated	History of travel to a country with high, intermediate, or low HAV endemicity (*18*)	Advise on future travel precautions
Person-to-person	Local contact with HAV-infected person	Widen contact tracing to identify risk groups and vaccination (e.g., school, health care setting, homeless, travel group)
MSM	Male-with-male sex	Widen contact tracing
Foodborne	Suspected food product or food handler	Trace sources (notify the food safety authority)
Unknown	No other applicable category	No further action

*In addition to hygiene measures, vaccination of household contacts, and restriction from school or work according to national guidelines; HAV, hepatitis A virus; MSM, men who have sex with men.

Table 2. Description of reported patients with hepatitis A virus infection, by age group, the Netherlands, 2008–2010

Age group, y	Reported	Male, no. (%)	Incidence 2008–09*	Incidence 2009–10*	Sequenced, no. (%)†
0–9	78	41 (52.6)	1.6	2.4	29 (37.2)
10–19	74	37 (50.0)	2.5	1.3	48 (64.9)
20–29	73	36 (49.3)	1.7	2.0	43 (58.9)
30–39	56	35 (62.5)	1.3	1.2	42 (75.0)
40–49	79	49 (62.0)	1.4	1.7	55 (69.6)
50–59	36	18 (50.0)	0.6	1.0	23 (63.9)
60–69	17	7 (41.2)	0.5	0.5	7 (41.2)
70–79	5	3 (60.0)	0.3	0.2	1 (20.0)
80–89	3	3 (100.0)	0.0	0.5	0 (0.0)
Total	421	229 (54.4)	1.2	1.3	248 (59.0)

*Cases/100.000 population, by age category.
†Number and proportion of cases for which typing data could be obtained.

from the background data were used to classify strain sequences from patients with unknown exposure to a probable source. This association was reported to the MHS only if the association was considered robust; robust clusters consisted of at least 3 identical sequences from independent patients with the same country of infection or MSM association, branching separately in a maximum-parsimony tree with >75% reproducibility of bootstraps. Clusters were defined when the following were found: at least 2 identical sequences branching separately in a maximum-parsimony tree with >75% reproducibility of bootstraps. Maximum-parsimony trees (phylogenetic trees based on finding the simplest or minimal evolutionary change between strains) were built by using Bionumerics, and reproducibility was tested by performing 1,000 bootstraps. Cases with strains meeting this cluster definition and sharing the same suspected mode of transmission were considered confirmed clusters within the assigned transmission category.

Descriptive and Statistical Analyses

Data analyses were performed by using SAS software version 9.2/9.3 (SAS Institute, Cary, NC, USA). We described the study population by age and sex, disease incidence, and the number and percentage of patients for whom the virus could be typed. We analyzed the representativeness of age distribution for patients for whom sequencing was performed. If date of onset of disease was unknown, we used the date of diagnosis as a proxy. We compared age distribution and, when available, lag time between onset of disease and PCR diagnosis of positive and negative cases to weigh a negative result. We described the number and percentage of most probable modes of transmission in 5 categories (Table 1) before and after inclusion of typing results.

Results

A total of 421 cases were reported. Of these, serum samples could be obtained from 292 (69%) patients; HAV RNA from 248 (59%) of these samples could be typed.

Description of Cases

The 421 cases reported over the 2-year period resulted in incidence rates of 1.2 cases/100,000 population during the study year 2008–09 and 1.3 cases during 2009–10 among a total population of 16.4 million at the start of the study period and 16.5 million at the start of the second year. Most Most reported patients were in age groups from 0–9 through 40–49 years (range 13.3%–18.8% per group; Table 2). For patients in the youngest age group (0–9 years), sequenced cases were underrepresented, although distributions for patients for whom sequencing was performed did not differ significantly from reported patients (data not shown). The overall proportion of male patients was 54.4%.

MHS determined the most probable modes of transmission for 268 of the 421 reported cases before typing (64%). Travel-associated transmission dominated (141 cases), followed by person-to-person transmission (76), male-to-male sexual contact (33), and foodborne transmission (18) (Table 3).

Table 3. Hepatitis A transmission modes, the Netherlands, 2008–2010*

Transmission	No. reported	Sequenced, no. (%)			
		Total	Assigned category confirmed	Unresolved	Assigned category misclassified and reassigned
Travel-associated	141	66 (47)	40 (61)	23 (35)	3 (5)
Person-to-person	76	53 (70)	53 (100)	0	0
Male-with-male sex	33	25 (76)	23 (92)	2 (8)	0
Foodborne	18	17 (94)	7 (41)	9 (53)	1 (6)
Unknown	153	87 (57)	NA	45 (52)	42 (48)
Total	421	248 (59)	123 (50)	79 (32)	46 (19)

*NA, not applicable; if a category was assigned after sequencing, then the assumption "unknown" was misclassified.

Sequence Analyses

Of 292 samples received (69% of reported cases) PCR results for HAV were negative for 39 and positive for 253 (5 of which could not be typed and were excluded). The remaining 248 (59% of reported cases) were included in the final analysis. For 21 strains, sequencing was limited to 402–458 nt instead of the goal of 460 nt; for 1 strain, sequencing was limited to 100 nt.

Logistic regression showed that a longer lag time between onset of disease and diagnosis and belonging to the youngest or oldest age groups correlated with negative PCR results for HAV. This finding was expected because of unclear date of disease onset (data not shown).

Combined Analysis

Typing results confirmed all clusters of suspected person-to-person transmission, nearly all reported cases of male-to-male sexual transmission, and a large proportion of travel-associated infections (Table 3). One third of patients with travel-associated infections had traveled to countries with insufficient HAV sequence information in the public databases for reference. Therefore, the strain sequences for the virus in these patients could not be definitively assigned (category unresolved, Table 3).

In the category of suspected foodborne infections, nearly half of the cases for which sequencing had been performed could be confirmed. Only 1 case was misclassified; this infection was assigned to male-to-male sexual contact because the strain from this patient matched the dominant strain for MSM and the patient's sexual orientation was concordant with this finding. The remaining cases were considered unresolved because the virus sequences did not cluster with known sequence clusters in the database.

For almost half of the 87 patients with unknown mode of transmission for whom sequencing was performed, the mode of transmission was resolved according to interpretation of the typing results. A remarkably high proportion (52%) of these infections were foodborne (Table 4).

Table 4. Hepatitis A virus transmission categories after typing of 42 cases previously assigned to transmission category "unknown," the Netherlands, 2008–2010

Transmission mode	No. (%)
Travel-associated	2 (5)
Person-to-person	12 (29)
Male-with-male sex	6 (14)
Foodborne	22 (52)
Total	42 (100)

Probable Foodborne Outbreaks

Cluster 1 began with 2 cases linked to the same restaurant according to notification alone. A cook working in the restaurant had been infected by the dominant strain usually identified in MSM. He had continued working during his illness and was the probable source of infection. After genotyping and additional questioning, 2 more cases were added to this cluster.

Cluster 2 consisted of 2 cases clustered in time. Each patient had a unique genotype IA strain not previously detected, and both patients had eaten mussels.

Clusters 3 and 4 were associated with 2 consecutive outbreaks related to semidried tomatoes (12 and 5 primary cases, respectively). Cluster 3 turned out to be part of the largest foodborne outbreak thus far reported in the Netherlands, reaching 17 cases (including primary and secondary cases). The cases were clustered in time (reported in February and March) but were geographically dispersed, and the national notification rate was at an expected low level for this time of year, according to the 5 previous years. The strain sequences clustered with those from a large outbreak (at least 144 cases) in Australia and an outbreak (59 cases) in France, both of which were associated with consumption of semidried tomatoes (*22–24*). Cluster 4 was caused by a genotype IB strain closely resembling the strain involved in cluster 3.

Cluster 5 consisted of 1 case in a food handler of a dinner and 5 secondary cases. Cluster 6 consisted of 5 cases that were clustered strongly in time and for which virus strain sequences were identical, but the cases were geographically dispersed. Although the strain sequences were similar to those of strains typically detected in travelers returning from Morocco, the patients reported no travel history and no contact with patients with HAV infection imported from Morocco. Moreover, they clustered in April, a time of year when secondary or tertiary infections following travel-related imported cases are rare (*8*). Therefore, this cluster was considered a point-source—and very probably foodborne—cluster, although a source could not be determined.

Of 29 foodborne cases confirmed by a combination of epidemiologic and typing information (7 previously suspected foodborne and 22 previously unknown source), 20 additional reports were made. These cases were reported to the national food safety authority and international alerts through the Rapid Alert System for Food and Feed and Early Warning and Response System of the European Commission and the European Centre for Disease Prevention and Control.

Unresolved Cases

For 45 (52%) cases initially reported as having no known source of infection (Table 3), conclusive evidence for a source was not found despite molecular typing. Nevertheless, some clustering occurred among these unresolved cases. The dominant MSM strain was found in 11 patients; however, these patients were not epidemiologically linked (time, place, food consumption), and among them were women and children, indicating spillover from the MSM

risk group to the general population. Several other strains matched background strains previously imported from or known to circulate in Morocco and Egypt and even an outbreak strain from the Czech Republic (*25*). None of these patients had a history of travel. This finding could indicate unnoticed endemic transmission from persons with imported cases, although transmission through food or food handlers could not be excluded.

Discussion

Use of real-time enhanced molecular surveillance of HAV infections for 2 years enabled us to identify food-associated infections that had not been recognized through regular investigations by MHS. We confirmed almost half of the suspected foodborne cases and resolved a quarter of cases with initially unknown source of infection as probable foodborne infections. Among these infections was an outbreak associated with semidried tomatoes, which was part of an international outbreak. This outbreak would not have been detected without genotyping because baseline surveillance did not generate a signal (*22*). Together, confirmed and unresolved foodborne infections explained 16% of 248 cases for which typing had been performed as opposed to the 4% that had been suspected on the basis of epidemiologic investigation alone. Furthermore, we were able to lower the proportion of cases with unknown mode of transmission from 35% to 18%. On the basis of these findings, we conclude that virus typing is useful for the detection of foodborne outbreaks and, more generally, for the explanation of cases with unknown mode of transmission.

A strength of our study is the representativeness of the study population. In the Netherlands, HAV incidence remained steady at a very low endemic level of ≈200 reported cases per year during 2005–2011 (*26*). Not only did we gather all notification data; we received 69% of patient serum samples. Age distribution was in accordance with the susceptibility of the population of the Netherlands (*27*) and with the distribution described in neighboring Germany (*28*). This study provides a realistic estimation of the incidence of foodborne infection in the Netherlands and maybe in industrialized countries with low HAV endemicity in general, although varying between years with typical epidemic rather than endemic occurrence. The age distribution indicates a risk that food handlers will have an infection and become a source of foodborne infections. Of note, the proportion of foodborne infections was comparable to the proportion of infections among MSM; both types of infection can be epidemic and sporadic.

Real-time investigation of cases enabled us to compare the conclusions that were drawn on interviews alone before typing with those drawn after receiving typing results. The hierarchy of assigning the most probable mode of transmission based on interviews was not standardized, supporting the need for more robust information. Previous studies conducted in industrialized countries have provided insight into nationwide epidemiology supported by molecular typing data (*11*,*28*–*32*), although these studies have not been set up to direct the source tracing. Our study was able to detect foodborne clusters despite the long lag time between infection and notification (average 6 weeks). Routine implementation of standard food-consumption questionnaires at first patient contact and prompt forwarding of serum samples from HAV IgM–positive patients for typing can probably reduce the lag time.

Although we focused on foodborne infections, we have other findings to share. Nearly 100% of suspected cases of person-to-person and male-to-male sexual transmission of HAV could be confirmed; however, additional cases and previously undetected clusters surfaced after sequencing from the category "unknown." Interventions were altered accordingly, which resulted in 8 additional screening or vaccination actions (data not shown). Only 61% of cases of travel-associated transmission could be confirmed. We have no reason to doubt the patients' travel history. The most likely explanation is a lack of robust molecular information from many countries. Secondary or additional cases acquired through contact with persons with unnoticed primary cases indeed proved to be part of the explanation for cases with unknown transmission, as expected, although we have shown that this was not the only explanation. In another study, we will aim to combine our data on travel-related risk with data on travel behavior.

A proportion of cases left with unknown source of infection could still have been sporadic foodborne infections or part of undetected international clusters. We are only marginally able to detect such clusters, despite the existence of a shared database provided by GenBank and the early warning networks among public health services and food authorities (Rapid Alert System for Food and Feed and Early Warning and Response System). This marginal ability at least partly results from the fact that typing is often not a structural part of a national surveillance system, and if it is, there is no international consensus on the location and length of the sequenced part of the HAV genome. GenBank offers many more strain sequences for comparison based on shorter sequences (<300 nt) or from different gene fragments, but the robustness of clustering decreases with fragment length (*16*). In addition, metadata in GenBank are often lacking, thereby limiting the usefulness of this repository for molecular epidemiologic studies.

A weakness of our study is that it was not designed to provide estimates for the number of cases prevented. For foodborne outbreaks, altering production processes with risk for contamination or withdrawal of (frozen) products from the market can substantially reduce the number of new cases. The described international outbreak did result

in the evaluation of the manufacturing process of semidried tomatoes and a warning published by the food safety authority to inform retailers about risky products. As further illustration of a possible cost benefit, 2 previously healthy persons who were part of a foodborne cluster each needed liver transplantation because of fulminant hepatitis; the costs associated with this treatment alone greatly exceed the costs of 2 years of typing all HAV cases.

A challenge associated with responding to foodborne illness outbreaks is that detection of pathogens in food products typically is requested as support for control activities by a food safety authority. The national food safety authority was not able to confirm any of the suspected foodborne clusters for several reasons but particularly because food leftovers were sparsely available (in part because of the long incubation period), and virus detection in food is challenging (*13*). Nevertheless, contamination of semidried tomatoes with HAV was actually confirmed in the related outbreak in Australia (*23*), and there are examples of HAV infection caused by consumption of food that was contaminated through contact with an infected food handler (*33*) or fecal contamination during food production (e.g., for shellfish or green onions) (*34–36*). The largest known outbreak, in Shanghai in 1988, resulted in >250,000 cases linked to the consumption of clams (*37*).

Molecular typing of HAV in patient serum is not routinely performed, and strain typing information is not included in notifications. Combining typing results with anonymized notification data proved to be challenging in our surveillance system. We might have been unable to merge some cases with their typing results because of a lack of unique identifiers, although we believe that this inability to merge cases and typing information occurred randomly and would not have substantially influenced the study results. According to our data, we advise revision of HAV surveillance so that it also provides baseline information to support foodborne illness detection. The revised system should also include mechanisms for rapid exchange of this information internationally, to enhance the ability to detect diffuse outbreaks (*38*). With the fast development and decreasing cost of sequencing technology, routine collection of these types of data will become realistic in the near future and will provide added value for public health work provided such data-sharing mechanisms are developed (*39*). We have recently implemented this recommendation in our national guidelines.

We also recommend that strains uploaded to GenBank be accompanied, at least, by information about time (date of diagnosis or disease onset rather than by date of submission) and space (country where infection most likely was acquired rather than country from which infection was reported). Sufficient molecular background information is needed to be able to notice a distinct cluster. Therefore, broad sampling, data sharing, and centralized interpretation of data should be part of an enhanced surveillance system. The previously described foodborne outbreaks have already proved the usefulness of national and international exchange of epidemiologic and sequence data.

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Dr Petrignani is a medical doctor who works as a liaison officer for the Dutch Center of Infectious Disease Control at the Municipal Health Service of Rotterdam. She is also a PhD candidate at the Erasmus Medical Center, Rotterdam. Her research interests are public health and infectious disease control.

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Address for correspondence: Mariska Petrignani, GGD Rotterdam-Rijnmond, Schiedamsedijk 95, Postbus 70032, 3000 LP Rotterdam, the Netherlands; email: m.petrignani@rotterdam.nl

Travel-associated Antimicrobial Drug–Resistant Nontyphoidal Salmonellae, 2004–2009

Russell S. Barlow, Emilio E. DeBess, Kevin L. Winthrop, Jodi A. Lapidus, Robert Vega, and Paul R. Cieslak

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Describe the epidemiology and clinical characteristics of nontyphoidal *Salmonella* (NTS) infections
- Distinguish antibiotic resistance associated with NTS cases in Oregon between 2004 and 2009
- Identify factors positively and negatively associated with antibiotic resistance among NTS cases in Oregon between 2004 and 2009.

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Authors

Disclosures: ***Russell S. Barlow, MPH; Emilio E. DeBess, DVM, MPVM; Jodi A. Lapidus, PhD; Robert Vega, MS(SM,AAM);*** *and* ***Paul R. Cieslak, MD,*** *have disclosed no relevant financial relationships.* ***Kevin L. Winthrop, MD, MPH,*** *has disclosed the following relevant financial relationships: served as an advisor or consultant for Pfizer, UCB, Genentech, AbbVie, Regeneron; received grants for clinical research from Pfizer.*

To evaluate trends in and risk factors for acquisition of antimicrobial-drug resistant nontyphoidal *Salmonella* infections, we searched Oregon surveillance data for 2004–2009 for all culture-confirmed cases of salmonellosis. We defined clinically important resistance (CIR) as decreased susceptibility to ampicillin, ceftriaxone, ciprofloxacin, gentamicin, or trimethoprim/sulfamethoxazole. Of 2,153 cases, 2,127 (99%) nontyphoidal *Salmonella* isolates were obtained from a specific source (e.g., feces, urine, blood, or other normally sterile tissue) and had been tested for drug susceptibility. Among these, 347 (16%) isolates had CIR. The odds of acquiring CIR infection significantly increased each year. Hospitalization was more likely for patients with than without CIR infections. Among patients with isolates that had been tested, we analyzed data from 1,813 (84%) who were interviewed. Travel to eastern or Southeast Asia was associated with increased CIR. Isolates associated with outbreaks were less likely to have CIR. Future surveillance activities should evaluate resistance with respect to international travel.

Author affiliations: Oregon Health Authority, Portland, Oregon USA (R.S. Barlow, E.E. DeBess, P.R. Cieslak); Oregon Health and Science University, Portland (R.S. Barlow, K.L. Winthrop, J.A. Lapidus); and Oregon State Public Health Lab, Hillsboro, Oregon, USA (R. Vega)

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Each year, nontyphoidal salmonellae (NTS) are responsible for >1 million infections in the United States and an estimated 98 million cases globally (*1*–*4*). Each year in the United States, infections result in an estimated 168,000 physician visits, 19,000 hospitalizations, and 380 deaths at a cost of $US 2.3 billion (*1*–*3*,*5*). Data suggest that 85.6% of NTS infections are foodborne and that the remaining infections occur by the fecal–oral route in human-to-human transmission and zoonotic transmission (*2*). For healthy persons, infections commonly result in self-limiting acute gastroenteritis that resolves without antimicrobial drug therapy. However, antimicrobial drugs can be life saving for immunologically vulnerable populations, such as infants, elderly persons, immunocompromised persons, and persons with invasive infection (*6*–*8*). The drugs most commonly prescribed in developing countries are ampicillin and chloramphenicol; those most commonly prescribed in the United States are trimethoprim/sulfamethoxazole, fluoroquinolones, and cephalosporins (*9*).

In the 1980s, studies demonstrated alarming increases in the prevalence of antimicrobial drug resistance among NTS infections (*10*,*11*). This increase was associated with indiscriminate use of antimicrobial drugs in animal husbandry and in humans (*10*–*12*). A retrospective study conducted during 1996–2001 associated antimicrobial drug resistance with increased disease severity, highlighting the risk to public health (*13*).

During the past decade, few population-level analyses have identified risk factors for acquiring a resistant NTS infection outside of outbreak clusters and retail meat supplies. A recently identified risk factor is international travel (*14*,*15*). Bacteriologic studies from Europe identified differences in resistance among *Salmonella enterica* serotypes Stanley, Concord, and Typhimurium isolated from patients with a history of international travel (*16*–*20*). However, these studies did not estimate the magnitude of or risk for antimicrobial drug–resistant NTS acquisition among international travelers. We hypothesized that international travel is a risk factor for acquisition of a resistant NTS infection. To test our hypothesis, we analyzed surveillance data from Oregon for 2004–2009 and quantified trends in antimicrobial drug resistance, investigated the relationship between resistance and case outcomes, and assessed whether international travel was associated with acquisition of NTS infections with clinically important resistance (CIR).

Methods

Reportable Infectious Disease Surveillance in Oregon

The Oregon Health Authority conducts active, laboratory-based surveillance for all cases of NTS infection. Physicians and laboratories are required by law to report laboratory-confirmed and clinically suspected cases of salmonellosis to the patient's local health department; reports should contain the patient's date of birth, sex, diagnosis, date of symptom onset, date of specimen collection, and laboratory test results. All *Salmonella* isolates are forwarded to the Oregon State Public Health Laboratory (OSPHL), where they are serotyped. Local health department officials interview patients about hospitalization, clinical outcomes, additional demographic information, and exposure history for the 7 days before illness onset. Risk-factor questions ask about specific travel, human, animal, and high-risk food exposures. International travel was considered a risk factor only if it had taken place in the 30 days before illness onset. Patients with recurrent infection or multiple *Salmonella* isolates (of same serotype within a plausible time frame for the original infection) are interviewed only once, at the time of initial illness onset.

During 2004–2009, the population of Oregon was 3.6–3.8 million persons, which is ≈1.2% of the US population (*21*,*22*). The surveillance system in Oregon is estimated to capture >99% of laboratory-confirmed cases of salmonellosis; however, for every 1 case confirmed, an estimated 25 additional cases are not detected (*2*).

Antimicrobial Drug Susceptibility Testing

For 2004 and 2005, all confirmed isolates were forwarded to the Oregon State University Veterinary Diagnostic Laboratory for susceptibility testing. From 2006 through 2009, susceptibility testing was performed by OSPHL. All isolates were tested by using broth microdilution to determine MICs for the following 10 antimicrobial agents: ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, nitrofurantoin, sulfamethoxazole, tetracycline, and trimethoprim/sulfamethoxazole. Susceptibilities were determined according to Clinical and Laboratory Standards Institute interpretative criteria (*23*). To ascertain cephalosporin resistance, OSPHL tested isolates for ceftriaxone susceptibility; and the Oregon State University Veterinary Diagnostic Laboratory tested for susceptibility to cefuroxime and cephalothin by using analogous broth microdilution methods. MIC results were dichotomized as susceptible or resistant.

Analyses

Isolates were included in analyses only if they were cultured from specific specimens, such as feces, urine, or blood or other normally sterile tissues (i.e., cerebrospinal fluid). CIR was defined as resistance to at least 1 of the following: ampicillin, ceftriaxone, ciprofloxacin, gentamicin, or trimethoprim/sulfamethoxazole (*13*). We used the Cochran-Armitage test for trend to analyze NTS case data for 2004–2009 to determine whether the proportion of *Salmonella* isolates with CIR increased significantly. Demographic and

exposure risk factors, specifically international travel, were evaluated as risk factors for acquisition of a resistant isolate. The 9 most common *Salmonella* serotypes were fixed (included in all models regardless whether they met the $p<0.05$ level of significance) in all analyses, and remaining serotypes were grouped as "all other." Serotype Enteritidis is the most frequently isolated serotype in Oregon and was therefore used as the referent for all comparisons.

We sought to evaluate whether resistance was associated with increased disease severity, including hospitalization and invasive infection. Invasive infection was defined as isolation of *Salmonella* from a normally sterile body site or tissue, such as blood (*13*). Multiple logistic regression models were constructed with variables that were significant at the $p<0.25$ level in unadjusted analyses. *Salmonella* serotype and patient race, age, and year of illness onset were fixed in all models. Other variables were given further consideration according to disease severity or relevance for external validity. Predictor variables significant at $p<0.05$ were retained in the final model, and adjusted log odds ratios (aORs) were calculated. Model fit was assessed by using the Hosmer–Lemeshow goodness-of-fit test.

All analyses were performed by using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Because this study involved more extensive analysis only of data collected routinely as part of public health surveillance, it was not considered human subjects research.

Results

Descriptive Epidemiology and Resistance Trends

From 2004 through 2009, a total of 2,255 laboratory-confirmed cases of nontyphoidal salmonellosis were reported in Oregon. In accordance with Oregon law, 2,153 isolates were forwarded to OSPHL, and 2,127 (98.8% of all NTS isolates) were cultured from a specific source and had antimicrobial drug susceptibility testing information (Figure). Of these isolates, 26 (1.2%) were obtained through a nonspecific source, such as lesions or sputum, and were excluded from analysis.

The most common *Salmonella* serotypes detected were Enteritidis (18.4%), Typhimurium (14.3%), Heidelberg (8.2%), Typhimurium var. Copenhagen (5.1%), and Newport (4.5%). Cases that were part of identified outbreaks represented 24.1% of the cohort; the remaining 75.9% were considered sporadic cases. The median age of patients was 29 years (interquartile range 9–51 years), and 53.1% of patients were female. Information about race was not available for 9%; of patients for whom race was known, 91.8% were white and 9.2% were not white. Similarly, information about ethnicity was not available for 10.5% of patients. Among patients for whom ethnicity was known, 87.4% were not Hispanic and 12.6% were Hispanic. Isolates from 1,213 (57%) patients were susceptible to all antimicrobial drugs screened (pansusceptible), and isolates from 347 (16.3%) had CIR (Table 1). Of the 2,127 patients,

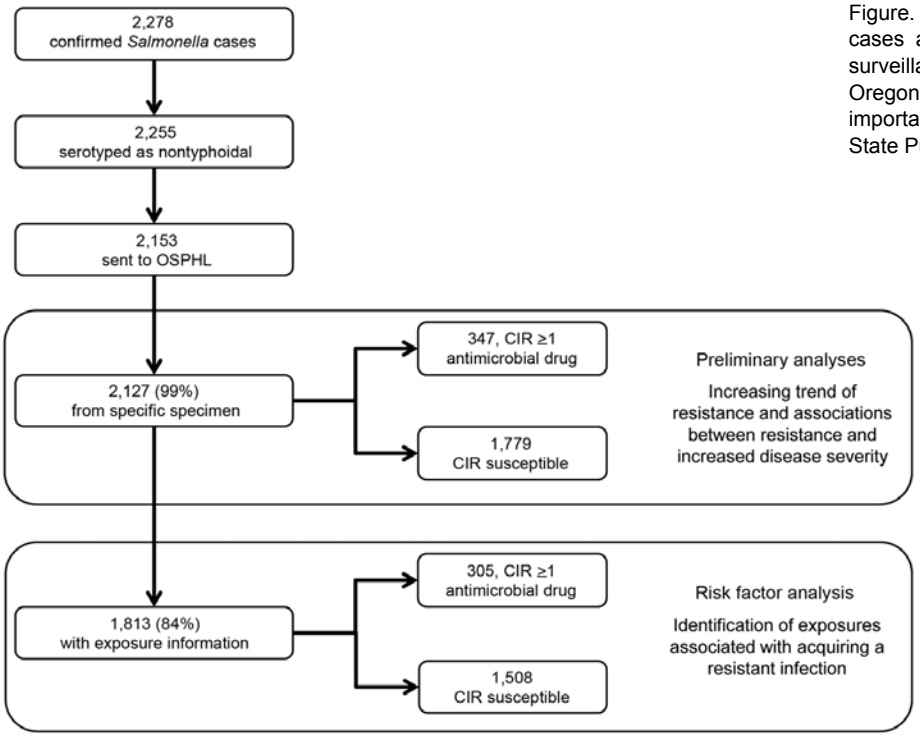

Figure. Culture-confirmed salmonellosis cases ascertained by statewide active surveillance and included in analyses, Oregon, USA, 2004–2009. CIR, clinically important resistance; OSPHL, Oregon State Public Health Laboratory.

Table 1. Frequencies of antimicrobial drug resistance among 2,127 *Salmonella* isolates, Oregon, USA, 2004–2009*

Variable	Resistance, no. (%)
Drug	
Ampicillin	285 (13.4)
Ceftriaxone	109 (5.1)
Chloramphenicol	177 (8.3)
Ciprofloxacin	13 (0.6)
Gentamicin	84 (4.0)
Nalidixic acid	135 (6.4)
Nitrofurantoin	283 (13.3)
Sulfamethoxazole	411 (19.3)
Tetracycline	574 (27.0)
Trimethoprim/ sulfamethoxazole	60 (2.8)
Resistance profiles	
Pansusceptible	1,213 (57.0)
CIR	347 16.3)

*CIR, clinically important resistance to ≥1 of the following: ampicillin, ceftriaxone, ciprofloxacin, gentamicin, or trimethoprim/sulfamethoxazole.

412 (19.4%) were hospitalized and 110 (5.2%) had invasive disease.

The proportion of isolates that were pansusceptible significantly decreased from 69.5% in 2004 to 53.6% in 2009 (p<0.01). CIR did not significantly increase during this study period (p = 0.27). Stratification by serotype revealed that CIR increased among the 3 most common serotypes: Enteritidis (3% to 8%, p = 0.02), Typhimurium (19% to 34%, p = 0.03), and Heidelberg (6% to 30%, p<0.01). Significant in-creases were identified for resistance to ciprofloxacin (p<0.05), nalidixic acid (p<0.01), sulfamethoxazole (p<0.01), tetracycline (p<0.01), and trimethoprim/sulfamethoxazole (p<0.01). Cephalosporin resistance increased, although not significantly (p = 0.06).

We suspected that these findings were confounded by serotype and therefore used logistic regression to model the odds of acquiring a resistant infection for each of the clinically important antimicrobial drugs (ampicillin, ceftriaxone, ciprofloxacin, gentamicin, or trimethoprim/sulfamethoxazole) as well as CIR. Serotype-adjusted log odds ratios were generated with year of infection entered as a discrete continuous variable. After adjusting for serotype, we found that with each subsequent year, patients were 30% more likely to acquire an infection that was resistant to quinolones (nalidixic acid or ciprofloxacin) and trimethoprim/sulfamethoxazole (Table 2). Resistance to ampicillin and cephalosporin also increased, although not significantly. We also found that with each year, odds of acquiring an infection with CIR increased by 13%.

CIR was associated with hospitalization (odds ratio [OR] 1.5, 95% CI 1.1–2.0). This association was preserved after adjustment for serotype, patient age, patient race, and year (aOR 1.7, 95% CI 1.2–2.1). For patients with CIR infections, odds of invasive infection were increased, although not significantly, according to unajusted or adjusted analyses (OR 1.4, 95% CI 0.9–2.2 and aOR 1.5, 95% CI 0.9–2.5, respectively).

Risk Factors

Of the 2,127 patients included in the previous analyses, 1,813 (84.2% of all Oregon patients with NTS) were interviewed. For 305 (16.8%) of these patients, isolates had CIR, and for the remaining 1,508 (83.2%), isolates were susceptible to all clinically important antimicrobial drugs (Figure). Of the 1,508 isolates susceptible to clinically important drugs, 1,002 (55.3%) were susceptible to all drugs screened and 506 (27.9%) were resistant to at least 1 non-CIR drug.

According to the unadjusted analysis, several serotypes were more likely than the referent serotype, Enteritidis, to be resistant to ≥1 clinically important antimicrobial drug (Table 3). Patient sex, race, age, and ethnicity were not significantly associated with resistance.

CIR was not associated with any other demographic risk factors or high-risk food or animal exposures. However when international travel was examined by individual countries or applicable United Nations region, CIR was significantly associated with travel to Southeast Asia (*24*). Associations of resistance with travel to Mexico and eastern Asia also approached significance (Table 4). The most common travel destinations in Asia where resistant infections were acquired were Thailand, China, and Malaysia/ Indonesia. On the basis of these findings, we analyzed international travel by 3 destinations: Central America, including Mexico (135 patients), eastern and Southeast Asia (46 patients), and all other international travel destinations (77 patients).

Patients who were part of identified outbreak clusters were significantly less likely than patients with sporadic infections to have a resistant infection (OR 0.5, 95% CI 0.4–0.8). During our study period, 131 outbreaks (406 cases) occurred, and for 25 of these outbreaks a causative vehicle was successfully identified. To assess whether oversampling of cases from outbreak clusters could explain this association, we first restricted cases to 1 isolate per outbreak where a causative vehicle was implicated while retaining all cases from outbreaks for which a vehicle was not implicated (302 cases). Second, we further restricted cases to 1 isolate per outbreak, regardless whether a vehicle was

Table 2. Serotype-adjusted odds of salmonellosis with resistance to specific antimicrobial drugs per year, Oregon, USA, 2004–2009*

Variable	Odds ratio (95% CI)†
Ampicillin	1.1 (1.0–1.1)
Cephalosporins	1.2 (1.0–1.6)
Gentamicin	1.0 (0.9–1.2)
Quinolones‡	**1.3 (1.1–1.5)**
Trimethoprim/sulfamethoxazole	**1.3 (1.1–1.5)**
CIR	**1.1 (1.0–1.2)§**

*Multiple logistic regression analysis of 2,127 isolates. Boldface indicates statistical significance at p<0.05.
†Serotype adjusted; increased odds of resistance per year, 2004–2009.
‡Resistant to naladixic acid or ciprofloxacin.
§p<0.01.

Table 3. Associations of salmonellosis with CIR, Oregon, 2004–2009*

Variable	No. patients	% CIR isolates	Odds ratio (95%CI)	Adjusted odds ratio (95% CI)
Patient travel history				
No international travel	1,571	16.2	Referent	Referent
Travel to Asia	**46**	**39.1**	**3.3 (1.8–6.1)**	**5.2 (2.6–10.4)**
Case type				
Sporadic	1407	18.6	Referent	Referent
Outbreak	**406**	**10.8**	**0.5 (0.4–0.8)**	**0.5 (0.4–0.7)**
Year (odds of CIR cases/y)	**1,813**	**16.8**	**1.0 (0.9–1.1)**	**1.1 (1.0–1.2)**
Salmonella serotype				
Enteritidis	334	6.0	Referent	Referent
Typhimurium	**260**	**25.0**	**5.2 (3.1–8.9)**	**6.2 (3.6–10.7)**
Heidelberg	**149**	**27.5**	**6.0 (3.3–10.6)**	**7.4 (4.1–13.5)**
Typhimurium var. Copenhagen	**92**	**53.3**	**17.9 (9.7–32.9)**	**20.2 (10.7–38.0)**
Newport	**82**	**41.5**	**11.1 (5.9–20.9)**	**10.8 (5.6–20.5)**
I 4, 5, 12:i:-	**79**	**19.0**	**3.7 (1.8–7.6)**	**4.1 (2.0–8.7)**
Montevideo	72	4.2	0.7 (0.2–2.4)	0.8 (0.2–2.7)
Saintpaul	**52**	**15.4**	**2.9 (1.2–6.9)**	**3.4 (1.4–8.3)**
Paratyphi B var. L+ Tartrate+	**50**	**24.0**	**5.0 (2.2–10.9**	**5.8 (2.6–13.1)**
All other	643	9.0	1.6 (0.9–2.6)	1.5 (0.9–2.5)
Patient age, y				
18–64	999	16.0	Referent	Referent
<1	122	18.9	1.2 (0.8–2.0)	1.4 (0.9–2.4)
1–4	220	16.4	1.0 (0.7–1.5)	0.8 (0.5–1.2)
5–17	279	20.1	1.3 (0.9–1.8)	1.0 (0.7–1.5)
≥65	193	15.5	1.0 (0.6–1.5)	0.9 (0.6–1.4)
Patient race				
White	1,662	16.7	Referent	Referent
Not white	151	18.5	1.1 (0.7–1.8)	1.0 (0.6–1.6)

*Multiple logistic regression analysis of 1,813 patients; CIR, clinically important resistance to ≥1 of the following: ampicillin, ceftriaxone, ciprofloxacin, gentamicin, or trimethoprim/sulfamethoxazole. Boldface indicates statistical significance at p<0.05.

identified (131 cases). In each of these analyses the magnitude, direction, and significance of the association was preserved (OR 0.6, 95% CI 0.4–0.8 and OR 0.5, 95% CI 0.3–0.9, respectively), suggesting that oversampling could not have explained this association. Furthermore, 53.6% of outbreaks had intra-outbreak cases for which the antimicrobial drug susceptibility profiles of the isolates differed.

The resultant main-effects model included the fixed variables of serotype, patient age, year of onset, and patient race, along with travel to eastern or Southeast Asia, and outbreak association (Table 3). We hypothesized that effect modification occurred between the variables of outbreak cases and travel to eastern or Southeast Asia, as well as between travel to eastern or Southeast Asia, serotypes, and outbreaks. However, no significant interactions at the p<0.25 level were identified. No other variables or exposures were significantly associated with CIR. This model had excellent goodness-of-fit (p = 0.87). The association between resistance and travel to eastern or Southeast Asia was preserved after exclusion of all outbreak-associated cases (aOR 4.6, 95% CI 2.3–9.4; Table 5). Similarly, the association between resistance and outbreak-associated cases was preserved after exclusion of patients with a history of international travel (aOR 0.5, 95% CI 0.4–0.8; Table 6). Therefore, these results suggest that inclusion of patients with a history of travel to Asia, as well as patients with outbreak-associated infections for the main analysis, was appropriate.

Patients with a history of recent travel to eastern or Southeast Asia were >5 times more likely to acquire a CIR infection than were patients with no history of recent international travel. The most common serotypes acquired among persons with a history of travel to Asia were Enteriditis (n = 13, 54% CIR), Typhimurium (n = 5, 60% CIR), Newport (n = 4, 25% CIR), I 4, 5, 12:i:- (n = 4, 50% CIR), Stanley (n = 3, 33% CIR), and Typhimurium var. Copenhagen (n = 2, 100% CIR). Patients with outbreak-associated infections were half as likely as those with sporadic infections to have CIR (Table 3).

To identify risk factors for resistance to individual antimicrobial drugs, we constructed models with each of the clinically important antimicrobial drugs. Travel to eastern or Southeast Asia was significantly associated with resistance to ampicillin, quinolones (nalidixic acid or ciprofloxacin), and trimethoprim/sulfamethoxazole (Table 7). Only individual serotypes were associated with resistance to cephalosporins or gentamicin, and no other risk factors were significantly associated with resistance to ampicillin, quinolones, or trimethoprim/sulfamethoxazole.

Discussion

We found that NTS infections were more likely to have CIR with each subsequent year of our study. In Oregon during 2004–2009, the proportion of isolates susceptible to all antimicrobial drugs significantly decreased. Travel to eastern and Southeast Asia was associated with

Table 4. Unadjusted associations of CIR of *Salmonella* isolates among 1,813 patients, by travel destination, Oregon, 2004–2009*

Destination	No. patients	% CIR isolates	Odds ratio (95% CI)
None	1,571	16.8	Referent
Mexico	119	9.2	0.5 (0.3–1.0)
Southeast Asia	**29**	**41.4**	**3.5 (1.7–7.4)**
Europe	25	16.0	0.9 (0.3–2.8)
East Asia	17	35.3	2.7 (1.0–7.4)
Caribbean	16	12.5	0.7 (0.2–3.1)
Central America†	16	6.3	0.3 (0.1–2.5)
Africa	10	20.0	1.2 (0.3–5.9)
Oceania	5	20.0	1.2 (0.1–11.1)
Canada	5	40.0	3.3 (0.5–19.8)
Any travel	242	16.9	1.0 (0.7–1.4)

*CIR, clinically important resistance to ≥1 of the following: ampicillin, ceftriaxone, ciprofloxacin, gentamicin, or trimethoprim/sulfamethoxazole. Boldface indicates statistical significance at p<0.05.
†Excludes Mexico.

acquisition of *Salmonella* with CIR. Such travel was specifically associated with resistance to ampicillin, quinolones, and trimethoprim/sulfamethoxazole. Isolates from patients who were part of identified outbreak clusters were significantly less likely to be resistant, suggesting that resistance estimates based on outbreak cases alone may underestimate the true level of resistance. We also report that resistance is associated with increased hospitalization (*13*,*25*).

Our analysis was performed by using *Salmonella* susceptibility data from a surveillance system that captures ≈100% of confirmed infections, has antimicrobial drug susceptibility information for >95% of confirmed cases, and includes exposure histories for >84% of patients. This study is strengthened by having collected data on several known and potential confounders before the drug-susceptibility profiles were known. Our study design complements a previous National Antimicrobial Resistance Monitoring System/FoodNet study that analyzed antimicrobial drug resistance and increased disease severity (*13*). However, we determined where resistant infections were acquired (exposures) and patient outcomes associated with resistant infections by using an entire population. The ability to integrate resistance and serotype data with case-specific demographic and risk-factor data improves the generalizability and plausibility of our study and provides population-level risk estimates (*14–20*).

Widespread quinolone resistance in Southeast Asia has been reported (*26*); a better understanding of global use of antimicrobial drugs might suggest where resistant salmonellae are prevalent. Examination of serotype profiles among patients who had traveled to eastern or Southeast Asia and multivariate analyses adjusted for serotype provided strong evidence that the increased resistance in this region is widespread and not specifically attributable to a single serotype or regional serotype differences.

Table 5. Associations of salmonellosis with CIR among 1,407 sporadic cases only, Oregon, 2004–2009*

Variable	No. patients	% CIR isolates	Odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Patient travel to Asia				
No	1,363	17.9	Referent	Referent
Yes	**44**	**38.6**	**2.9 (1.6–5.4)**	**4.6 (2.3–9.4)**
Year (odds of CIR cases/y)	**1,407**	**18.6**	**1.0 (0.9–1.1)**	**1.1 (1.0–1.2)**
Salmonella serotype				
Enteritidis	254	7.5	Referent	Referent
Typhimurium	**179**	**31.8**	**5.8 (3.3–10.2)**	**6.4 (3.6–11.5)**
Heidelberg	**94**	**33.0**	**6.1 (3.2–11.5)**	**6.9 (3.6–13.2)**
Typhimurium var. Copenhagen	**81**	**54.3**	**14.7 (7.8–27.9)**	**17.3 (8.9–33.4)**
Newport	**78**	**42.3**	**9.1 (4.7–17.3)**	**9.6 (4.9–18.6)**
I 4, 5, 12:i:-	**60**	**23.3**	**3.8 (1.8–8.0)**	**4.2 (1.9–9.0)**
Montevideo	48	2.1	0.3 (0.0–2.0)	0.3 (0.0–2.2)
Saintpaul	35	14.3	2.1 (0.7–5.9)	2.3 (0.8–6.7)
Paratyphi B var. L+ Tartrate+	**36**	**19.4**	**3.0 (1.2–7.7)**	**3.2 (1.2–8.5)**
All other	542	9.2	1.3 (0.7–2.2)	1.2 (0.7–2.1)
Patient age, y				
18–64	785	17.7	Referent	Referent
<1	94	21.3	1.3 (0.7–2.1)	1.6 (0.9–2.8)
1–4	156	18.0	1.0 (0.6–1.6)	0.7 (0.4–1.2)
5–17	209	21.1	1.2 (0.8–1.8)	0.9 (0.6–1.4)
≥65	163	18.4	1.0 (0.7–1.6)	1.1 (0.7–1.7)
Patient race				
White	1,295	18.1	Referent	Referent
Not white	112	24.1	1.4 (0.9–2.3)	1.3 (0.8–2.2)

*Multiple logistic regression analysis. CIR, clinically important resistance to ≥1 of the following: ampicillin, ceftriaxone, ciprofloxacin, gentamicin, or trimethoprim/sulfamethoxazole. Boldface indicates statistical significance at p<0.05.

Table 6. Associations of salmonellosis with CIR for 1,571 patients, excluding patients with history of international travel, Oregon, 2004–2009*

Variable	No. patients	% CIR isolates	Odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Salmonella serotype				
Enteriditis	232	4.3	Referent	Referent
Typhimurium	**245**	**22.5**	**5.6 (2.8–11.5)**	**6.9 (3.4–14.0)**
Heidleberg	**147**	**27.2**	**6.9 (3.3–14.6)**	**9.1 (4.3–19.0)**
Typhimurium var. Copenhagen	**85**	**52.9**	**19.7 (8.9–43.4)**	**26.0 (12.0–56.4)**
Newport	**66**	**48.5**	**16.1 (7.0–36.8)**	**19.3 (8.7–43.1)**
I 4, 5, 12:i:-	**77**	**18.2**	**3.3 (1.4–8.0)**	**5.1 (2.2–12.1)**
Montevideo	70	4.3	0.7 (0.2–2.8)	1.0 (0.3–3.8)
Saintpaul	**43**	**18.6**	**5.8 (2.0–16.7)**	**5.5 (2.0–15.1)**
Paratyphi B var. L+ Tartrate+	**45**	**26.7**	**6.3 (2.5–16.1)**	**9.0 (3.5–22.6)**
All other	561	8.0	1.6 (0.8–3.3)	1.8 (0.9–3.6)
Case type				
Sporadic	1190	18.7	Referent	Referent
Outbreak	**381**	**11.0**	**0.6 (0.4–0.8)**	**0.5 (0.4–0.8)**
Year (odds of CIR cases/y)	**1571**	**16.8**	**1.1 (1.0–1.2)**	**1.1 (1.0–1.2)**
Age, y				
18–64	817	16.5	Referent	Referent
<1	119	18.5	1.1 (0.7–1.9)	1.3 (0.8–2.3)
1–4	204	16.2	0.9 (0.6–1.4)	0.8 (0.5–1.2)
5–17	245	18.8	1.1 (0.8–1.6)	0.9 (0.6–1.4)
≥65	186	15.1	0.8 (0.5–1.3)	0.8 (0.5–1.4)
Race				
White	1662	16.7	Referent	Referent
Not white	151	18.5	1.2 (0.7–1.9)	1.1 (0.7–1.8)

*Multiple logistic regression analysis. CIR, clinically important resistance to >1 of the following: ampicillin, ceftriaxone, ciprofloxacin, gentamicin, or trimethoprim/sulfamethoxazole.clinically important resistance. Boldface indicates statistical significance at p<0.05.

Increasing antimicrobial drug resistance has widespread implications for human health. We confirm the results of Varma et al. and Lee et al., who found antimicrobial drug resistance to be associated with increased likelihood of hospitalization (*13*,*25*). More severe infections can lead to treatment failure, sepsis, meningitis, and even death. If resistance to clinically important antimicrobial drugs continues to increase by 13% per year, as our data suggest, we can expect more severe illnesses, hospitalizations, and deaths, along with the accompanying higher economic costs.

The association between resistance and outbreak cases persisted after restricting the data in unadjusted and adjusted analyses. The lack of effect modification between outbreak cases and a history of travel to Asia in the multiple logistic regression modeling suggests that this finding is independent of travel. Resistant isolates might be less infectious and therefore less likely to cause recognizable outbreaks. Alternatively, common sources of resistant isolates might be less likely to cause widespread contamination.

Our study had limitations. We did not have information about previous antimicrobial drug use (*11*,*27*,*28*). However, this exposure would be expected to confound the observed associations nondifferentially, thereby resulting in lower point estimates. Reporting lags could have delayed risk-factor interviews, resulting in nondifferential recall bias. This bias would not be expected to explain the association between resistance and international travel and would ultimately lead to underestimation of the true effect size. Case ascertainment among persons with a history of travel to eastern or Southeast Asia could have been biased. This bias could have affected our analyses if more severe illness developed in travelers with resistant infections, who were more likely to seek health care or be reported than were travelers without resistant infections. However, according to a subanalysis, not presented here, we found that patients who traveled to eastern or Southeast Asia were less likely to be hospitalized than were those who had not recently traveled internationally (OR 0.4, 95% CI 0.2–1.2). This finding might be explained by a healthy traveler effect; thus, the association between resistance and travel to eastern and Southeast Asia cannot be explained by biased case ascertainment (*29*). The use of 2 laboratories for

Table 7. Associations of salmonellosis with resistance to specific antimicrobial drugs and travel to Asia, Oregon, 2004–2009*

Drug	Adjusted odds ratio (95% CI)†
Ampicillin	**5.9 (2.9–11.8)**
Cephalosporins	1.0 (0.2–5.4)
Gentamicin	0.7 (0.1–5.3)
Quinolones‡	**22.0 (10.1–47.9)**
Trimethoprim/sulfamethoxazole	**4.5 (1.4–14.5)**

*Multiple logistic regression analysis for 1,813 patients, comparing odds of resistance for those with a history of travel to Asia with those with no history of international travel. Boldface indicates statistical significance at p<0.05.
†Adjusted by serotype, year, patient age, patient race, and outbreak status.
‡Resistance to nalidixic acid or ciprofloxacin.

susceptibility testing could have resulted in systematic bias. Both laboratories were licensed and were using Clinical and Laboratory Standards Institute standardized methods, suggesting that this bias, if present, would be minimal and could not explain the observed associations.

This study demonstrates that antimicrobial drug resistance among NTS is increasing and has clinical and public health implications. Our analyses elucidated that travel to Asia is strongly associated with antimicrobial drug resistance. When considering antimicrobial drug therapy, providers should evaluate patient travel history and *Salmonella* serotype. Our results highlight the need for enhanced domestic surveillance for antimicrobial drug resistance and suggest a need for increased prudence regarding the use of antimicrobial drugs.

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Mr Barlow is a graduate student in the Departments of Immunology and Global Health, University of Washington, Seattle, Washington, USA. His research interests include host–pathogen interactions and disease surveillance relating to emerging infectious diseases.

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Address for correspondence: Emilio E. DeBess, Acute and Communicable Disease Prevention, Oregon Health Authority, 800 NE Oregon St., Portland, OR 97232, USA; email: emilio.e.debess@state.or.us

Ciprofloxacin Resistance and Gonorrhea Incidence Rates in 17 Cities, United States, 1991–2006

Harrell W. Chesson, Robert D. Kirkcaldy, Thomas L. Gift, Kwame Owusu-Edusei, Jr., and Hillard S. Weinstock

Antimicrobial drug resistance can hinder gonorrhea prevention and control efforts. In this study, we analyzed historical ciprofloxacin resistance data and gonorrhea incidence data to examine the possible effect of antimicrobial drug resistance on gonorrhea incidence at the population level. We analyzed data from the Gonococcal Isolate Surveillance Project and city-level gonorrhea incidence rates from surveillance data for 17 cities during 1991–2006. We found a strong positive association between ciprofloxacin resistance and gonorrhea incidence rates at the city level during this period. Their association was consistent with predictions of mathematical models in which resistance to treatment can increase gonorrhea incidence rates through factors such as increased duration of infection. These findings highlight the possibility of future increases in gonorrhea incidence caused by emerging cephalosporin resistance.

Each year, the estimated 820,000 incident cases of gonorrhea in the United States result in lifetime direct medical costs of $162 million (*1*,*2*). Although substantial, the incidence of gonorrhea in the United States has decreased since the 1970s in part because of sexually transmitted disease (STD) prevention programs (*3*–*5*). However, treatment and control efforts can be hindered by antimicrobial drug resistance (*6*–*8*).

Neisseria gonorrhoeae has been remarkably adept at acquiring and maintaining resistance to antimicrobial drugs used for treatment, such as penicillin, tetracyclines, and fluoroquinolones (e.g., ciprofloxacin). After first spreading in Hawaii and California during the late 1990s and early 2000s, ciprofloxacin-resistant gonococcal strains became increasingly prevalent in the United States during the 2000s. By 2007, the Centers for Disease Control and Prevention (CDC) no longer recommended ciprofloxacin or other fluoroquinolones for treatment of gonorrhea, which make the cephalosporins cefixime or ceftriaxone the only remaining recommended treatment option (*9*).

During the past several years, gonococcal susceptibility to the cephalosporins has been decreasing (*6*–*8*). In response to increasing cefixime MICs in the United States, CDC recently updated its treatment recommendations for gonococcal infections (*10*). CDC now recommends dual therapy with ceftriaxone (an injectable cephalosporin) and a second antimicrobial drug as the only remaining recommended first-line treatment option for gonorrhea (*10*). However, the possible emergence and spread of cephalosporin resistance could eventually threaten the effectiveness of this regimen and pose a major public health challenge (*6*–*8*).

Although the course of emerging cephalosporin resistance and the possible effect on gonorrhea incidence are difficult to predict, it is possible to analyze historical trends in gonorrhea incidence during periods of increasing resistance to previously recommended antimicrobial drugs. In this study, we analyzed historical ciprofloxacin resistance data and gonorrhea incidence data to examine the possible effect of antimicrobial drug resistance on gonorrhea incidence at the population level. Assessing the historical population-level association between ciprofloxacin resistance and gonorrhea incidence can provide information about cephalosporin resistance in the future.

Methods

Overview

We first focused on simple comparisons of trends in gonorrhea incidence rates in 2 groups of cities in the United

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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States: those with relatively high prevalence and those with relatively low prevalence of ciprofloxacin resistance. After performing these illustrative comparisons, we used regression analyses to examine the association between ciprofloxacin resistance and gonorrhea incidence in a more robust manner. For simplicity, we describe our study as a city-level analysis, although as described in more detail below, the data we analyzed comprised a mixture of sources at the city, county, and metropolitan statistical area levels.

Gonococcal Isolate Surveillance Project

We used antimicrobial drug susceptibility data from the Gonococcal Isolate Surveillance Project (GISP) to analyze the association between ciprofloxacin resistance and gonorrhea incidence over time at the city level. GISP has been described in detail by Schwarcz et al. (*11*) and Kirkcaldy et al. (*12*). In brief, GISP is a sentinel surveillance system established in 1986 to monitor antimicrobial drug susceptibility among *N. gonorrhoeae* isolates. Each month, urethral gonococcal isolates and clinical data are systematically collected consecutively from up to the first 25 men at participating STD clinics in each city in whom urethral gonorrhea was diagnosed (*11*,*12*). Cities may have a single participating clinic or multiple clinics. The gonococcal isolates are tested for antimicrobial drug susceptibility by using agar dilution method. Ciprofloxacin susceptibility has been monitored in GISP since 1990 (*9*). The prevalence of ciprofloxacin resistance increased during the late 1990s and 2000s (*9*). By 2007, ciprofloxacin resistance was prevalent in all regions of the United States, prompting CDC to no longer recommend fluoroquinolones for treatment of gonorrhea (*9*). Given this time line and availability of data for additional variables described later, we included the years 1991–2006 in our analysis.

Cities with ≥1 STD clinic participating in GISP were included in the study if annual ciprofloxacin resistance prevalence data were available from that city for ≥13 years during 1991–2006 and if city-level gonorrhea incidence rates during the same 16-year period were available from gonorrhea case report surveillance data maintained by the Division of STD Prevention at CDC. Seventeen cities met the inclusion criteria: Albuquerque (New Mexico), Atlanta (Georgia), Baltimore (Maryland), Birmingham (Alabama), Cincinnati (Ohio), Cleveland (Ohio), Denver (Colorado), Honolulu (Hawaii), Minneapolis (Minnesota), New Orleans (Louisiana), Philadelphia (Pennsylvania), Phoenix (Arizona), Portland (Oregon), San Diego (California), San Francisco (California), Seattle (Washington), and St. Louis (Missouri). For most cities in our analysis, the city-specific STD rates we obtained were derived from county data and may only approximate city jurisdictions. Our dataset consisted of 272 observations, and each observation included the annual prevalence of city-level gonococcal ciprofloxacin resistance (prevalence in 17 cities each year over a 16-year period).

Gonorrhea Incidence Rates in Cities Grouped by Ciprofloxacin Resistance

We calculated the median percentage of isolates resistant to ciprofloxacin in 2004 and labeled the 8 cities above the median as higher resistance cities and the 9 cities at or below the median as lower resistance cities. For each group, we calculated gonorrhea incidence rates during 1991–2006. The rate for each group of cities was calculated as the sum of reported gonorrhea cases in the cities divided by the sum of the populations of the cities and multiplied by 100,000. The percentage of isolates resistant to ciprofloxacin for each group of cities was calculated as the average across all cities in the group.

Regression Analyses—Description of Data and Model Overview

We performed regression analyses in which the dependent variable was the city gonorrhea incidence rate (log) and the independent variable of interest was the percentage of GISP isolates resistant to ciprofloxacin in GISP clinic(s) located in the given city. The regression also included sociodemographic variables (percentage of persons who were black, percentage of persons 15–29 years of age, unemployment rate, per capita income, robbery rate) and binary (dummy) variables for each city and year to control for city-specific factors and national trends in factors that influence city-level gonorrhea incidence rates (Table 1).

We included percentage of persons who were black and percentage of persons 15–29 years of age as explanatory variables because reported STD rates are often disproportionately high among black persons and youth (*13*). We included unemployment, income, and robbery rates as explanatory variables because STD rates also have been linked to social determinants of health (*14*,*15*). Sociodemographic variables, such as these, have been shown to correlate with STD rates at the population level over time (*16*,*17*).

Gonorrhea and syphilis incidence rates, percentage of persons who were black, and percentage of persons 15–29 years of age were obtained from surveillance records and US Census Bureau data maintained by CDC (*13*). Robbery rates, unemployment rates, and per capita income data were obtained online from various federal agencies (Table 1). Of our 272 observations, 11 had missing values for ≥1 variable. Missing values for the variables were replaced with estimated values, and we assumed a linear trend from one year to the next. For example, if the unemployment rate for a given city in 2004 was missing, the average of the unemployment rate for the given city in 2003 and 2005 was assigned for 2004.

Table 1. Variables used in regression analyses of ciprofloxacin resistance and gonorrhea incidence rates in 17 cities, United States, 1991–2006*

Variable	Mean (SD)	Description	Source
Ciprofloxacin resistance	0.028 (0.070)	Fraction of GISP isolates resistant to ciprofloxacin (MIC ≥1 μg/mL) in GISP clinic(s) in given city	GISP
Gonorrhea incidence rate (log)	5.60 (0.911)	Log of city's reported gonorrhea incidence rate (cases/100,000 persons)	CDC
Syphilis rate (log)	2.07 (1.22)	Log of city's reported primary and secondary syphilis rate (cases/100,000 persons)	CDC
% Black	24.3 (21.6)	% of city population that is black	Census
% 15–29 y of age	21.7 (1.6)	% of city population that is 15–29 y of age	Census
Robbery rate	589 (356)	No. reported offenses/100,000 persons	FBI
Unemployment rate	6.07 (1.99)	% of city's labor force not employed	BLS
Per capita income	$36,483 ($5,788)	Per capita personal income in the city's respective metropolitan statistical area (2006 dollars)	BEA
City variables	NA	Binary (dummy) variables for each city	Created
Year variables	NA	Binary (dummy) variables for each year	Created

*For simplicity, we describe our study as a city-level analysis, although the data we analyzed were comprised of a mixture of sources at the city level, county level, and metropolitan statistical area. The dataset consisted of 1 observation/city/year during 1991–2006. Gonorrhea and syphilis incidence rates, % Black, and % 15–29 y of age were obtained from surveillance records and US Census Bureau data maintained by CDC (Atlanta, GA, USA) (*13*). We added 1 to the syphilis rate before taking the log. The city-specific data obtained from CDC were derived from county data and may only approximate city jurisdictions. City-specific resistance was based on resistance reported in GISP. Robbery rates and unemployment rates were based on city-level data, and per capita income was based on metropolitan statistical area data (www.ucrdatatool.gov, http://www.bls.gov/data and http://bea.gov/, respectively). Per capita income was updated to 2006 US dollars by using the all items component of the consumer price index (www.bls.gov/cpi/data.htm). For ease of display of regression coefficients, the unemployment rate was entered into the regression analyses as the no. persons unemployed/100 in the labor force, robbery rates were entered as the no. offenses/100 population, and income was entered in $100,000s (e.g., $36,483 was entered as 36.48). GISP, Gonococcal Isolate Surveillance Project; CDC, Centers for Disease Control and Prevention; FBI, Federal Bureau of Investigation; BLS, Bureau of Labor Statistics; BEA, Bureau of Economic Analysis.

Regression Model Details

A common problem with regression analysis of data consisting of multiple observations over time is serial correlation, in which the error term in a given year correlates with the error term in the previous year. We used 2 approaches to address the issue of serial correlation. First, we calculated SEs that are robust to the serial correlation. Second, we corrected for the autocorrelated error terms when computing the regression (*18*). Specifically, we used ordinary least squares (OLS), included the lagged dependent variable as an exploratory variable, and used the Newey-West procedure to calculate heteroskedasticity- and autocorrelation-consistent SEs for the regression coefficients. We also estimated a linear regression with correction for first-order autocorrelated errors (AR1) by using the AR1 procedure.

The specific equation we estimated with OLS was $G_{i,t} = \alpha + \beta_1 G_{i,t-1} + \beta_2 R_{i,t} + \gamma X_{i,t} + C_i + Y_t + \varepsilon_{i,t}$, in which $G_{i,t}$ is the log of the gonorrhea incidence rate in city *i* in year *t*, α is a constant, $R_{i,t}$ is the percentage of isolates resistant to ciprofloxacin in GISP clinic(s) of city *i* in year *t*, $X_{i,t}$ is a vector of sociodemographic variables listed earlier, C denotes city dummy variables, Y denotes year dummy variables, and ε is the error term. The equation we estimated with AR1 was the same as the previous equation except that the lagged value of the dependent variable ($G_{i,t-1}$) was not included in the model. Thus, the differences between the 2 approaches we used to address serial correlation can be summarized as follows. The OLS regression includes the lagged value of gonorrhea incidence rates as an independent variable and calculates SEs that are robust to autocorrelation in the error terms. The AR1 regression is corrected for first-order correlation in the error terms and does not include the lagged value of the gonorrhea incidence rate. Analyses were conducted by using WinRATS version 8.01 (Estima, Evanston, IL, USA).

Additional Regression Analyses

We performed additional analyses to examine the robustness of our results. First, we repeated our regression analysis by substituting the log of the syphilis rate for the log of the gonorrhea incidence rate as the dependent variable, thereby testing to determine whether our model would suggest an implausible link between gonococcal ciprofloxacin resistance and changes in the incidence of syphilis. In performing this procedure, we added 1 to the syphilis rate before taking the log so as not to exclude observations in which the syphilis rate was 0. Second, we examined temporal aspects of the association between ciprofloxacin resistance and gonorrhea incidence rates to determine whether gonorrhea incidence rates could be better predicted on the basis of past values of gonorrhea incidence rates and ciprofloxacin resistance rather than past values of gonorrhea incidence rates alone (as in Granger causality tests) (*18*,*19*). To do so, we modified our model so that 3 lagged values of the resistance variable ($R_{i,t-1}$, $R_{i,t-2}$, and $R_{i,t-3}$) were included as explanatory variables rather than the current year value of the resistance variable ($R_{i,t}$). We also included 3 lagged values of gonorrhea incidence (specifically, the log of the gonorrhea incidence rate in years t – 3, t –2, and t –1) as explanatory variables rather than 1 lag. We examined the joint significance of the 3 lagged resistance variables ($R_{i,t-1}$, $R_{i,t-2}$, and $R_{i,t-3}$)

by using an F test to compare this model with a restricted model in which the coefficients of these 3 variables were set to 0. The joint significance of the 3 lagged values of gonorrhea incidence was calculated in an analogous manner. We then reversed the model such that ciprofloxacin resistance was the dependent variable. Third, we tested the sensitivity of our results to functional form by using the gonorrhea incidence rate rather than the log of the gonorrhea incidence rate as the dependent variable. Fourth, we tested for the effect of influential observations by using 2 approaches: deleting observations with a residual >2 SEs and repeating the main analysis 17 times, each time omitting 1 of the 17 cities from the analysis.

Results

The average fraction of GISP isolates resistant to ciprofloxacin across the 17 cities during the 16 years examined was 0.028 (Table 1) (range 0–0.445). The average logged value of the gonorrhea incidence rate was 5.6 cases/100,000 persons (Table 1), which corresponds to a rate of 270 cases/100,000 persons (range 49–2,265 cases/100,000 persons). The average city population was 24.3% black and 21.7% were 15–29 years of age (Table 1).

Gonorrhea Incidence Rates in Cities with Ciprofloxacin Resistance

In 2004, a median percentage of 3.3% of isolates were resistant to ciprofloxacin in the 17 cities in our analysis. We classified the 8 cities above the median in 2004 as higher resistance cities and the 9 cities at or below the median in 2004 as lower resistance cities. Cities with higher resistance were Denver, Honolulu, Minneapolis, Phoenix, Portland, San Diego, San Francisco, and Seattle. Cities with lower resistance were Albuquerque, Atlanta, Baltimore, Birmingham, Cincinnati, Cleveland, New Orleans, Philadelphia, and St. Louis. In our simple comparison of higher resistance and lower resistance cities, we found divergent trends in gonorrhea incidence rates in the 2000s (Figure). Although gonorrhea incidence rates were much lower overall in the higher resistance cities, gonorrhea incidence rates generally increased in the higher resistance cities and decreased in the lower resistance cities during 2000–2006 (Figure, panel A). The timing of the divergent trends in gonorrhea incidence rates coincided with the divergent trends in ciprofloxacin resistance (Figure, panel B).

Regression Analyses

The coefficient of the ciprofloxacin resistance variable was positive and significant across all 4 models we estimated ($p<0.01$) (Table 2). Ciprofloxacin resistance in a given city in a given year was associated with higher gonorrhea incidence rates in that city in the given year. This finding was consistent regardless of estimation procedure (OLS in models 1 and 2 and AR1 in models 3 and 4) and regardless of the exclusion (models 1 and 3) or inclusion (models 2 and 4) of the additional sociodemographic variables.

Additional Regression Analyses

We found no association between ciprofloxacin resistance and syphilis incidence. When we examined the temporal association between ciprofloxacin resistance and gonorrhea incidence, the coefficients of the lagged individual ciprofloxacin resistance variables were not all significant individually when the dependent variable was the log of the gonorrhea incidence rate (Table 3). However, the sum of the coefficients of the lagged ciprofloxacin resistance variables was positive and these coefficients were jointly significant ($p<0.01$). When we reversed the model such that ciprofloxacin resistance was the dependent variable, lagged values of the gonorrhea incidence coefficients (specifically the coefficients of the logs of the gonorrhea incidence rate in years $t-3$, $t-2$, and $t-1$) were not jointly significant (Table 3). Although past levels of ciprofloxacin resistance helped to predict current gonorrhea incidence rates, past gonorrhea incidence rates did not help to predict current ciprofloxacin resistance levels. Our results were generally consistent across the range of additional analyses we conducted, including applying gonorrhea incidence rates in non-log form, omitting outliers, and omitting any given city from the analysis.

Discussion

We found a strong positive association between ciprofloxacin resistance and gonorrhea incidence rates at the city level during 1991–2006. However, ecologic studies, such as ours, of the population-level association between ciprofloxacin resistance and gonorrhea incidence cannot establish that this association is causal. Nonetheless, our study offers evidence consistent with that of a causal association between drug resistance and increased incidence. In focusing on the temporal order of the association between ciprofloxacin resistance and gonorrhea incidence rates, we found a strong association between ciprofloxacin resistance and subsequent gonorrhea incidence rates. In contrast, we did not find a robust association between gonorrhea incidence rates and subsequent ciprofloxacin resistance. Nor did we did find an association between ciprofloxacin resistance and syphilis incidence. If the association we observed between ciprofloxacin resistance and gonorrhea incidence rates were spurious, we might also expect to find an association between ciprofloxacin resistance and syphilis incidence rates, given a strong association between syphilis rates and gonorrhea incidence rates among the cities in our analysis for most years during 1991–2006.

Although we found that ciprofloxacin resistance may have contributed to increases in gonorrhea incidence,

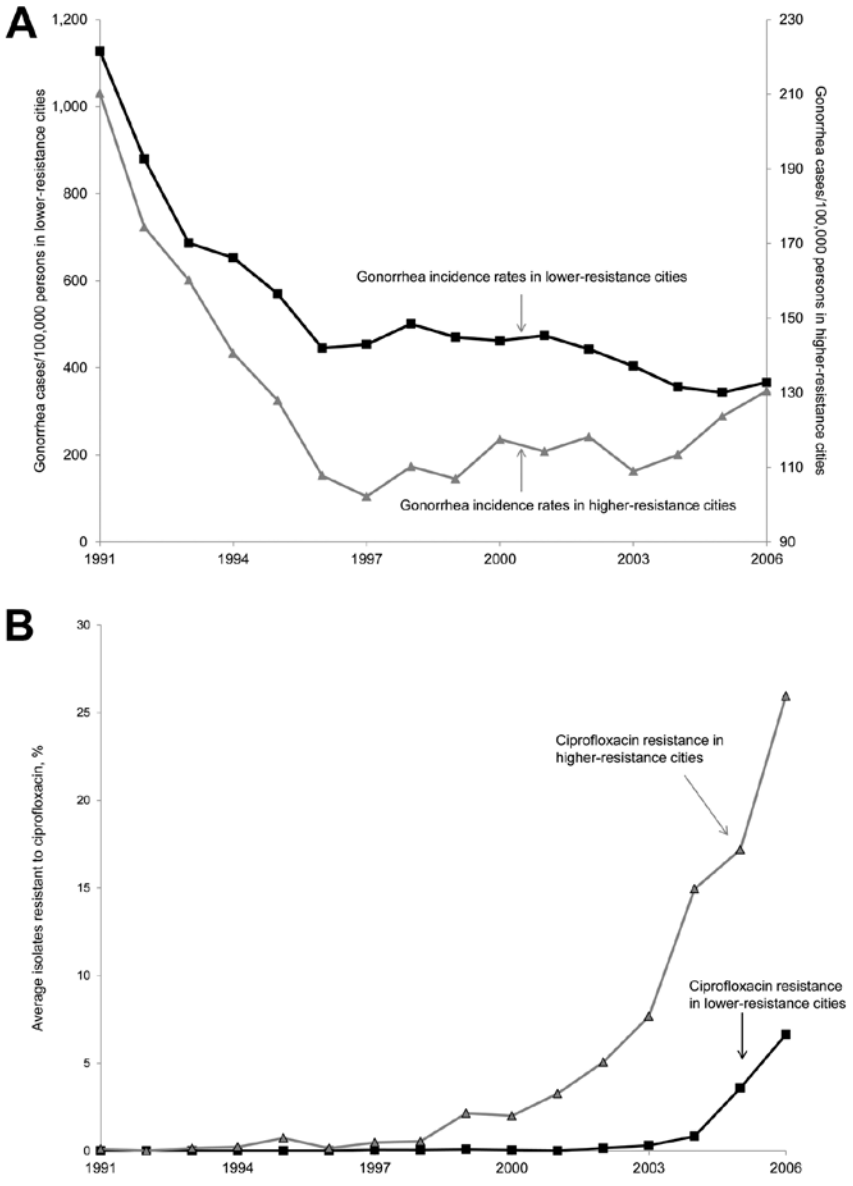

Figure. Ciprofloxacin resistance and gonorrhea incidence rates in 17 cities, United States, 1991–2006. A) Gonorrhea incidence rates and B) average percentage of isolates resistant to ciprofloxacin for 2 groups of cities with higher (above the median) and lower (at or below the median) percentages of isolates resistant to ciprofloxacin as of 2004. Cities with higher resistance were Denver (Colorado), Honolulu (Hawaii), Minneapolis (Minnesota), Phoenix (Arizona), Portland (Oregon), San Diego (California), San Francisco (California), and Seattle (Washington). Cities with lower resistance were Albuquerque (New Mexico), Atlanta (Georgia), Baltimore (Maryland), Birmingham (Alabama), Cincinnati (Ohio), Cleveland (Ohio), New Orleans (Louisiana), Philadelphia (Pennsylvania), and St. Louis (Missouri).

reported gonorrhea incidence rates were generally lower in cities that had higher levels of ciprofloxacin resistance than in cities that had lower levels of ciprofloxacin resistance. Thus, any effect that increased ciprofloxacin resistance might have had on gonorrhea incidence rates during the late 1990s and early 2000s would likely be relatively minor compared with all other factors that influence gonorrhea incidence at the population level.

Our results can help to quantify the possible effect of antimicrobial drug resistance on the incidence of gonorrhea at the population level. In model 2, the resistance coefficient was 0.710, which suggested that a change of 0.1 in the resistance variable would be associated with an increase in gonorrhea of ≈7%. Thus, our findings suggest that gonorrhea incidence rates in a scenario in which 10% of isolates were resistant to treatment would be ≈7% higher than in a scenario of no drug resistance, although the cumulative effect of resistance over time could be more substantial.

At least 2 possible explanations exist for the observed association. First, treatment failures or delays in clearance of infections caused by ciprofloxacin resistance might have increased the duration of infectivity and facilitated transmission to partners. Second, mutational changes in the organism that conferred resistance or co-occurred with resistance determinants might have supported gonococcal transmission. This possibility is suggested in the study reported by Kunz et al. that mutant gyrase $(gyrA)_{91/95}$ alleles in *N. gonorrhoeae* appeared to provide fitness benefit (*20*).

Table 2. Results of regression analysis of gonorrhea incidence rates in 17 cities, United States, 1991–2006*

Independent variable	Model 1	Model 2	Model 3	Model 4
Ciprofloxacin resistance	0.739 (0.172)†	0.710 (0.201)†	0.892 (0.322)†	0.926 (0.322)†
Lagged dependent variable	0.597 (0.052)†	0.553 (0.053)†	–	–
% Black	–	–0.143 (0.962)	–	0.991 (1.67)
% 15–29 y of age	–	–0.381 (1.20)	–	–1.60 (2.49)
Robbery rate	–	0.247 (0.058)†	–	0.336 (0.125)†
Unemployment rate	–	–0.660 (1.20)	–	–0.724 (1.83)
Per capita income	–	0.449 (0.656)	–	0.324 (1.19)
Adjusted R^2	0.969	0.970	0.967	0.967

*Values are coefficients (SEs) unless otherwise indicated. All of the above regressions also included a constant term and binary (dummy) variables for city and year (not reported in table). Models 1 and 2 included the lagged value of the dependent variable and were estimated by using ordinary least squares. Models 3 and 4 were estimated by using linear regression corrected for first-order autocorrelated errors. –, variables were not included in the regression.
†p<0.01.

Our assessment of the association between ciprofloxacin resistance and gonorrhea incidence offers evidence that emerging cephalosporin resistance could lead to higher gonorrhea incidence rates at the population level than would have been observed in the absence of cephalosporin resistance. However, because *N. gonorrhoeae* ciprofloxacin resistance might differ in several ways from cephalosporin resistance, the possible effect of cephalosporin resistance on gonorrhea incidence rates might differ substantially from that of ciprofloxacin resistance. Whereas ciprofloxacin resistance is conferred by amino acid substitutions in the A subunits of DNA gyrase and parC, the A subunit of DNA topoisomerase IV (*21*), cephalosporin resistance, particularly ceftriaxone resistance, may require acquisition of an unusual *penA* mosaic allele and mutations in *mtrR*, *penB*, and *ponA1* (*22*,*23*). The ease with which *N. gonorrhoeae* can acquire these resistance determinants is unclear, and the biologic fitness of ceftriaxone-resistant strains is unknown. During the emergence of ciprofloxacin resistance, non-fluoroquinolone treatment options were readily available. However, few, if any, alternative options are available to treat ceftriaxone-resistant infections. In this scenario of limited treatment options, the population-level effect of ceftriaxone resistance could be more substantial.

STD surveillance data are subject to limitations, such as incomplete reporting of cases and differences across jurisdictions in how data are collected (*13*). Furthermore, for most cities in our analysis, the city-specific STD rates we obtained were derived from county data and might only approximate data for city jurisdictions. However, our use of binary (dummy) variables for each city helps to guard against possible biases that arise because of constant differences across cities in STD reporting practices and the use of county-level data to approximate data for city jurisdictions. The sociodemographic variables we included might likewise only approximate those for city jurisdictions because some of these variables were based on county data and some were based on metropolitan statistical area data. However, biases in the sociodemographic variables are unlikely to have influenced our findings substantially because the association we observed between ciprofloxacin resistance and gonorrhea incidence was consistent regardless of whether the sociodemographic variables were included in the model.

Table 3. Selected results of regression analyses of the temporal association of ciprofloxacin resistance and gonorrhea incidence rates in 17 cities, United States, 1991–2006*

Independent variable	Gonorrhea incidence rate (log), year t	Ciprofloxacin resistance rate, year t
Gonorrhea incidence rate (log), year t – 1	0.571 (0.057)†	0.015 (0.012)
Gonorrhea incidence rate (log), year t – 2	0.043 (0.080)	0.000 (0.012)
Gonorrhea incidence rate (log), year t – 3	–0.057 (0.077)	0.009 (0.010)
Ciprofloxacin resistance, year t – 1	–0.096 (0.488)	0.854 (0.154)†
Ciprofloxacin resistance, year t – 2	1.41 (0.538)†	0.395 (0.192)‡
Ciprofloxacin resistance, year t – 3	0.793 (0.492)	–0.127 (0.177)
Sum of gonorrhea incidence rate (log) coefficients	0.557 (0.070)	0.024 (0.014)
Joint significance of gonorrhea incidence rate (log) coefficients: F test	F = 46.6†	F = 1.09
Sum of ciprofloxacin resistance coefficients	2.11 (0.506)	1.12 (0.146)
Joint significance of ciprofloxacin resistance coefficients: F test	F = 8.88†	F = 66.7†
Adjusted R^2	0.971	0.859

*Values are coefficients (SEs) unless otherwise indicated. Both of the above regressions also included a constant term and binary (dummy) variables for city and year (not reported in table) and were estimated by using ordinary least squares. These findings, that past levels of ciprofloxacin resistance helped to predict current gonorrhea incidence rates but that past gonorrhea incidence rates did not help to predict current ciprofloxacin resistance levels, were generally consistent when linear regression corrected for first-order autocorrelated errors was used rather than ordinary least squares and/or when including additional covariates (% Black, % 15–29 y of age, robbery rate, unemployment rate, and per capita income).
†p<0.01.
‡p<0.05.

We assumed that the ciprofloxacin resistance in isolates collected from STD clinic(s) in a given city in a given year reasonably represent resistance for the entire city in the given year. Although overall prevalence of gonorrhea in STD clinics is not representative of the overall population because STD clinic attendees are generally at higher risk, those infected with gonococcal infections with lower (or greater) antimicrobial drug susceptibility are unlikely to preferentially attend these clinics. Because our analysis was limited to cities for which GISP susceptibility data and city-level gonorrhea incidence were available, the cities in our study might not be representative of other US cities.

Although we controlled for sociodemographic factors, city effects, and year effects, we were unable to control for all city-specific factors that might influence gonorrhea incidence rates. For example, we were unable to control for city-specific changes in gonorrhea treatment regimens over time because of lack of data.

This study helps to quantify the association between ciprofloxacin resistance and gonorrhea incidence and can inform assessments of the possible effect of emerging resistance to current gonorrhea treatment. The association we observed is consistent with predictions of mathematical models in which resistance to treatment can increase gonorrhea incidence rates through factors such as increased duration of infection (*24*,*25*).

Ciprofloxacin resistance was associated with increases in gonorrhea incidence rates during the late 1990s and 2000s despite availability of other well-studied recommended treatment options. Correspondingly, emerging cephalosporin resistance could have even more substantial health and economic consequences, particularly as the number of available treatment options decreases. Efforts to control the spread of drug-resistant strains may mitigate this possible effect.

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Dr Chesson is a health economist at the Centers for Disease Control and Prevention, Atlanta, Georgia. His research interests include assessments of the impact and cost-effectiveness of STD prevention programs and policies.

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Address for correspondence: Harrell W. Chesson, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E80, Atlanta, GA 30333, USA; email: hbc7@cdc.gov

Contact Investigation for Imported Case of Middle East Respiratory Syndrome, Germany

Annicka Reuss, Annette Litterst, Christian Drosten, Michael Seilmaier, Merle Böhmer,[1] Petra Graf, Hermann Gold, Clemens-Martin Wendtner, Arina Zanuzdana, Lars Schaade, Walter Haas, and Udo Buchholz

On March 19, 2013, a patient from United Arab Emirates who had severe respiratory infection was transferred to a hospital in Germany, 11 days after symptom onset. Infection with Middle East respiratory syndrome coronavirus (MERS-CoV) was suspected on March 21 and confirmed on March 23; the patient, who had contact with an ill camel shortly before symptom onset, died on March 26. A contact investigation was initiated to identify possible person-to-person transmission and assess infection control measures. Of 83 identified contacts, 81 were available for follow-up. Ten contacts experienced mild symptoms, but test results for respiratory and serum samples were negative for MERS-CoV. Serologic testing was done for 53 (75%) of 71 nonsymptomatic contacts; all results were negative. Among contacts, the use of FFP2/FFP3 face masks during aerosol exposure was more frequent after MERS-CoV infection was suspected than before. Infection control measures may have prevented nosocomial transmission of the virus.

Middle East respiratory syndrome coronavirus (MERS-CoV) infection was initially reported to the World Health Organization (WHO) in September 2012 (*1*,*2*). By November 11, 2013, a total of 153 laboratory-confirmed cases of human infection with MERS-CoV had been identified; 64 (42%) of those with confirmed cases had died (*3*). Most (63%) case-patients had severe respiratory disease; 76% also had ≥1 underlying chronic condition (*4*). The median age of case-patients was 50 years (range 14 months to 94 years). All cases were directly or indirectly related to countries in the Middle East or on the Arabian Peninsula.

MERS-CoV shows a close genetic relationship with coronaviruses found in bats (*1*,*5*–*10*), but no zoonotic link has been confirmed. Person-to-person transmission has been reported in the work environment, among family contacts, or to health care workers (HCWs) (*11*–*13*). Although situations involving consecutive human transmission events have been documented (*13*), none of the known clusters have led to sustained person-to-person transmission in the general population. In Europe, single imported infections have been reported in the United Kingdom, Germany, France, and Italy, and secondary cases have been reported in the United Kingdom, France, and Italy (*12*,*14*,*15*). Because a large proportion of cases are fatal and the virus could acquire the ability to spread more efficiently (as was the case with severe acute respiratory syndrome coronavirus), WHO has recommended thorough contact investigations for confirmed human cases to identify, quantify, and prevent person-to-person transmission (*16*).

In Germany, MERS-CoV infection was initially reported in a person from Qatar (*17*). He was in his third week of illness and was already on mechanical ventilation when he was admitted to a hospital in Essen in October 2012. A retrospective contact investigation found no indication of person-to-person transmission to contacts in Germany (*17*).

On March 23, 2013, the Institute for Virology of the University of Bonn reported an imported case of MERS-CoV infection to the Department of Health and

Author affiliations: Robert Koch Institute, Berlin, Germany (A. Reuss, M. Böhmer, A. Zanuzdana, L. Schaade, W. Haas, U. Buchholz); Department of Health and Environment, Munich, Germany (A. Litterst, P. Graf, H. Gold); University of Bonn Medical Centre Institute of Virology, Bonn, Germany (C. Drosten); Hospital Schwabing, Munich, Germany (M. Seilmaier, C.-M. Wendtner); and Bavarian Health and Food Safety Authority, Oberschleißheim, Germany (M. Böhmer)

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[1]Postgraduate Training for Applied Epidemiology, Robert Koch Institute, Berlin, Germany, associated with European Programme for Intervention Epidemiology Training, European Centre for Disease Prevention and Control, Stockholm, Sweden.

Environment in Munich (City Health Department). A 73-year-old man from Abu Dhabi, United Arab Emirates, had been admitted to a hospital in Munich and had positive test results for MERS-CoV infection (Figure 1). Clinical details and virologic findings have been reported elsewhere (*18*). Briefly, the patient had underlying multiple myeloma and had received several modes of treatment, including high-dose chemotherapy and autologous stem-cell transplantation in 2009. On March 8, 2013, influenza-like illness with fever and cough developed in the patient. After his symptoms worsened, he was hospitalized in his country on March 10 with a diagnosis of pneumonia; he was intubated on March 17 and transferred by flight ambulance services to Germany on March 19, eleven days after illness onset, for further intensive care treatment and mechanical ventilation.

General infection control guidelines of the Munich hospital required that patients from areas such as the Middle East, where prevalence of multidrug-resistant pathogens is high, be isolated until colonization or infection with a multidrug-resistant pathogen is ruled out. This rule is particularly enforced when patients have been previously hospitalized in the country of origin. Thus, at the time of hospital admission in Germany, the patient was isolated from other patients. When MERS-CoV infection was suspected and included in the differential diagnosis on March 21, standard hygiene measures for HCWs were changed to infection control measures as recommended for severe acute respiratory syndrome patients, including the use of FFP2 face masks for usual patient care (*19*).

MERS-CoV infection was diagnosed in the patient on March 23; he died on March 26 of multiorgan failure and acute respiratory distress syndrome. After MERS-CoV infection was diagnosed, the City Health Department, in cooperation with the state health department, the Institute for Virology in Bonn, and the Robert Koch Institute, initiated an investigation to 1) monitor all contacts of the patient to identify possible person-to-person transmission, 2) assess infection control measures, and 3) explore possible sources for the patient's infection to prevent further cases.

Methods

Contact Investigation

For the investigation, the City Health Department assessed all contact persons (contacts) retrospectively and monitored them prospectively. All contacts received a questionnaire for retrospective documentation and prospective daily self-monitoring of symptoms, exposure to the patient, and infection control measures applied. For every day from March 19 through April 5, information was collected about the contacts' distance from the patient (<2 meters vs. ≥2 meters); type of contact with the patient (aerosol-producing procedures, non–aerosol-producing procedures, care of patient, handling of urine catheter, handling of respiratory samples in the laboratory, handling of urine samples in the laboratory); type of protection used (surgical mask, FFP1 mask, FFP2 mask, FFP3 mask, gown, gloves, protective glasses); and symptoms experienced by the contacts (cough, fever, temperature, sore throat, diarrhea, shortness of breath). An aerosol-producing procedure was defined as respiratory suction, bronchoalveolar lavage, intubation, or bronchoscopy.

On the basis of the self-reported information in the questionnaires and personal interviews with the contacts, we divided contacts into 2 groups. Close-distance contacts had face-to-face contact with the patient (<2 meters from the patient) or direct contact with secretions or body fluids of the patient, irrespective of protective measures worn. All other contacts were classified as less-close-distance contacts. According to WHO recommendations on the duration of follow-up at that time, close-distance contacts were asked to contact the City Health Department daily for 10 days after the last exposure to the patient. Those who failed to do so were contacted by the City Health Department, supported by the occupational health service of the hospital. Less-close-distance contacts were asked to report to the City Health Department only in case of onset of symptoms.

Respiratory illnesses in contacts that occurred 1–10 days after exposure to the patient were assessed through the City Health Department by telephone contact with the contact; a respiratory tract sample was taken from any contact

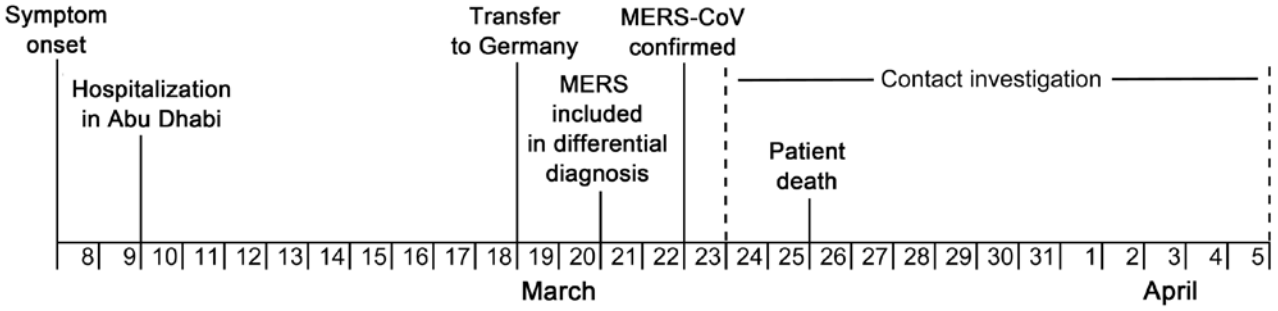

Figure 1. Timeline for patient history and contact investigation in imported case of Middle East respiratory syndrome (MERS), Germany, 2013.

with respiratory illness. In addition, attempts were made to obtain paired serologic samples from all contacts, the first taken immediately after contact and the second ≥28 days after the last exposure.

Patient Questionnaire

Because the MERS-CoV patient was on mechanical ventilation and could not be interviewed, family contacts who had accompanied him to Germany were interviewed about the onset of his symptoms and possible exposures in the 10 days before disease onset. For the interview, a structured questionnaire was used, and information collected was documented on paper.

Laboratory Methods

PCR testing and serologic testing were done as described (*17*,*20*). Serum samples from contacts were tested for MERS-CoV antibodies if a serum sample was taken ≥28 days after last exposure. In addition, serum samples were tested for antibodies against influenza A, B, and C; rhinovirus A, B, and C; parainfluenzavirus 1, 2, 3, and 4; respiratory syncytial virus A and B; human metapneumovirus; coronavirus 229E, NL63, OC43, and HKU1; and adenovirus. All samples were analyzed at the Institute for Virology of the University of Bonn.

Data Analysis

Data from the City Health Department's contact monitoring, the contacts' questionnaires, and the laboratory findings were integrated in 1 database. Results were validated and analyzed by using Stata version 12.0 (StataCorp, College Station, TX, USA).

Results

Contact Investigation

The City Health Department identified 83 contacts. Of these, 69 (83%) were classified as close-distance contacts and 14 (17%) as less-close-distance contacts (Table). Four (5%) of the contacts were members of the patient's family, 16 (19%) were physicians, 25 (30%) were nursing staff, 20 (24%) were laboratory personnel, and 18 (22%) were part

Table. Results of contact investigation of patient with imported MERS-CoV infection, Germany, 2013*

Data category	No. (%) contacts†	No. (%) close-distance contacts, n = 69‡	No. (%) less-close-distance contacts, n = 14‡	p value§
Contacts, n = 83				0.103
Physicians	16 (19)	11 (69)	5 (31)	
Nursing staff	25 (30)	24 (1)	1 (4)	
Laboratory personnel	20 (24)	17 (85)	3 (15)	
Family members	4 (5)	4 (100)	0	
Other	18 (22)	13 (72)	5 (28)	
Response to questionnaire	61 (73)	55 (90)	6 (10)	0.004
Aerosol exposure	15 (18)	15 (100)	0	0.054
Symptoms				0.006
Symptomatic	10 (12)	9 (90)	1 (10)	
Nonsymptomatic¶	71 (86)	60 (85)	11 (15)	
Unknown	2 (2)	0	2 (100)	
Swab samples, symptomatic contacts, n = 10				0.725
Swab sample collected	9 (90)	8 (89)	1 (11)	
No swab sample collected	1 (10)	1 (100)	0	
PCR results for symptomatic contacts with swab samples, n = 9				NA
MERS-CoV positive	0	NA	NA	
MERS-CoV negative	9 (100)	8 (89)	1 (11)	
HCoV-NL63 positive	1 (11)	1 (100)	0	
Rhinovirus positive	2 (22)	2 (100)	0	
Serologic test results				0.007
MERS-CoV positive	0	NA	NA	
MERS-CoV negative	60 (72)	54 (90)	6 (10)	
Not done	23 (28)	15 (65)	8 (35)	
Serologic testing among symptomatic contacts, n = 10				0.107
MERS-CoV positive	0	NA	NA	
MERS-CoV negative	7 (70)	7 (100)	0	
Not done	3 (30)	2 (67)	1 (33)	
Serologic testing among nonsymptomatic¶ contacts, n = 71				0.095
MERS-CoV positive	0	NA	NA	
MERS-CoV negative	53 (75)	47 (89)	6 (11)	
Not done	18 (25)	13 (72)	5 (28)	

*Definitions of close-distance and less-close-distance contacts provided in article text. MERS-CoV, Middle East respiratory syndrome coronavirus; NA, not applicable.
†Percentages are of contacts (N = 83) unless otherwise indicated.
‡Percentages are of category total.
§Probability that the distribution as indicated occurs by chance given the column and row totals.
¶Nonsymptomatic contacts are asymptomatic persons and those who were symptomatic before exposure.

of other professional groups. Clinical follow-up was available for 81 (98%) contacts.

A respiratory symptom or fever developed in 10 (12%) contacts. Of these, swab specimens were collected from 9 (90%) and blood samples from 7 (70%). All 9 swab specimens were negative for MERS-CoV; 1 (11%) was positive for CoV NL-63, and 2 (22%) were positive for rhinovirus. All 7 serum samples were negative for MERS-CoV antibodies. All symptomatic contacts had ≥1 sample type (respiratory swab or serum) collected for laboratory testing; results of PCR and serologic testing were available from 6 (60%), PCR only from 3 (30%), and serologic testing only from 1 (10%). In addition, serologic test results were available for 53 (75%) of the 71 nonsymptomatic contacts; all were negative for MERS-CoV antibodies. Overall, persons for whom serologic testing results were available were more likely to be close-distance contacts than were persons without available serologic results (p = 0.007; Table).

The 4 family members who accompanied the patient were his wife, daughter, son, and son-in-law. Their ages were 35–37 years, and none reported symptoms. The patient's children and son-in-law had their last contact with the patient on March 20 and his wife on March 23. Because no protection measures had been used until after March 20, the family members were considered at high risk for infection. All 4 provided respiratory swab and serum samples on March 24; all samples had negative results. Serum samples taken ≥28 days after last exposure to the patient were not available.

MERS-CoV infection was added to the differential diagnosis for the patient on March 21. The daily numbers of HCWs who had any contact with him (regardless of protection measures) and of those who had aerosol exposure were lower after that date than before (Figure 2): 4.4 HCW per illness day vs. 7.5 HCW per illness day (p = 0.05) and 2.8 HCW per illness day vs. 6 HCW per illness day (p = 0.03). Among HCWs with aerosol exposure, 1 (8%) of 12 daily exposures occurred while FFP2 or FFP3 masks were being used before March 21; after that date, 11 (79%) of 14 daily exposures occurred while FFP2 or FFP3 masks were being used (p<0.01).

Patient Questionnaire

The patient was a 73-year-old married man from Abu Dhabi, United Arab Emirates; he had a medical history of multiple myeloma. At the time of his MERS-CoV infection, he was receiving corticosteroid therapy. His profession was camel breeding; in the 2 weeks before his onset of illness, 1 of his camels was reported to have had a respiratory illness. In the questionnaire, we did not differentiate between dromedary (*Camelus dromedaries*) and Bactrian (*C. bactrianus*) camels. His neighborhood had palm trees, and bats were known to dwell in the area. The patient had no known contact with other MERS-CoV patients, had no personal contacts in Qatar or Jordan, and had no travel history in the 10 days before illness onset. He consumed different types of fruit juices and cooked goat meat, beef, and sheep meat, but no raw meat. He ate dates from his region, but he reportedly did not consume date or palm syrup. Other than the camels on his farm, he had no contact with animals; he did not practice falconry and did not visit camel racetracks or animal markets.

Discussion

We describe the case and contact investigation of a confirmed case of MERS-CoV infection that was imported to Germany. We did not identify person-to-person transmission from the patient to any of the contacts. As with the previous imported case in this country, the patient was already on mechanical ventilation when he was transferred to Germany. However, whereas the previous case was in the late third week of illness, this patient was in the second week of illness. Sample from this patient taken from different body locations and at different times were positive

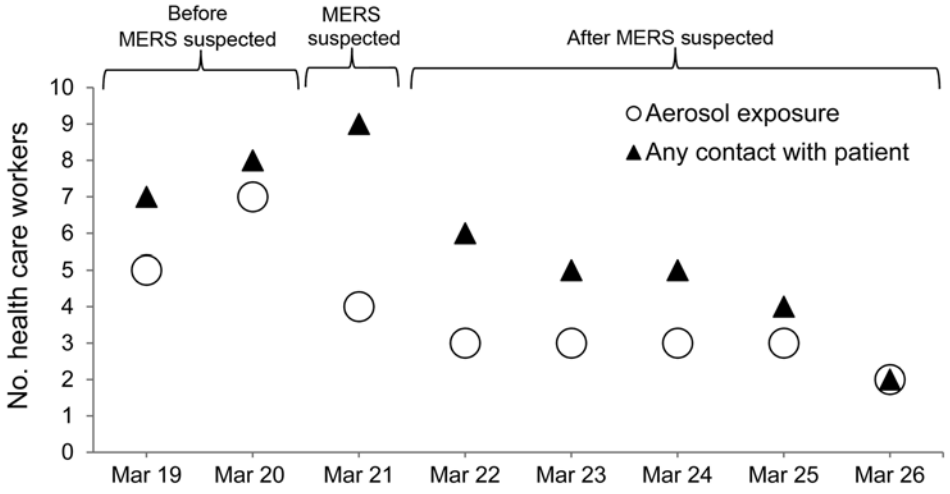

Figure 2. Daily number of health care workers who had contact with a patient infected with Middle East respiratory syndrome (MERS) coronavirus who was hospitalized in Germany, March 19–26, 2013.

for MERS-CoV by PCR, and the viral load detected was several logs higher than in samples from the patient with the previous imported case (*18*,*20*). These results indicate that this patient may have been more infectious than the previous patient.

Nosocomial transmission from MERS patients to HCWs has been documented (*13*,*21*,*22*). In our study, the patient was isolated during the first 2 days of his hospital stay (before MERS was suspected), although the reason for this intervention was the hospital's policy to isolate every patient from the Middle East, irrespective of the assumed diagnosis, because of perceived increased risk of carrying drug-resistant pathogens, rather than any special measures taken because of the patient's respiratory illness. After MERS was suspected, HCWs used FFP2 masks significantly more frequently than they had before, and fewer HCWs had daily contact with the patient. Our result suggest that, in the later stages of this disease, the combination of standard protection measures (use of surgical masks for potentially aerosol-generating procedures), cautious handling of the patient (because of his potential to harbor drug-resistant bacteria), and possible decreased infectiousness compared with the first week of illness may have prevented transmission to HCWs. These findings also underline the importance of following WHO recommendations on infection prevention and control when managing a patient who may be infected with a pathogen that could lead to nosocomial transmission (*23*).

Regarding possible sources of infection, an extensive interview was conducted with family members because the patient could not be interviewed. The patient's illness was likely a primary case, and possible exposures that might have caused the MERS-CoV infection were explored. Of note were the presence of bats in the neighborhood of his residence, the patient's profession as camel breeder, and his contact with a camel that was reported to have had a respiratory illness before his own illness onset. Bats are a likely reservoir for MERS-related CoV (*5*,*8*), and serum samples from Omani racing camels have shown to have neutralizing antibodies against MERS-CoV (*24*). These findings suggest these animals' possible relevance (e.g., as intermediate hosts) for human acquisition of MERS-CoV.

Two complementary monitoring instruments for contact persons were used: active follow-up with daily telephone contact and a self-administered monitoring questionnaire. Both methods have merits, and a combination of both is likely to ensure the most thorough contact follow-up. Advantages of personal interviews on the telephone are immediacy and the possibility for the interviewer to receive intangible information, such as the self-assessment of symptoms, as well as the opportunity to answer questions from the contacts. This process enables a more specific way to judge a person's health status. On the other hand, a daily monitoring questionnaire provides detail in clinical information, exposure, and protection measures that might be used for more in-depth analyses (e.g., when a few contacts have become infected). Such a questionnaire could be expanded to include a section for contact persons to fill in the names of persons with whom they had face-to-face contact during each day. This information might become crucial for second-generation contact tracing when contacts under observation become infected. Rapid availability of this type of information is essential for efficient investigation of clusters or outbreaks similar to those that have been reported already (*13*).

In conclusion, we conducted a contact investigation of an imported case of MERS-CoV infection in Germany. Laboratory testing of symptomatic and asymptomatic contacts of the index case-patient did not indicate transmission of the virus. Furthermore, we documented the change from standard hygiene to infection control measures after MERS-CoV was suspected, an adaptation that may have prevented nosocomial transmission. Exposure to camels as a possible etiologic mechanism for human MERS-CoV infection requires further evidence from other studies.

Dr Reuss is an epidemiologist at the Respiratory Infections Unit, Robert Koch Institute, Berlin, Germany. Her research interests include emerging infectious respiratory diseases, pandemic preparedness, and influenza vaccination.

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Address for correspondence: Annicka Reuss, Robert Koch Institute, Department for Infectious Disease Epidemiology, Division 36 Respiratory Diseases, DGZ-Ring 1, 13086 Berlin, Germany; email: reussa@rki.de

Rapid Increase in Pertactin-deficient *Bordetella pertussis* Isolates, Australia

Connie Lam, Sophie Octavia, Lawrence Ricafort, Vitali Sintchenko, Gwendolyn L. Gilbert, Nicholas Wood, Peter McIntyre, Helen Marshall, Nicole Guiso, Anthony D. Keil, Andrew Lawrence, Jenny Robson, Geoff Hogg, and Ruiting Lan

Acellular vaccines against *Bordetella pertussis* were introduced in Australia in 1997. By 2000, these vaccines had replaced whole-cell vaccines. During 2008–2012, a large outbreak of pertussis occurred. During this period, 30% (96/320) of *B. pertussis* isolates did not express the vaccine antigen pertactin (Prn). Multiple mechanisms of Prn inactivation were documented, including IS*481* and IS*1002* disruptions, a variation within a homopolymeric tract, and deletion of the *prn* gene. The mechanism of lack of expression of Prn in 16 (17%) isolates could not be determined at the sequence level. These findings suggest that *B. pertussis* not expressing Prn arose independently multiple times since 2008, rather than by expansion of a single Prn-negative clone. All but 1 isolate had *ptxA1*, *prn2*, and *ptxP3*, the alleles representative of currently circulating strains in Australia. This pattern is consistent with continuing evolution of *B. pertussis* in response to vaccine selection pressure.

Bordetella pertussis is the gram-negative coccobacillus that causes the respiratory disease pertussis, also known as whooping cough. The incidence of pertussis infection and related deaths decreased dramatically after implementation of immunization with a whole-cell vaccine (WCV) during the 1950s. Because of side effects of WCV, such as high rates of fever and local reactions, and variable efficacy of WCVs, a less reactogenic acellular vaccine (ACV) was developed in the 1980s. ACVs have now replaced WCVs in many industrialized countries for primary and booster vaccinations against pertussis.

Although ACV formulations differ in the number of component pertussis antigens, the vaccine used in Australia contains pertussis toxin (Ptx), pertactin (Prn), and filamentous hemagglutinin (Fha). A 5-component (Ptx, Prn, Fha, fimbrial antigen [Fim] 2, and Fim3) ACV is used for short periods in some regions (*1*). ACVs were introduced for the fourth and fifth doses in most states in Australia during 1997 and for all doses during 1999 (Figure 1). South Australia introduced ACVs for all doses in 1997. The current vaccination schedule for pertussis comprise 3 primary doses of ACV at 2, 4, and 6 months of age, and a booster vaccination at 4 years of age. A booster vaccination with ACV at 18 months of age, which was introduced in 1985, was removed from the National Immunization Program in Australia in 2003, and an adult-formulated ACV was introduced for children at 12–17 years of age in school-based programs in 2004 (*2*,*3*).

Since 1991, data on reported pertussis cases show that outbreaks occurred in Australia in 1996–1997, 2001, and 2004, and a series of outbreaks occurred in different regions starting in 2008 (Figure 1) (*2*,*3*). Multiple factors probably contributed to the resurgence of pertussis in high-income countries that had long-standing pertussis immunization programs. These factors include waning immunity (exacerbated by the change from WCVs to ACVs and, in Australia, cessation of the booster vaccination at 18 months

Author affiliations: University of New South Wales, Sydney, New South Wales, Australia (C. Lam. S. Octavia, L. Ricafort, R. Lan); University of Sydney, Sydney (V. Sintchenko, G.L. Gilbert); Westmead Hospital, Sydney (V. Sintchenko, N. Wood, P. McIntyre); University of Adelaide, Adelaide, South Australia, Australia (H. Marshall); Institut Pasteur, Paris, France (N. Guiso); Princess Margaret Hospital for Children, Perth, Western Australia, Australia (A.D. Keil); Women's and Children's Hospital, Adelaide (A. Lawrence); Sullivan Nicolaides Pathology, Brisbane, Queensland, Australia (J. Robson); and University of Melbourne, Parkville, Victoria, Australia (G. Hogg)

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of age) and increased use of more sensitive diagnostic tests, such PCR (*4*).

An additional possible contributing factor is evolution of *B. pertussis* through vaccine-driven adaptation (*5*). The most prominent recent changes in circulating *B. pertussis* strains are polymorphisms within genes encoding 2 of the 3 main virulence factors (Ptx and Prn) contained in the vaccine. Variations have also been reported in *ptxP*, the promoter of the *ptx* operon (*6*). In Australia, we have shown by single nucleotide polymorphism (SNP) typing that among *B. pertussis* isolates, *ptxP3*–containing strains predominate (*7*), and these strains belong to SNP cluster I (*8*,*9*).

Surveillance of recent *B. pertussis* isolates in several countries has identified *prn* deletions and gene disruptions, which lead to lack of expression of mature Prn (*10*–*13*). This protein is a 69-kDa adhesin that aids *B. pertussis* attachment to epithelial cells and is one of the most polymorphic virulence genes within *B. pertussis* (it has 13 documented alleles) (*5*). SNPs and differences in the number of amino acid (GGFGP and PQP) repeats contribute to variation within the *prn* gene; variations are usually limited to 2 regions known as region 1 and region 2.

In this study, we identified *B. pertussis* isolates that do not express Prn (Prn negative) from a set of isolates collected in Australia during 1997–2012. We also characterized the causes of their lack of expression and evaluated trends in the proportion of Prn-negative isolates over this period.

Methods

Bacterial Strains and Growth

A total of 453 *B. pertussis* isolates were available for this study; 133 isolates collected during 1997–2008 and 194 collected during 2008–2010 have been described (*9*). A total of 126 additional isolates collected from Westmead Hospital (Sydney, New South Wales, Australia) and Princess Margaret Hospital for Children (Perth, Western Australia, Australia) during 2011–2012 were also included this study. Although specific clinical information about the source of isolates was not available, isolates were obtained from patients who lived in large urban areas and who had PCR-confirmed pertussis infections. The number of available isolates in 2011–2012 was relatively small because several participating laboratories discontinued pertussis culture in favor of only direct PCR testing.

All *B. pertussis* isolates were grown on Bordet Gengou agar (Becton Dickinson, Sparks, MD, USA) supplemented with 10% defibrinated horse blood (Oxoid, Basingstoke, UK) at 37°C for 3–5 days before subculture and incubation at 37°C for 24 h. All cultures were examined for hemolytic activity indicating expression of the virulent (Bvg+) phase before being collected and resuspended in saline to an optical density at 650 nm = 1 for Western immunoblotting.

Western Immunoblotting

The Ptx, Prn, and Fha proteins were detected by Western immunoblotting as described (*10*,*11*,*14*). Bacterial suspensions were mixed with Laemmli buffer containing 5% β-mercaptoethanol and boiled for 5 min. Proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis were transferred to a polyvinylidene difluoride membrane at 100 V for 1 hr. Membranes were blocked with 5% (wt/vol) skim milk powder in wash buffer for 1 hr and incubated overnight with mouse polyclonal antibodies against Ptx, Fha, and Prn diluted 1:1,000 with Tris-buffered saline (TBS) containing 1% Tween 20. After 3 washes with TBS containing 1% Tween 20, membranes were incubated for 1 h with sheep antimouse monoclonal

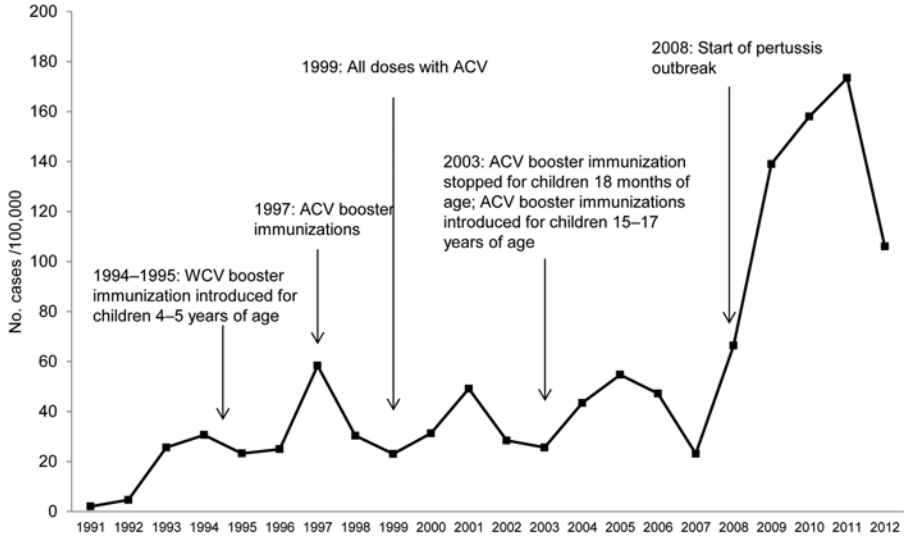

Figure 1. Pertussis cases/100,000 population in Australia, 2008–2012, since mandatory reporting was instituted in 1991 and changes to pertussis vaccination schedule, including introduction of whole-cell vaccine (WCV) booster vaccinations for 4–5-year-old children in 1994–1995 and introduction of acellular vaccine (ACV) booster vaccinations in 1997. By 1999–2000, ACVs were used for all pertussis vaccinations. In 2003, the booster vaccinations for children 18 months of age was removed and replaced with a booster vaccination for children 15–17 years of age (*3*).

antibodies in TBS plus 5% skim milk and 0.1% Tween 20. Antigen–antibody complexes were visualized by chemiluminescence on a LAS3000 imager (Fujifilm, Tokyo, Japan). The minimum detectable amount with this method was 1 ng of specific protein.

Genotyping and *prn* Gene Sequencing

Isolates were genotyped for *fim3*, *prn*, and *ptxP* alleles as described (*7*–*9*). Isolates that had not already been typed were characterized by SNP cluster and SNP profile as described by Octavia et al. (*8*), multilocus variable number tandem repeat analysis (MLVA) as described by Kurniawan et al. (*1*) typing of *prn, fim3*, and *ptxP* alleles (*6*,*15*). Relationships among SNP profiles and clusters were defined by Octavia et al. (*8*) and are shown in online Technical Appendix Figure 1 (wwwnc.cdc.gov/EID/article/20/4/13-1478-Techapp1.pdf).

For isolates that did not express Prn, overlapping primers reported by Fry et al. (*16*) were used to amplify a predicted 2,869-bp region that included the signal peptide region and the *prn* gene. The *prn* promoter region was also sequenced to detect any changes. Each PCR mixture contained ≈30 ng DNA, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 100 μmol/L of each deoxynucleotide, 10 pmol/L of each primer, 2.5 units of *Taq* polymerase, and milliQ water (Millipore, Billerica, MA, USA). Products were then sequenced on an Automated DNA Sequence Analyzer ABI3730 (Applied Biosystems, Foster City, CA, USA) to determine the complete *prn* gene, which included region 1 and region 2. All sequences were aligned against *prn* gene sequences identified by Mooi et al. (*17*).

Results

Identification and Distribution of *B. pertussis* Not Expressing Prn

The 320 *B. pertussis* isolates obtained during 2008–2012 were from 5 states in Australia: New South Wales (116 isolates), Queensland (37), South Australia (47), Victoria (30), and Western Australia (90). All 96 (30) isolates identified by Western immunoblot as not expressing Prn were obtained after 2008. Examples of Western immunoblots are shown in online Technical Appendix Figure 2. The other 133 isolates obtained before 2008 expressed Prn and were from SNP clusters I and II or were unclustered. The distribution of Prn-negative isolates in individual states is shown in the Table. Only isolates from Western Australia and New South Wales were available for all years during 2008–2012; no isolates were available from South Australia or Victoria during 2011–2012 or from Queensland during 2008–2009 and 2012. All isolates expressed Ptx and Fha.

The Prn-negative strains were first identified in isolates collected in 2008, when they made up 5% (2/39) of the isolates. By 2012, the proportion of Prn-negative isolates had increased to 78% (28/36) (Figure 2). In Western Australia and New South Wales, where isolates were available for all years, there was a progressive increase from 3% in 2009 to 78% in 2012 (online Technical Appendix Figure 3). Lack

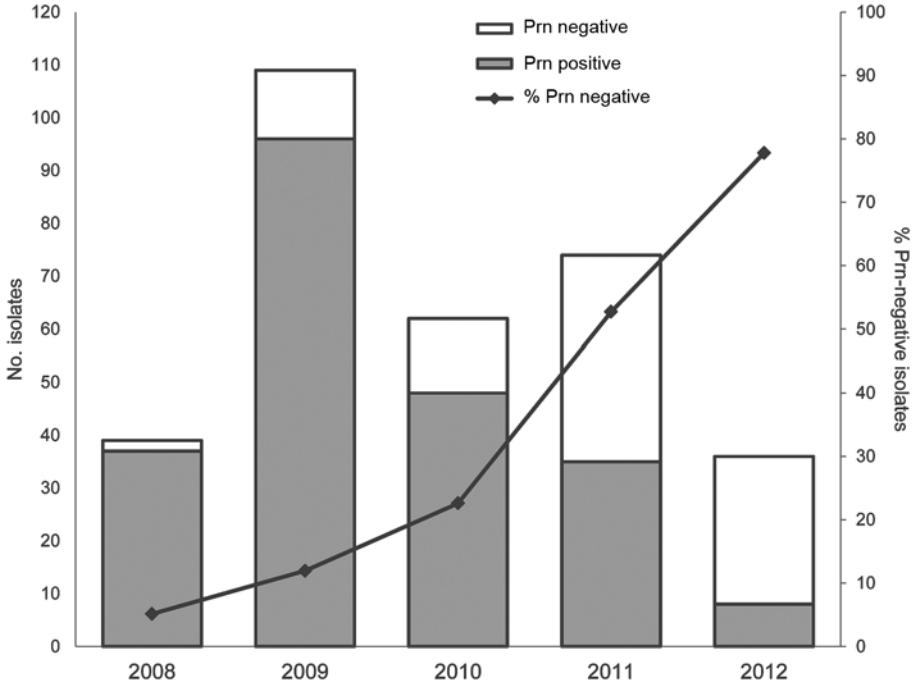

Figure 2. Number and percentage of pertactin (Prn)–negative *Bordetella pertussis* isolates in Australia, 2008–2012. During this period, 320 *B. pertussis* isolates obtained in New South Wales, Queensland, South Australia, Victoria, and Western Australia were identified as expressing Prn or not expressing Prn by using Western immunoblotting. The increasing percentage of Prn-negative isolates each year during 2008–2012 was 5%, 12%, 23%, 53%, and 78% respectively. Data for individual states and years can be found in the Table. Gray bars indicate number of isolates expressing Prn, and white bars indicate number of isolates not expressing Prn.

Table. Distribution of pertactin-positive and protactin-negative *Bordetella pertussis* isolates in 5 states, Australia, 2008–2012*

	State														
	New South Wales			Queensland			South Australia			Victoria			Western Australia		
Year	No. pos	No. neg	% Neg	No. pos	No. neg	% Neg	No. pos	No. neg	% Neg	No. pos	No. neg	% Neg	No. pos	No. neg	% Neg
2008	18	0	0	–	–	–	13	2	13	1	0	0	5	0	0
2009	52	0	0	–	–	–	17	9	35	10	1	9	18	2	10
2010	8	6	43	3	6	67	5	1	17	17	1	6	14	1	7
2011	6	17	74	21	7	25	–	–	–	–	–	–	8	15	65
2012	2	7	78	–	–	–	–	–	–	–	–	–	6	21	78

*Pos, positive; Neg, negative; –, no isolates were obtained.

of isolates from Queensland, Victoria, and South Australia in various years was related to changes in laboratory practice (cultures no longer obtained) or decreased numbers in a post-epidemic period, rather than any systematic differences in collection. It is unlikely that different patterns of circulating *B. pertussis* differed in these regions.

The increase in Prn-negative isolates during 2011–2012, in comparison with 2008, was significant ($p<0.05$, by Fisher exact test with multiple test correction). All but 1 Prn-negative isolate had the *ptxA1*, *prn2*, and *ptxP3* alleles and belonged to SNP cluster I; the exception, L1378, had *ptxA1* and *prn1* but not *ptxP3*, and was not assigned to any SNP cluster. In addition, the Prn-negative isolates had new MLVA types that were closely related to MT27 and MT114, both of which are currently circulating in Australia (*9*), although MT27 still predominates.

Sequence Analysis of *prn* Gene of Prn-deficient Isolates

Mechanisms of disruption, identified by sequencing the *prn* region, including the signal peptide, of 80/96 Prn-negative isolates, are shown in Figure 3. Seventy-seven (82%) isolates had IS elements located between region 1 and region 2; in *prn*, a 1049-bp IS*481* was inserted in the forward direction in 13 isolates and in the reverse direction in 58 isolates. A 1,037-bp IS*1002* was inserted in the forward direction in 6 isolates, which has not been described in the *prn* region. All IS element disruptions were at position 1613 and were flanked by a 6-bp repeat (ACTAGG) at the 5′ end and AGGCAG at the 3′ end (Figure 3).

One isolate had no IS within *prn* but had an additional guanine residue at position 1185 between region 1 and region 2, which resulted in a stop codon at amino acid position 749. Two isolates from South Australia that had SNP profile SP13 were nontypeable. For *prn*, multiple pairs of PCR primers specific for the *prn* gene (*15*–*19*) failed to amplify a product, which indicated deletion of the entire gene. IS disruptions, deletions, or other variations were not detected in *prn* or the *prn* promoter region of 16 Prn-negative isolates. Details of the 96 Prn-negative isolates, including individual *prn*, *fim*, and *ptxP* alleles, SP, MLVA type, and mechanism of *prn* disruption, are shown in the online Technical Appendix Table.

Discussion

In the 2 regions of Australia where isolates were available for all years during 2008–2012, Prn-negative *B. pertussis* isolates increased from >10% to ≈80% of *B. pertussis* isolates over this period. Prn-negative strains have been isolated in several countries that have high coverage for vaccination but have not been shown to constitute such a high proportion of circulating *B. pertussis* (*12*,*13*,*20*,*21*). Japan was the first country to implement ACVs against pertussis in 1981, and the proportion of Prn-negative isolates reported from countrywide surveillance during 2005–2009 was 32% (18/57). In France, where ACVs have been used since 1998, originally as booster vaccinations, and then for all doses since 2002 (*11*), Ptx-negative and Fha-negative isolates were first obtained in 2003, although only Prn-negative isolates have increased and were reported to make up 13.3% of 120 isolates analyzed in 2011 (*10*).

The Prn-negative *B. pertussis* isolates have also been identified in Finland and the United States (*13*,*21*,*22*). The United States introduced ACVs as booster vaccinations in 1991, but not until 1997 were all 5 primary doses replaced with ACVs (*23*). Although Finland replaced WCVs with ACVs at a later time (booster vaccinations in 2003 and primary vaccinations in 2005), both countries detected Prn-negative isolates during 2011–2012. Long-term temporal analysis has not been performed to determine whether such isolates are increasing over time.

In comparison, until 2001 and 2009, respectively, Russia (*24*) and Senegal (*25*), which currently use only WCVs, have not reported Prn-negative isolates. However, it is difficult to draw a definitive conclusion on the correlation of timing of emergence of Prn-negative strains with timing of introduction of ACVs. Extensive analysis of isolates from earlier years from different countries would be required.

Multiple mechanisms of lack of expression of Prn have been reported (*11*,*12*). Insertion of IS*481* into the *prn* gene in either the forward or reverse direction was still the main mechanism of disruption (73.9%). This disruption occurred at the same conserved site identified in 3 isolates from the United States (nt position 1613) (*21*) and 9 isolates from Japan (nt position 1598) (*12*). The 15-bp difference in position is caused by an additional GGFGP repeat in *prn2* in the isolates in our study and those from

the United States, compared with those from Japan, which have *prn1*.

Six isolates in our study had an additional IS*1002* disruption at nucleotide position 1613 (Figure 2), which confirmed that the 6-bp repeat site flanking IS elements is conserved (*26*). The lower number of isolates with IS*1002* disruptions could be caused by fewer copies of IS*1002* than IS*481* in the genome (6 for IS*1002* in Tohama I compared with 238 for *IS481*). Disruption of virulence genes by IS*1002* has been reported; unlike B. parapertussis and B. bronchiseptica, B. pertussis does not express O antigen because of an IS1002-mediated deletion of the O-antigen locus (*27*). All isolates in this study that had the IS*1002* disruption were collected in 1 state in Australia (New South Wales) and might have arisen from a single outbreak. More isolates are needed to determine whether this finding is indicative of an expanding clone.

Another mechanism of disruption is an 84-bp deletion of the signal peptide (nucleotide position 26). This deletion was observed in 24 isolates from Japan (*12*) and 2 isolates from Finland (*13*) but was not observed in any of the isolates in our study. However, 2 of the isolates apparently had the entire *prn* gene deleted.

In 1 isolate (L1502), an additional G residue in a homopolymeric tract of G residues resulted in a downstream stop codon. Truncations caused by stop codons in the *prn* gene were reported in 7 isolates from the United States (*21*), but they were at nucleotide position 1273 and the actual base change was not specified. Phase variation has been associated with variation in other *B. pertussis* genes, including *fim2*,

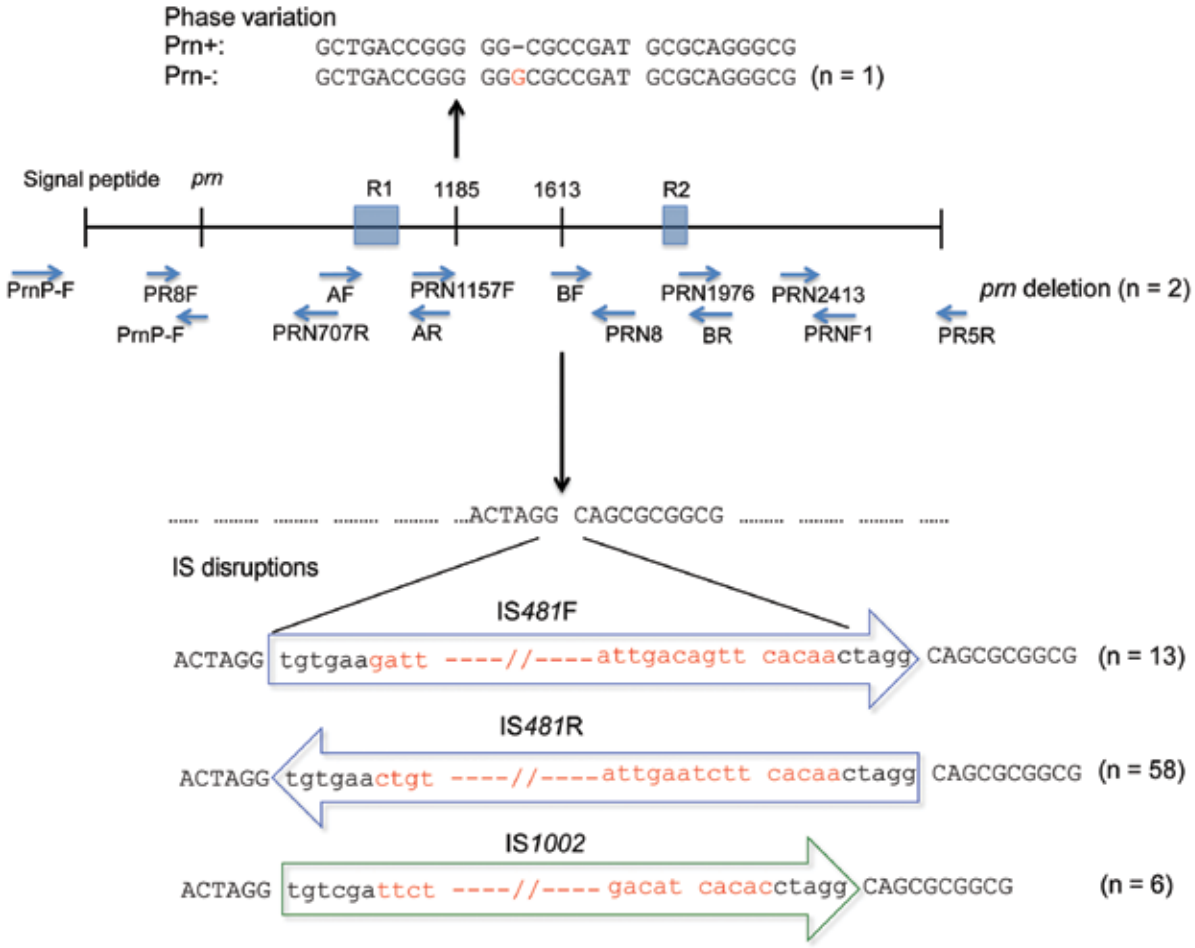

Figure 3. Variations in pertactin (*prn*) gene of Prn-negative *Bordetella pertussis* isolates, Australia, 2008–2012, Ninety-six *B. pertussis* isolates were identified as Prn negative. Eighty of these isolates had 1 of 4 mechanisms of *prn* disruption: IS*481* (in forward and reverse directions) and IS*1002,* which were inserted at the ACTAGG motif within *prn*, or an extended homopolymeric tract of G residues (n = 1). Lower case letters indicate residues that are conserved in all IS disruptions, and red letters indicate differences in IS disruptions. Positions of nucleotides have been numbered relative to the first start codon of sequence AJ011092 (*17*). The *prn* gene of 2 isolates was not amplified by PCR with a combination of primers from published studies (*15*–*19*), which indicated a deletion of the entire gene. Sixteen isolates that had no gene disruptions were also observed.

fim3, *fimX*, and *bapC* (*28*), and is a common mechanism of phase variation in other pathogenic microorganisms (*29,30*).

A large proportion (17%, 16/96) of Prn-negative isolates had no sequence change detected in the *prn* gene or its promoter upstream, which indicated that other mechanisms must have been responsible for inactivating Prn expression. These 16 isolates belong to 3 SNP profiles; 8, 7, and 1 isolates belonging to SP13, SP14, and SP18, respectively, which suggests 3 independent inactivating events. Inactivation of expression could have occurred at the transcriptional or translational level. Our preliminary investigations showed that 3 of these Prn-negative isolates produced *prn* gene transcripts. A consequence of Prn inactivation without sequence variation of the *prn* gene is that it can be detected only at the protein level. Until mechanisms are identified, culturing of isolates will still be needed to monitor *B. pertussis*.

The increase in isolates that do not express a specific antigen has been documented only recently in Australia and other countries that use ACVs. The predominant isolates we identified are from SP13, SP14, or SP16, and all but 1 had the *ptxA1*, *prn2*, *ptxP3* genotype. We have also shown that isolates with different SNP profiles can be affected by the same IS disruptions, and conversely, different IS disruptions can affect isolates with the same SNP profiles.

Most of the recently isolated Prn-negative strains from the United States have the *prn2* allele, which has been the predominant type since the 1990s (*21,22*). However, mutations causing inactivation of expression of the *prn* gene differ from those reported in this study and elsewhere. Prn-negative isolates characterized by Otsuka et al. (*12*) had the *prn1*, *ptxA2*, *ptxP1* genotype and were from MT186 or related MT194 or MT226. Our previous analysis showed that MT186 belongs to SNP cluster V; this type is unrelated to isolates examined in the current study, which belong to SNP cluster I (online Technical Appendix Figure 1), but was affected by the same IS disruption mechanisms. Two isolates from Finland that had *prn1* were also reported to be Prn negative because of deletions, although *prn2* is the current predominant allele (*13*). Thus, the combination of SNP typing, antigen gene typing, and *prn* gene disruption mechanisms clearly demonstrates that isolates that do not express the *prn* gene from Australia and other countries do not belong to the same clone and that the recent almost simultaneous appearance and expansion of Prn-negative isolates in several countries were independent events rather than global spread of a single clone.

The multiple origins of Prn-negative isolates also point strongly to selective pressure on the bacterium. Therefore, it is conceivable that these Prn-negative isolates are more likely to evade a vaccine-induced immune response. However, the relative contribution of Prn to pertussis disease has not been clearly established. Various studies using *prn* mutants have shown that mutants that do not express Prn do not colonize mouse lungs as well as isolates that express Prn (*31*) but were more invasive in epithelial cells and persist for a longer period (*32*). The Prn-negative strains have a greater growth advantage in vitro than their Prn-positive counterparts (*12*). This growth advantage can be beneficial in maintaining a high level of transmissibility between hosts, which is consistent with increasing numbers of infections with Prn-negative isolates identified in Australia and elsewhere.

Whether these isolates have greater or lesser virulence than Prn-positive strains is unclear. In contrast to lack of production of Ptx, loss of Prn does not seem to affect *B. pertussis* lethality in mice, possibly because of the range of autotransporters within *B. pertussis* that can compensate for the role of Prn (*10*). In a retrospective study, no differences were found in severity of symptoms or duration of hospitalization between infants infected with Prn-positive and Prn-negative strains in France (*20*); the only major difference observed was the longer period from onset of pertussis symptoms to time of hospitalization among infants whose *B. pertussis* isolate was Prn negative. Regardless of Prn expression, vaccination reduced the severity of disease and the likelihood of being admitted to intensive care, which suggests that even an incomplete course of primary vaccination provides some protection against severe pertussis (*20*).

The results in this study highlight the emerging trend of Prn-deficient *B. pertussis* isolates circulating in Australia. In addition to changes observed in *prn*, *ptxA*, *ptxP*, and *fim* genes of currently circulating strains, this study and other studies have reported the increasing prevalence of isolates not expressing Prn in many countries that have a high uptake of ACV. The overall effect of lack of expression of an antigen on herd immunity is unknown. Emergence of Prn-negative isolates is a relatively recent phenomenon that has affected currently circulating *B. pertussis* isolates. Whether strains not expressing Prn continue to increase locally or globally and affect vaccine effectiveness and bacterial pathogenicity is unknown. Continued monitoring of genotypic and phenotypic properties of *B. pertussis* is required to better understand the effects of vaccination on the evolution of the organism.

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Ms Lam is a doctoral candidate at the University of New South Wales, Sydney, Australia. Her research interests include the epidemiology and evolution of human pathogens.

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Address for correspondence: Ruiting Lan, School of Biotechnology and Biomolecular Sciences, University of New South Wales, Biological Sciences Building D26, Sydney 2052, New South Wales, Australia; email: r.lan@unsw.edu.au

etymologia

Pertactin

Pertactin [*per-tak'tin*]

From *per-* (pertussis) + *tactus* (Latin, "to touch"), pertactin is a virulence factor of *Bordetella pertussis* that promotes adhesion to tracheal epithelial cells and resistance to neutrophil-mediated clearance and is a component of acellular pertussis vaccines. Pertactin-negative *B. pertussis* has been reported in several countries, and its prevalence in the United States has increased in recent years. However, evidence suggests that other components of current pertussis vaccines provide protection against pertactin-negative strains.

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Address for correspondence: Ronnie Henry, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E03, Atlanta, GA 30333, USA; email: boq3@cdc.gov

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Gnathostoma spp. in Live Asian Swamp Eels (*Monopterus* spp.) from Food Markets and Wild Populations, United States

Rebecca A. Cole, Anindo Choudhury, Leo G. Nico, and Kathryn M. Griffin

In Southeast Asia, swamp eels (Synbranchidae: *Monopterus* spp.) are a common source of human gnathostomiasis, a foodborne zoonosis caused by advanced third-stage larvae (AL3) of *Gnathostoma* spp. nematodes. Live Asian swamp eels are imported to US ethnic food markets, and wild populations exist in several states. To determine whether these eels are infected, we examined 47 eels from markets and 67 wild-caught specimens. Nematodes were identified by morphologic features and ribosomal intergenic transcribed spacer–2 gene sequencing. Thirteen (27.7%) *M. cuchia* eels from markets were infected with 36 live *G. spinigerum* AL3: 21 (58.3%) in liver; 7 (19.4%) in muscle; 5 (13.8%) in gastrointestinal tract, and 3 (8.3%) in kidneys. Three (4.5%) wild-caught *M. albus* eels were infected with 5 *G. turgidum* AL3 in muscle, and 1 *G. lamothei* AL3 was found in a kidney (both North American spp.). Imported live eels are a potential source of human gnathostomiasis in the United States.

In parts of Asia, wild-caught and aquaculture-reared swamp eels (Synbranchidae: *Monopterus* spp.) are widely consumed as food by humans (*1–3*) and are a common source of human gnathostomiasis, a foodborne zoonosis caused by advanced third-stage larvae (AL3) of *Gnathostoma* spp. nematodes. (*4–8*). Over the past 2 decades, many thousands of swamp eels (Synbranchidae: *Monopterus* spp.) have been legally shipped alive from Asia to North America, where they were distributed to numerous ethnic food markets in major cities in the United States and Canada (*9*; L.G. Nico, unpub. data). An earlier survey of live Asian swamp eels from ethnic markets in the United States and introduced wild populations in Florida found substantial parasite burden in both market and wild swamp eels sampled; however, the researchers did not examine eels for *Gnathostoma* spp. (*9*).

In US ethnic food markets, imported swamp eels from Asia, together with a variety of other native and nonnative fishes, are commonly displayed alive. Consumers are able to purchase the animals and have them processed on site (gutted/filleted) or they can butcher their live purchase at home (*9*). Most of these market fish are purchased for food, but some are introduced into the wild. For instance, in Asia and certain western countries, several live fish and other animals sold in food markets and other venues are subsequently released into open waters by groups conducting ceremonial religious practices (*10–12*) with some releases that apparently involved swamp eels (*9,13*; L.G. Nico, unpub. data). Because a large number of fishborne parasitic zoonoses are found throughout the world (*14*,*15*), the importation of live fish infected with parasites from their native waters poses a threat to humans (*14*,*16*). Moreover, releasing imported foreign fish infected with parasites into open waters may introduce and spread nonnative parasites harmful to native faunas (*17*,*18*).

Swamp eels are a group of eel-like percomorph fishes naturally distributed in tropical and temperate regions of the New and Old Worlds (*19*). They are not native to the United States or Canada, but at least 5 separate introduced

Author affiliations: US Geological Survey–National Wildlife Health Center, Madison, Wisconsin, USA (R.A. Cole, K.M. Griffin); St. Norbert College, DePere, Wisconsin, USA (A. Choudhury); and US Geological Survey–Southeast Ecological Science Center, Gainesville, Florida, USA (L.G. Nico)

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populations of Asian swamp eels (*Monopterus* spp.) have been established in open waters in the continental United States. These consist of 3 populations in peninsular Florida, 1 in northern Georgia, and 1 most recently established population in southern New Jersey (*9*,*20*). The live food trade is the suspected source of all or many of these introductions (*9*). Genetic analysis revealed that the introduced wild populations are composed of 3 genetically distinct clades within the *M. albus* (Zuiew, 1793) complex, a widely-distributed group native to eastern and southeastern Asia (*20*,*21*). A separate Asian swamp eel species, *M. cuchia* (Hamilton-Buchanan, 1822), also referred to as *Amphipnous cuchia*, is native to northern and northeastern India, Bangladesh, Nepal, Myanmar, and Pakistan (*22*). *M. cuchia* and members of the *M. albus* complex have been documented in animals in the live food trade and in ethnic food markets in the USA, but *M. cuchia* has not yet been documented in the United States in wild populations (L.G. Nico, unpub. data). All swamp eel species sold in the live food trade have behavioral and physiologic adaptations that make them attractive for live import and increase the risk for their invasion success in the wild. For example, both *M. albus* and *M. cuchia* eels are air breathers and, if kept moist, they can survive for months out of water and without food (*23*; L.G. Nico, unpub. data). Some *M. albus* swamp eels are protogynous hermaphrodites and change naturally from female to male, supposedly in response to environmental cues (*24*).

Farmed and wild *M. albus* eels in Asian countries are reported to have a high prevalence of infection with *G. spinigerum* nematodes (*4*–*8*). This nematode is native to Asia and the most commonly reported cause of gnathostomiasis in humans in Asia (*6*,*25*). Species of *Gnathostoma* have a 3-host life cycle. Cyclopoid copepods act as first intermediate host and consume stage 2 larvae (L2) that develop into early L3) in the copepod's hemocoel. The copepod infected with the early L3 is then consumed by second intermediate hosts such as freshwater or saltwater fish, amphibians, reptiles, or birds, in which it migrates from the stomach into other organs (most commonly the liver and striated muscle) where it develops to AL3. Felids and canids are typical definitive hosts (*7*). Humans become infected by consuming raw or undercooked meat from second intermediate hosts. Once in the human host, AL3 do not develop further, but continue to migrate through tissues, including subcutaneous spaces, visceral organs, and the central nervous system (*26*).

As many as 13 species of *Gnathostoma* are currently recognized as valid (*27*). Although it has been hypothesized that all species of *Gnathostoma* can infect humans, only 6 species have been reported to infect humans: *G. binucleatum*, *G. doloresi*, *G. hispidum*, *G. malaysiae*, *G. nipponicum,* and *G. spinigerum* (*27*). These zoonotic species use a variety of animals as definitive hosts: cats (*G. binucleatum* and *G. spinigerum*), pigs (*G. doloresi* and *G. hispidum*), rats (*G. malaysiae*), weasels (*G. nipponicum*) and dogs (*G. spinigerum*). Four species of *Gnathostoma* have been reported from wildlife in the United States. Among the 4, *G. procyonis* (raccoons) is widely distributed in the United States, whereas *G. turgidum* (opossums), *G. miyazakii* (otter), and *G. socialis* (mink) have patchy distributions (*27*).

To determine whether imported *M. cuchia* swamp eels were infected with *Gnathostoma* spp., we examined live eels obtained from various ethnic food markets in 3 major metropolitan areas in the eastern United States. We also examined individual wild *M. albus* eels, from populations introduced into open waters in Florida and New Jersey, for the presence of AL3 to determine their ability to host endemic or introduced *Gnathostoma* spp.

Materials and Methods

Fish Sampling and Examinations

Asian swamp eels examined for *Gnathostoma* spp. infection included 47 specimens from 5 ethnic market in 3 major metropolitan areas in the eastern United States and 67 wild-caught specimens from 4 of the 5 known introduced populations established in the continental United States (Table 1). All market specimens identified as *M.* (*Amphipnous*) *cuchia* eels purchased live from ethnic food markets during 2010–2012 included the following: 1) 10 specimens obtained from 3 markets in New York's Chinatown in Manhattan; 2) 12 specimens from a single market in the Atlanta, Georgia, area; and 3) 25 specimens from a single market in the Orlando, Florida, area. On the basis of species identification and information in US Fish and Wildlife Service Law Enforcement Management Information System (USFWS-LEMIS) live-animal shipment records, we concluded that all or most of the market *M. cuchia* eels likely originated in Bangladesh and were shipped by air to the United States.

Wild-caught swamp eel specimens collected during 2011–2012 were members of the *M. albus* species complex and included 3 geographically disjunct populations in peninsular Florida and 1 in New Jersey. Each wild population is a distinct clade (20; L.G. Nico, unpub. data). Populations and sites sampled included the following: 1) Tampa area population (clade C), 14 specimens from 2 sites in the Frog Creek drainage, Tampa Bay Basin, in Manatee County (near 27°35′18″N, 82°30′35′W and 27°35′20″N, 82°32′28″W); 2) North Miami population (clade C), 11 specimens from 2 sites in the Snake Creek Canal (canal C-9) drainage, Broward and Dade counties (near 25°58′36″N, 80°13′46″W and 25°57′36″N, 80°12′18″W); 3) Florida Homestead population (clade B), 23 specimens collected from canals C-111 and L-31N, Dade County, near Everglades National Park (near 25°30′19″N, 80°33′35″W and 25°23′14″N,

Table 1. Summary information on live Asian swamp eels from market and wild populations in the United States examined for larval stages of *Gnathostoma* spp. in 47 *Monopterus cuchia* swamp eels purchased from 5 ethnic food markets and 67 wild-caught *M. albus* (clades A, B, and C) from 4 introduced populations*

Sources and eel identifications (dates sampled)	No. samples	Total length, mm, min/max, (mean)	Body weight, g, min/max (mean)	No. eels (%) infected with *Gnathostoma* spp.	Eel specimen, parasite species, intensity, and tissue infected
Market samples: all *M. cuchia*					
New York Chinatown, 3 markets (2011 Aug 22)	10	631–850 (707)	208–693 (359)	3 (30): *G. spinigerum*	Mc 28 Gs 1K, 1M Mc 30 Gs 1G Mc 32 Gs 1M
Orlando, Florida, 1 market (2011 Jan 27, Oct 17, Oct 31; 2012 Jan 9)	25	546–781 (669)	173–565 (350)	5 (20): *G. spinigerum*	Mc 17 Gs 4L Mc 21 Gs 1G Mc 37 Gs 1L Mc 58 Gs 1G, 2M Mc 59 Gs 2G, 1M
Atlanta, Georgia, 1 market (2010 Oct 25)	12	663–825 (730)	316–796 (486)	5 (41.7): *G. spinigerum*	Mc 3 Gs 1G Mc 9 Gs 12L, 2M Mc 10 Gs 1L Mc 11 Gs 1L Mc 12 Gs 2L
All market samples (2010–2012)	47	546–850 (692)	174–796 (386)	13 (27.7): *G. spinigerum*	
Wild population samples					
Florida,Tampa area: *M. albus* clade C (2011 Nov 29–30)	14	140–912 (347)	5–693 (95)	3 (21.4): *G. turgidum; G. lamothei*	Ma 48 Gt 4M Ma 49 Gt 1M Ma 54 Gl 1K
Florida, North Miami area: *M. albus* clade C (2012 Feb 6)	11	292–710 (522)	22–343 (168)	0	
Florida, Homestead area: *M. albus* clade B (2012 Mar 10 & 12)	23	230–650 (431)	6–309 (91)	0	
New Jersey: *M. albus* clade A (2012 Apr 18)	19	190–630 (314)	4–192 (35)	0	
All wild population samples (2011–2012)	67	140–912 (395)	4–693 (89)	3 (4.5): *G. turgidum; G. lamothei*	

*Min, minimum; max, maximum; Mc, *M. cuchia*; Gs, *G. spingerum*; K, kidney; M, muscle; G, gut; L, liver; Ma, *M. albus*; Gt, *G. turgidum*; Gl, *G. lamothei*.

80°33′29″W); and 4) New Jersey population (clade A), 19 specimens from Silver Lake in Gibbsboro, Camden County (near 39°50′21″N, 74°57′44″W). All sampled sites were inland, freshwater systems, and eels were collected in stream, canal, and lake habitats by using electrofishing gear.

Within 1–3 days of purchase or collection, swamp eels were transported to the US Geological Survey facility in Gainesville, Florida, where groups of ≤10 live swamp eels from each sampled population (i.e., market source or wild population) were held in large, clean indoor fiberglass tanks (120 cm long × 60 cm wide × 60 cm high) in 15 cm of non-circulating water from a tap source. Water in holding tanks had a pH of 7, salinity of 0.2 ppt, and temperature or 24–31°C. Large eels were separated from small eels to prevent cannibalism. Captive swamp eels held for more than several days were intermittently offered live commercially-raised earthworms (*Lumbricus terrestris*) as food, although few individuals fed on the worms. After a holding period from 1 day to several weeks, small numbers of swamp eels (1–9 individuals) were shipped live at selected intervals from November 2010 through May 2012 by overnight courier to the US Geological Survey, National Wildlife Health Center, where they were immediately euthanized with a solution of MS-222 (475 mg/L water) (tricaine methanesulfonate; Sigma, St. Louis, MO, USA) Each eel was then assigned a unique identifier code and the specimen's total length (TL) from tip of snout to posterior end of tail was measured to the nearest mm, and weighed to the nearest gram..

Eels were decapitated, then skinned and filleted; all muscle was removed and liver, kidney, and gastrointestinal tracts were removed and separated. All organs were examined for AL3 stages of *Gnathostoma* spp. by using a dissection stereomicroscope (magnification ×4–7) then placed in a commercial grade food blender in a 5% pepsin hydrochloric acid solution and macerated. Tissue digest solution was placed in a shaking hot water bath at 37°C for overnight up to 24 hours. All digested samples were centrifuged at 3,000 *g;* solid residue was then rinsed in phosphate-buffered solution, and residue was examined for AL3 with a dissection stereomicroscope (magnification ×4–7).

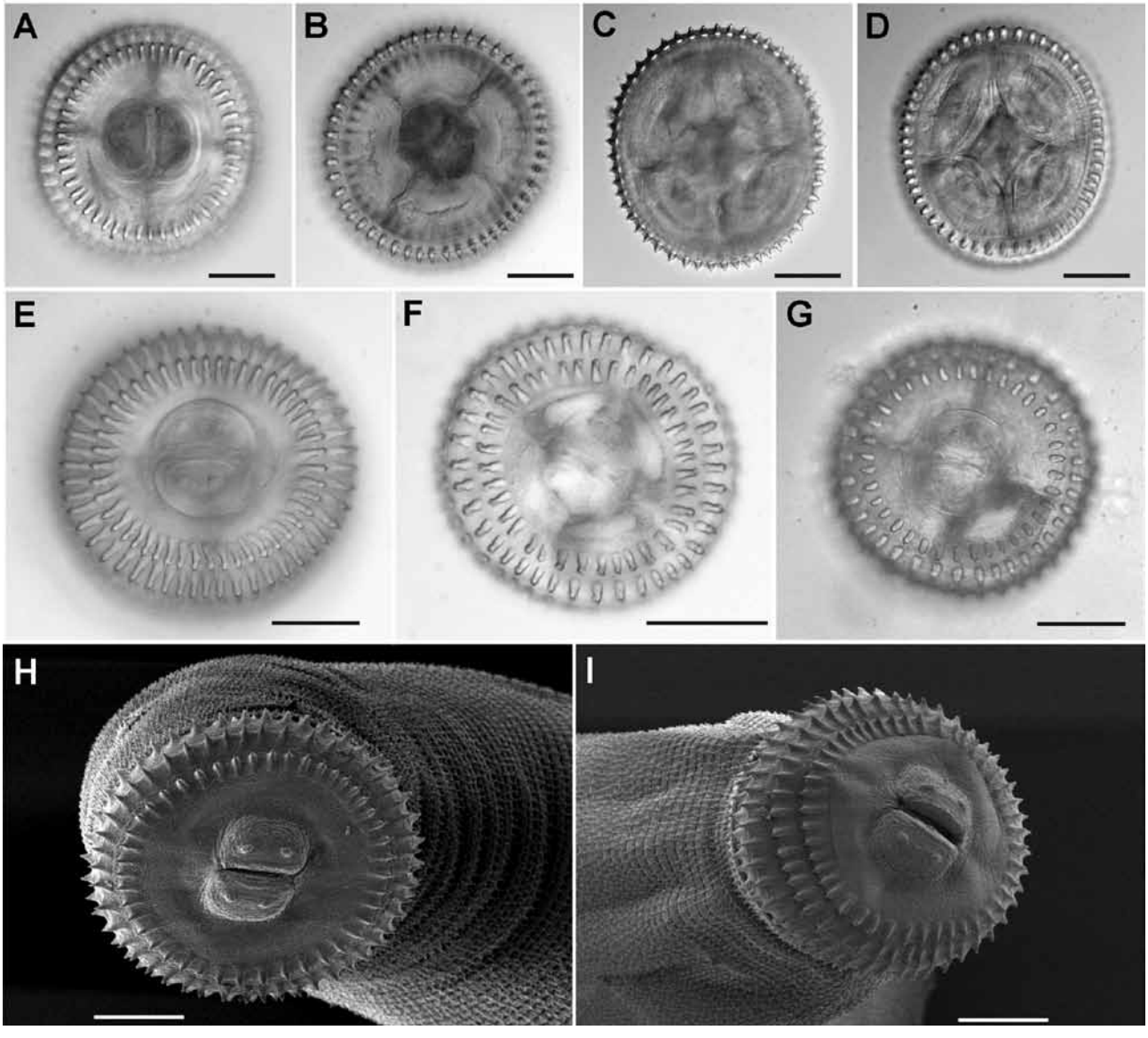
Fixation and Morphologic Identification of Nematodes

A subset of AL3 were fixed in toto in warm, 10% neutral-buffered formalin and then stored in 70% ethanol with 0.5% glycerin for morphologic identification. For all other AL3s, the posterior 2/3 of the worm was excised and

fixed in cold molecular grade 100% ethanol and stored at 4°C for up to 5 months for subsequent DNA extraction and sequencing. The remaining anterior portions were fixed for whole mounts as before. Morphologic identifications were made on the basis of published keys (*7*,*28*,*29*).

To obtain cephalic bulb hooklet counts, we placed cephalic bulbs in a 20% ethanol, 2% glycerin solution in which bulbs were severed and oriented in an en face position in a drop of the same medium and placed under a coverslip. Hooklets on the first 2 rows (Figure 1, panels A, B) were counted by using an Olympus BX 51 microscope with brightfield and Nomarski DIC optics (Olympus Corp., Center Valley, PA, USA). The cephalic bulb was reoriented with the lips facing down for counting the 3rd and 4th rows (Figure 1, panels C, D). Images were captured digitally.

For scanning electron microscopy, the anterior portions of roundworms were post-fixed in osmium tetroxide in phosphate buffer, dehydrated through a graded ethanol



Figures 1. A–D) Views showing the technique used for hook counts of *Gnathostoma* spp., United States, En face (panels A, B) and posterior (panels C,D) views showing the technique used for hook counts; specimen shown here is of *Gnathostoma spinigerum* from eel 59 specimen b from gastrointestinal digestion. E–G) En face mounts of the cephalic bulbs of specimens identified as 3 different species on the basis of molecular data: panel E, specimen eel 59 G, a, *G. spinigerum*; panel F, specimen eel 48 M, c, *G. turgidum*, and panel G, specimen eel 54 K, a, *G. lamothei*. Note the difference between the hook counts in row 1 between *G. spinigerum* and the 2 other species (Table 2). H–I) Scanning electromicrograph of specimens from eel 9, identified as *G. spinigerum* on the basis of cephalic bulb hook counts. Scale bars = 50 µm.

series, and infiltrated with hexamethyldisilizane, following which the hexamethyldisilizane was allowed to evaporate off the specimens that were then mounted on stubs, sputter coated with gold, and scanned by using a Philips XL-20 Scanning Electron Microscope (Philips, Andover, MA, USA). Images were captured digitally.

DNA Sequencing and Analysis

DNA was extracted following the Animal Tissue Protocol using QIAGEN's DNeasy Blood & Tissue Kit (QIAGEN Inc. Valencia, CA, USA). Primers, NEWS2 (forward) 5′-TGTGTCGATGAAGAACGCAG-3′ and ITS2-RIXO (reverse) 5′-TTCTATGCTTAAATTCAGGGG-3′ were used to amplify a 600-bp fragment of the 5.8S rRNA gene and the intergenic transcribed spacer 2 (ITS-2) by using PCR (*30*) to corroborate morphologic identifications. Five microliters of the reaction mixture was examined by 1% agarose gel containing 0.0001% Gel Red (Phenix Research Products, Candler, NC, USA) by gel electrophoresis. Primers and nucleotides were removed from the PCR products by using ExoSAP-IT for PCR Product Clean-Up (Affymetrix, Santa Clara, CA, USA) as specified in manufacturer's instructions. PCR products were sequenced at the University of Wisconsin–Madison Biotechnology Center's DNA Sequencing Facility using the BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) DNA sequencing system. Reaction products were analyzed by using an Applied Biosystems 3730xl automated DNA sequencing instrument. Sequences were examined with Finch TV 1.4.0 (Geospiza, Inc., Seattle, WA, USA; www.geospiza.com) and manually edited. A total of 23 individual worm sequences from 11 eels were available for analysis. These sequences were aligned along with 20 sequences of *Gnathostoma* spp. available on GenBank: *G. spinigerum*, *G. binucleatum*, *G. hispidum*, *G. nipponicum*, *G. miyazakii*, *G. lamothei*, *G. doloresi*, and *G. turgidum* from NCBI Database using ClustalW version 5.1 in MEGA (*30*) and manually trimmed to remove overhang. Sequences from this study were deposited in GenBank under accession nos. KF648531–KF648553.

Molecular analyses were conducted with MEGA version 5 (*31*). Cluster analyses were performed by using the unweighted pair group method with arithmetic mean (UPGMA) (*32*) and neighbor-joining algorithms. Statistical support for groupings was estimated by using bootstrap analysis.

Results

All 47 market swamp eels (*M. cuchia*) examined were adult-sized (Table 1). The 67 wild-caught specimens (*M. albus*) examined included juveniles and adults. Based on results from an age growth study conducted on *M. albus* swamp eels from a subtropical lake in China (*33*), sizes of swamp eels in current study corresponded to estimated ages ranging from <1 year (eels <200 mm TL) to ≥5 years (eels >500 mm TL).

Thirty-six AL3 of *G. spinigerum* roundworms were recovered from 13 (27.7%) *M. cuchia* swamp eels purchased from markets (5 from an Atlanta market; 5 from Orlando markets; 3 from New York markets). Five AL3 of *G. turgidum* roundworms and 1 AL3 of *G. lamothei* roundworms were collected from 2 (2.9%) and 1 (1.4%) of the *M. albus* eels obtained from wild populations in the Tampa, Florida, area. All AL3 were live and found in the digest residues of the following tissues: 21 in livers, 12 in muscles, 5 in gastrointestinal tracts and 4 in the kidneys. Only 1 eel had grossly visible white nodules on the liver, which contained AL3 of *G. spinigerum*. Among wild *M. albus* populations, gnathostomes were only collected from swamp eels from the Tampa population (Table 1).

All AL3 of *G. spinigerum* were found in imported eels from the markets, whereas *G. lamothei* and *G. turgidum* were only found in introduced wild swamp eels from open waters in the Tampa area of Florida. Matching the molecular data to the cephalic bulb hooklet counts (Table 2) corroborated that AL3 of *G. spinigerum* could be readily distinguished from *G. turgidum* and *G. lamothei* by the higher number of hooks in the first row (Figure 1, panels E–I). AL3 of *G. turgidum* and *G. lamothei* showed overlap of hook number in rows 1–3, but the 2 species could be distinguished by the fewer hooks in the 4th row of *G. lamothei*.

Assembled sequences of the amplicons varied from 511 to 659 bp. Mapping sequences to a reference sequence of *G. spinigerum*, (GenBank accession no. AB181155) comprising partial 18S, entire ITS-1, 5.8S, ITS-2, and partial 28S regions, demonstrated our sequences (see Figure 2 for accession nos.) comprised partial 5.8S, entire ITS-2 and partial 28S regions of the rRNA. After alignment and trimming, the resulting aligned database used for UPGMA and neighbor-joining analyses comprised only the ITS-2 region. Sequences from the aligned file mapped to a region between bp 1415 and 1790 on the reference sequence (AB181155), i.e., entirely within the reported ITS-2 region (Figure 2; Table 2). The UPGMA and neighbor-joining analyses resulted in the 23 isolates from this study falling into 3 distinct clusters, each representing a distinct species of *Gnathostoma*: *G. spinigerum* (17 isolates), *G. lamothei* (1 isolate), and *G. turgidum* (5 isolates) with high nodal support. Only the neighbor-joining tree is shown.

Discussion

During 2005–2008, more than 1 billion live animals were legally imported into the United States for food and pet trade markets (*34*). Possibly beginning in the 1990s, large numbers of live Asian swamp eels were shipped from several different countries in Asia to US ethnic food

Table 2. Hooklet numbers from the 4 rows of the cephalic bulbs of AL3 *Gnathostoma* spp. and corresponding GenBank sequences collected from live Asian eels from market and wild populations in the United States*

Eel species, individual no., tissue infected, AL3	Row 1	Row 2	Row 3	Row 4	Species identification and accession no.
Mc 3 G	46	46	49	51	*G. spinigerum* KF648531
Mc 9 L, a	49	50	ND	ND	*G. spinigerum* KF648532
Mc 9 L, b	48	53	55	56	*G. spinigerum* KF648533
Mc 9 L, c	42	45	43	52	*G. spinigerum* KF648534
Mc 9 L, d	43	44	47	49	*G. spinigerum*
Mc 9 L, e	43	49	50	52	*G. spinigerum*
Mc 9 L, f	45	49	50	52	*G. spinigerum*
Mc 9 L, g	43	44	49	51	*G. spinigerum*
Mc 10 L	42	47	48	54	*G. spinigerum*
Mc 11 L	40	44	50	44	*G. spinigerum*
Mc 12 L	44	41	45	49	*G. spinigerum*
Mc 17 L, a	45	43	45	50	*G. spinigerum*
Mc 17 L, b	44	48	49	50	*G. spinigerum*
Mc 17 L, c	41	44	47	49	*G. spinigerum*
Mc 17 L, d	ND	ND	ND	ND	*G. spinigerum* KF648535
Mc 17 K	43	44	44	46	*G. spinigerum*
Mc 21 G	42	45	47	52	*G. spinigerum* KF648536
Mc 26 M	33	39	40	53	G. spinigerum
Mc 28 K	43	47	50	54	*G. spinigerum* KF648552
Mc 28 M	42	43	45	46	*G. spinigerum* KF648551
Mc 30 G	40	43	42	47	*G. spinigerum* KF648550
Mc 32 M	44	45	50	–	*G. spinigerum* KF648553
Mc 37 L	42	47	50	54	*G. spinigerum* KF648549
Mc 58 M, a	ND	ND	44	52	*G. spinigerum* KF648542
Mc 58 M, b	46	49	51	52	*G. spinigerum* KF648540
Mc 58,G, c	45	47	50	52	*G. spinigerum* KF648541
Mc 59 G, a	47	48	47	52/53	*G. spinigerum* KF648538
Mc 59 G, b	48	50	52	55	*G. spinigerum* KF648539
Mc 59 M, c	42	46	47	52	*G. spinigerum* KF648537
Mean ± SD	41.8 ± 3.2	45.9 ± 3.0	47.6 ± 3.3	51 ± 2.9	*G. spinigerum* (this study)
Mean	42.9 ± 2.4	44.3 ± 2.0	44.9 ± 3.4	49.0 ± 2.9	*G. spinigerum*†
Ma 48 M, 2a	35	37	37	44	*G. turgidum* KF648547
Ma 48 M,1a	36	34	37	42	*G.turgidum* KF648548
Ma 48 M, b	32	39	40	48	*G. turgidum* KF648546
Ma 48 M, c	37	40	41	45	*G. turgidum* KF648545
Ma 49 M, a	35	37	36	42	*G. turgidum* KF648544
Mean ± SD	35 ± 1.9	37.4 ± 2.3	38.2 ± 2.2	44.2 ± 2.5	*G. turgidum* (this study)
Mean ± SD	30.8 ± 2.8	34.0 ± 2.4	36.7 ± 3.6	39.6 ± 2.7	*G. turgidum*‡
Ma 54, K a	36	38	36	36	*G. lamothei* (this study) KF648543

*AL3, advanced larval stage 3; Mc, *Monopterus cuchia*; G, gut; L, liver; ND, not determined; K, kidney; M, muscle; Ma, *M. albus*. Letters after tissue type indicate that multiple larvae were found in 1 sample.
†See (*29*).
‡See (*28*).

markets (*9*). Through a series of Freedom of Information Act requests, we obtained USFWS-LEMIS shipment records for live animal imports for July1996–February 2010. Of the 815,000 imported live swamp eels, >95% were listed as originating from wild populations, not aquaculture sources. LEMIS provides records for wildlife shipments transported through 18 ports especially designated for such commerce. In their countries of origin (e.g., Thailand, Vietnam, China, Cambodia), swamp eels are commonly consumed as food by humans and widely available in food markets. In their native ranges, swamp eels and various other Asian freshwater fishes are infected with *Gnathostoma* spp. larvae (*8*,*35*). Increased global trade of live fish increases the risk that gnathostomes and other fish-borne parasites will be introduced into regions along with their introduced hosts.

Gnathostomiasis is a major foodborne parasitic zoonosis and a notable public health problem in areas where raw or undercooked freshwater fish are consumed by humans. Most human infections are in Southeast Asia, especially in Thailand. Infected persons can exhibit intermittent migratory subcutaneous swellings, which often recur over several years because of larval migrans. In some instances, larvae migrate into deeper tissues, causing visceral gnathostomaisis, which can be fatal if the larvae invade the central nervous system (*7*). Because human gnathostomiasis has been documented with increased frequency in countries where the parasite is not endemic, it is currently regarded as an emerging imported disease (*26*). Travel to a gnathostone-endemic area (within the past 10 years) and consuming raw or undercooked fresh water fish, frogs, poultry, or

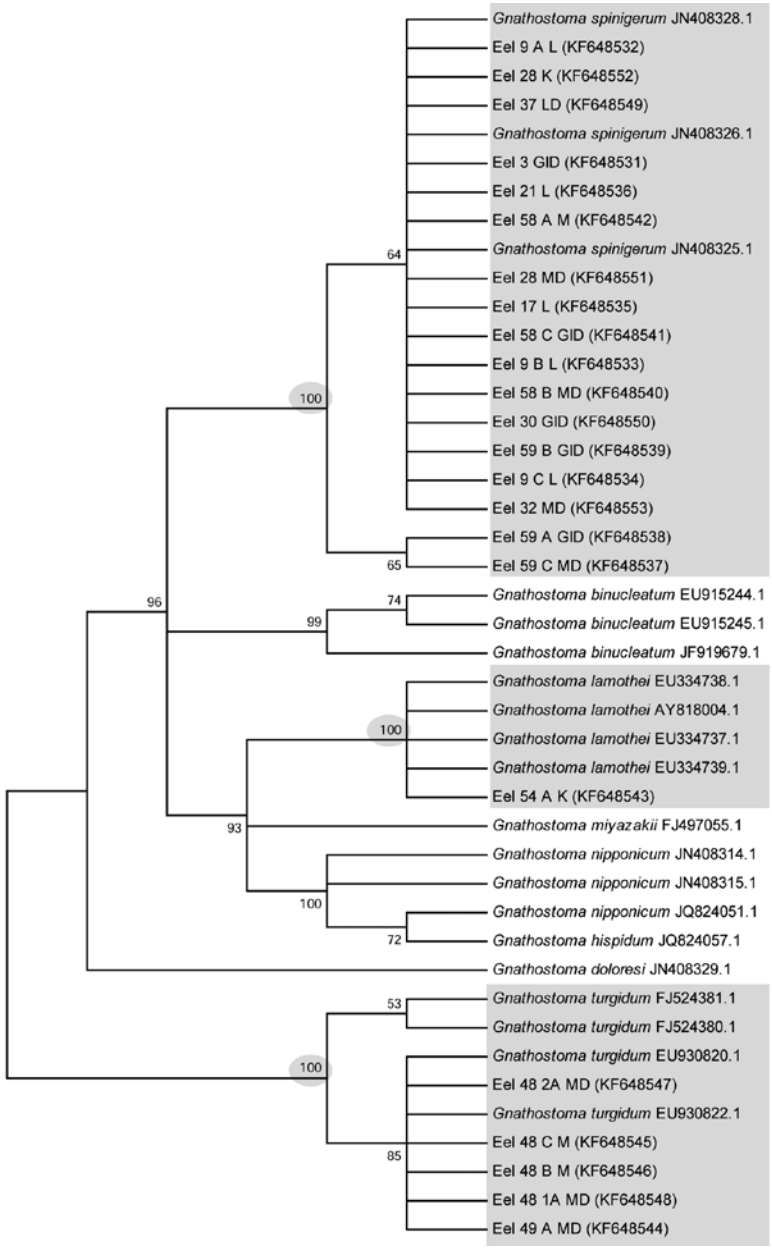


Figure 2. Dendogram showing the condensed bootstrap consensus tree (1,000 replicates) produced by neighbor-joining analysis for *Ganthostoma* spp. Partitions reproduced in <50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The sequences from the gnathostome larvae analyzed in this study fall within 3 distinct clusters (gray shading) corresponding to 3 species, with high nodal support (100%).

shellfish are key criteria used in diagnosing gnathostomiasis (*26*,*36*).

Our data show that live swamp eels imported to the United States from gnathostome-endemic areas could serve as a source of infection to humans in the United States. Therefore, travel outside of the United States to gnathostome-endemic areas may be limiting as a criterion in diagnosis. On rare occasions, an autochthonous infection has been reported in the United States (*37*,*38*). Several studies report a high prevalence of *G. spinigerum* in wild or farmed swamp eels in Southeast Asia (*4*–*8*).

Our recovery of live *G. spinigerum* from live swamp eels shipped from Asian sources to the United States is not surprising because the eels can survive for long periods in transport and at the market. In addition, AL3 are hardy and can remain alive for some time after the intermediate host is dead. The larvae can also survive 9–12 days at –9 to –4°C, 1 month at 4°C, and 8–9 days in 28% 35% ethanol (*7*). Considering food safety, evisceration of eels in this study rid the carcass of most of the worms (71.4%); however, 28.5% of larvae were found in the muscle, causing a risk for the consumer of raw or undercooked meat, or meat that was not frozen sufficiently before it was eaten raw. With respect to release of *G. spinigerum* roundworms into native fish and wildlife, through disposal of offal or the actual release of live swamp eels into open waters,

could facilitate parasite introduction because the US environment has all the components of the parasite's life cycle: canines and felines that serve as definitive hosts; cyclopoid copepods that serve as first intermediate hosts; fish, amphibians, and birds that serve as second intermediate hosts; and reptiles that serve as paratenic hosts (*7*).

Past studies have documented high prevalence of *G. spinigerum* larvae in live *M. albus* eels sampled from the wild, aquaculture settings, and markets in Thailand, Vietnam, and a few other Asian countries (*8*,*35*). However, *G. spinigerum* worms have not been reported in *M. cuchia*, a swamp eel also native to Asia but with a different natural geographic distribution than that of members of the *M. albus* species complex. *M. cuchia* eels are native to Bangladesh, the probable source of our US market specimens. In Bangladesh, although 40% of dogs surveyed were infected with *G. spinigerum*, human gnathostomiasis is reportedly uncommon (*39*,*40*).

The recovery of *G. turgidum* and *G. lamothei* from 2 and 1 *M. albus* eels, respectively, collected in open waters of Florida demonstrates that this introduced species of eel is a suitable host for North American species of *Gnathostoma*. Although these species have not been reported to be zoonotic, it has been suggested that all species of *Gnathostoma* can most likely infect humans (*7*). The previous record of *G. turgidum* infection in the United States is from the liver of a Florida black bear (*Ursus americanus floridanus*) in 1932 (*27*). Adult *G. turgidum* worms infect species of opossum (Didelphidae) and are prevalent in Mexico and South and Central America (*27*). Frogs (*Rana zweifeli*) and mud turtles (*Kinosternum integrum*) were the main second intermediate and paratenic hosts respectively of *G. turgidum* in Mexico (*28*). Adult *G. lamothei* infections in raccoons (*Procyon lotor hernandezii*) have been described in Mexico (*27*).

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Dr Cole is a research zoologist and the head of the Diagnostic Parasitology Laboratory at the US Geological Survey, National Wildlife Health Center in Madison, WI. Her research interests are parasites of native and introduced fish and wildlife.

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Address for correspondence: Rebecca A. Cole, US Geological Survey, National Wildlife Health Center, 6006 Schroeder Rd, Madison, WI, 53711, USA; email: rcole@usgsgov

Epidemic of Mumps among Vaccinated Persons, the Netherlands, 2009–2012

Jussi Sane, Sigrid Gouma, Marion Koopmans, Hester de Melker, Corien Swaan, Rob van Binnendijk, and Susan Hahné

To analyze the epidemiology of a nationwide mumps epidemic in the Netherlands, we reviewed 1,557 notified mumps cases in persons who had disease onset during September 1, 2009–August 31, 2012. Seasonality peaked in spring and autumn. Most case-patients were males (59%), 18–25 years of age (67.9%), and vaccinated twice with measles-mumps-rubella vaccine (67.7%). Nearly half (46.6%) of cases occurred in university students or in persons with student contacts. Receipt of 2 doses of vaccine reduced the risk for orchitis, the most frequently reported complication (vaccine effectiveness [VE] 74%, 95% CI 57%–85%); complications overall (VE 76%, 95% CI 61%–86%); and hospitalization (VE 82%, 95% CI 53%–93%). Over time, the age distribution of case-patients changed, and proportionally more cases were reported from nonuniversity cities (p<0.001). Changes in age and geographic distribution over time may reflect increased immunity among students resulting from intense exposure to circulating mumps virus.

Mumps is an acute illness caused by mumps virus (family *Paramyxoviridae*) and characterized by fever, swelling, and tenderness of ≥1 salivary gland, usually the parotid gland. Complications associated with mumps include orchitis (inflammation of the testes), meningitis, pancreatitis, and deafness. Mumps virus is spread in respiratory droplets, and the incubation period is 15–24 days (median 19) (*1*).

Author affiliations: National Institute for Public Health and the Environment, Bilthoven, the Netherlands (J. Sane, S. Gouma, M. Koopmans, H. de Melker, C. Swaan, R. van Binnendijk, S. Hahné); European Programme for Intervention Epidemiology Training, European Centre for Disease Prevention and Control, Stockholm, Sweden (J. Sane); and Erasmus Medical Centre, Rotterdam, the Netherlands (S. Gouma, M. Koopmans)

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Vaccination for mumps has been in use in industrialized countries for decades (*2*). The Netherlands began mumps vaccination in 1987, using the measles, mumps, and rubella combination vaccine (MMR). The vaccine, containing the Jeryl-Lynn mumps virus strain, is administered in a 2-dose schedule at 14 months and 9 years of age. Vaccination coverage of ≥1 dose of MMR has consistently been >93% since the introduction of the vaccination program (*3*). After the MMR program was launched, the incidence of mumps in the Netherlands decreased considerably; nevertheless, during the 2000s, several mumps outbreaks were detected. In 2004, an outbreak occurred among students at an international school (*4*), and in 2007–2008, an outbreak was detected mainly in a religious community that had low vaccination coverage (*5*). Since the end of 2009, a countrywide epidemic has been ongoing, affecting mainly student populations (*6*,*7*).

Mumps was notifiable in the Netherlands before 1999 and was made notifiable again in December 2008 (*5*). Mumps surveillance reports are released biweekly or monthly and include data on age and sex distribution, geographic distribution, vaccination, and contact status of case-patients. The report is distributed to public health professionals, including epidemiologists, virologists, and local-level health professionals, but comprehensive spatiotemporal characterization of the surveillance data has not been conducted. To provide information for future mumps prevention efforts, we used this surveillance data to assess the rates of illness and complications associated with the ongoing outbreak, to understand who is at risk for infection, and to assess whether transmission patterns have changed over time.

Methods

We reviewed data on mumps cases reported to the registration system for notifiable infectious diseases in the

Netherlands (OSIRIS) during September 1, 2009–August 31, 2012. Notification criteria for mumps include ≥1 related symptom (i.e., acute onset of painful swelling of the parotid or other salivary glands, orchitis, or meningitis) and laboratory confirmation of infection or an epidemiologic link to a laboratory-confirmed case (*7*). In addition to basic demographic information, notification data reported to OSIRIS included vaccination status and student or contact with student status. The questions on student/student contact status were made more specific on April 19, 2010. For cases reported before that date, the information for the new variable was obtained from open-format questions. Laboratory confirmation criteria included ≥1 of the following: detection of mumps-specific IgM; detection of viral RNA; or isolation of the virus on cell culture. Genotyping targeting the gene encoding the small hydrophobic protein was performed on specimens submitted to the National Institute for Public Health and the Environment by using an in-house method.

We used the χ^2 test for comparison of proportions and testing for trends over time and calculated a 3-week moving average to characterize trends and seasonality. Vaccine effectiveness (VE) was estimated as 1 – odds ratio. The odds ratio, which describes the association between complications/hospitalizations and vaccination status, was adjusted for age and sex (when outcome was orchitis, adjustment was done for age only) and estimated by using logistic regression. Associations with p values of <0.05 were considered statistically significant, and all reported p values are 2-tailed. Stata software version 12 (StataCorp, College Station, TX, USA) was used for the analyses.

Results

During September 1, 2009–August 31, 2012, a total of 1,557 cases of mumps were reported in the Netherlands (Figure); 1,254 (80.5%) of these were laboratory confirmed. Laboratory confirmation was most often by detection of viral RNA (68.8%), followed by antibody detection (21.9%) and virus isolation (7.3%). In 2% of cases, 2 methods were combined for diagnosis.

Most case-patients were male (59%) and 18–25 years of age (67.9%). The average annual incidence per 100,000 population was 0.5 for the 0–3-year age group, 0.8 for the 4–14-year age group, 4.5 for the 13–17-year age group, 21.4 for the 18–25-year age group, and 0.9 for the >25-year age group. Of the 1,474 cases for which patient vaccination status was reported, 998 (67.7%) case-patients had received 2 doses of MMR; 157 (10.6%) had received 1 dose, and 242 (16.4%) were unvaccinated. Genetic analysis of small hydrophobic gene sequences of 808 mumps-positive samples showed that most (98.5%) outbreak strains belonged to the G5 subtype.

Complications were reported in 126 cases (8.4% of 1,492 cases with known complication status) (Table 1). Most (78 [62%]) complications occurred in the 18–25-year age group. Orchitis was the most frequent complication (109 [12.7%] male case-patients ≥12 years of age) and occurred significantly more often among unvaccinated case-patients than among case-patients who had received 1 vaccine dose (p = 0.04); vaccination with 2 doses of MMR reduced the risk for orchitis even further (p<0.01). Other reported complications were meningitis (n = 6), pancreatitis (n = 3), thyroiditis (n = 1), and encephalitis (n = 1).

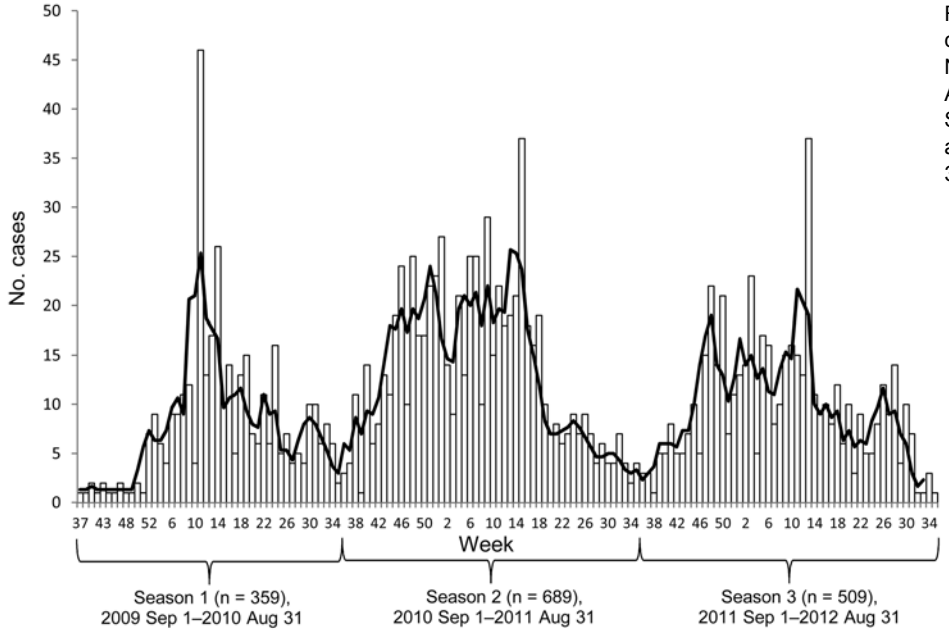

Figure. Numbers of notified mumps cases, by week of onset, The Netherlands, September 1, 2009–August 31, 2012 (N = 1,557 cases). Seasons and number of cases (n) are indicated; black line indicates 3-week moving average.

Table 1. Association between rates of mumps complications and hospitalization and MMR status, the Netherlands, September 1, 2009–August 31, 2012*

Complication	No. MMR doses received by case-patient	No. (%) case-patients with complications	Crude OR (95% CI)	p value	aOR† (95%CI)	p value	aVE† (95% CI)
Orchitis§	0	36 (15.5)	Ref		Ref	Ref	Ref
	1	10 (6.6)	0.46 (0.22–0.97)	0.04	0.46 (0.22–0.98)	0.04	54 (2–78)
	2	46 (4.7)	0.26 (0.16–0.41)	<0.01	0.26 (0.15–0.43)	<0.01	74 (57–85)
Deafness	0	2 (0.9)	Ref				
	1	0	NA	NA	–	–	–
	2	1 (0.1)	0.12 (0.01–1.3)	0.1	–	–	–
Meningitis	0	2 (0.8)	Ref				
	1	1 (0.6)	0.76 (0.07–8.5)	0.8	–	–	–
	2	2 (0.2)	0.24 (0.03–1.7)	0.2	–	–	–
All complications	0	44 (19.0)	Ref		Ref	Ref	Ref
	1	10 (6.6)	0.30 (0.15–0.62)	<0.01	0.29 (0.14–0.62)	<0.01	71 (38–86)
	2	55 (5.7)	0.26 (0.17–0.39)	<0.01	0.24 (0.14–0.39)	<0.01	76 (61–86)
Hospitalization	0	11 (4.8)	Ref		Ref	Ref	Ref
	1	3 (2.0)	0.41 (0.11–1.5)	0.18	0.43 (0.11–1.6)	0.2	57 (–60 to 89)
	2	10 (1.1)	0.22 (0.09–0.52)	<0.01	0.18 (0.07–0.47)	<0.01	82 (53–93)

*Only case-patients with known complications and vaccination status were included in the analyses. OR, odds ratio; VE, vaccine effectiveness; Ref, referent; NA, not applicable; –, not analyzed (insufficient sample size).
†Adjusted for age (age groups <18 y, 18–25 y, >25 y) and sex, except orchitis, which was adjusted only for age.
§Includes only male case-patients >12 y of age.

Three case-patients had permanent unilateral hearing loss that was probably caused by mumps virus infection. Deafness and meningitis occurred more frequently among unvaccinated than vaccinated persons, but those numbers were probably too low for statistical significance (Table 1).

A total of 31 patients (2.1% of 1,436 patients with known hospitalization status) were hospitalized. Risk for hospitalization was significantly lower among case-patients who had received 2 MMR doses than for unvaccinated case-patients ($p<0.01$); VE for preventing hospitalization was 82% (Table 1). Of the 31 hospitalized case-patients, 13 (42%) had orchitis. No deaths were reported.

Three distinct epidemic seasons occurred during the outbreak: seasonal peaks in spring and late autumn and a decline in number of cases during summer and, to some extent, during the Christmas holidays (Figure). Data on sex, age, vaccination status, residence in a city with a university, student status, and contact with student status by season are shown in Table 2. Overall, the age distribution of mumps case-patients differed significantly between the seasonal peaks ($p = 0.007$). The number of cases increased proportionally over time for the 13–17-year age group ($p = 0.003$) and the ≥25-year age group ($p = 0.042$) and decreased over time for the 18–25-year age group ($p<0.001$). The overall proportion of cases in vaccinated persons did not change (Table 2), and the proportion of complications or hospitalizations did not differ by season (data not shown).

We found significant seasonal differences in the proportion of cases occurring in students and in persons with student contacts ($p<0.001$). During early spring 2010, large clusters of cases were reported from university cities of Leiden and Delft, as described (*6*). However, during 2011 and 2012, proportionally more case-patients were not students and had no contact with students than during 2010 ($p<0.001$). The proportion of student case-patients enrolled in higher education other than university or case-patients who had contact with these nonuniversity students increased after 2010 ($p<0.001$). The absolute numbers of cases in these categories increased from 2010 to 2011 but stayed more or less constant, or decreased slightly, in 2012. The number of case patients who were university students or who had contact with university students decreased proportionally ($p<0.001$), and over time, proportionally more cases were reported from cities without universities ($p<0.001$). In addition, the total number of cases from nonuniversity cities was higher in 2012.

Discussion

The epidemic of mumps in the Netherlands during late 2009 through 2012 affected mainly vaccinated students. However, vaccination evidently offered protection against mumps-associated complications. The epidemic showed a seasonal trend, although cases were identified throughout the years. Over time, age, student status, and geographic distribution changed, which suggests a slight shift in transmission trends from student populations to younger and older nonstudent populations and to cities without a university. This shift may relate to increased immunity in the primarily affected high-risk student population; exposure to wild-type mumps virus may have boosted individual immunity and thus contributed to increased herd immunity.

Mumps outbreaks among vaccinated populations have been reported in other countries during recent years: a 2006 outbreak in the United States (*8*), a 2009–2010 outbreak in Canada (*9*), and a 2012 outbreak in the United Kingdom (*10*). Description of an outbreak in 2009–2010 in the northeastern United States among a highly vaccinated population of Orthodox Jews indicated that intense

Table 2. Demographic characteristics and student status for 1,557 patients with mumps, by annual epidemic season, the Netherlands, September 1, 2009–August 31, 2012*

Characteristic	No. (%) case-patients		
	Season 1, 2009 Sep 1–2010 Aug 31, n = 359	Season 2, 2010 Sep 1–2011 Aug 31, n = 689	Season 3, 2011 Sep 1–2012 Aug 31, n = 509
Sex			
M	205 (57.1)	416 (60.4)	296 (58.2)
F	154 (42.9)	271 (39.3)	213 (41.8)
Unknown	0	2 (0.3)	0
Age, y			
0–3	3 (0.8)	4 (0.6)	3 (0.6)
4–12	5 (1.4)	22 (3.2)	16 (3.1)
13–17	17 (4.7)	63 (9.1)	**54 (10.6)**
18–25	270 (75.2)	468 (67.9)	**318 (62.4)**
>25	64 (17.8)	131 (19)	**118 (23.2)**
Unknown	0	1 (0.2)	0
Vaccination status			
0 doses	57 (15.9)	115 (16.7)	70 (13.7)
1 dose	37 (10.3)	69 (10.0)	51 (10.0)
2 doses	225 (62.7)	436 (63.3)	337 (66.2)
≥3 doses	4 (1.1)	4 (0.6)	5 (1.0)
Vaccinated but unknown no. doses	24 (6.7)	25 (3.6)	15 (3.0)
Unknown	12 (3.3)	40 (5.8)	31 (6.1)
Residence in a city with university†			
Yes	258 (71.9)	351 (50.9)	**243 (47.7)**
No	92 (25.6)	322 (46.7)	**263 (51.7)**
Unknown	9 (2.5)	16 (2.3)	3 (0.6)
Student/contact with students			
Not a student and no contact with students	22 (6.1)	171 (24.8)	**118 (23.2)**
University student or contact with university students	229 (63.8)	275 (39.9)	**221 (43.4)**
Other student‡ or contact with other students	20 (5.6)	144 (20.9)	**88 (17.3)**
Unknown	88 (24.5)	99 (14.4)	82 (16.1)
Incidence estimates§			
University students	92.9	93.9	80.2
Other students	2.0	14.7	9.8
Secondary school students	0	0.7	5.4

*Boldface indicates significance trends by χ^2 test, calculated by using proportions excluding unknowns.
†University cities: Amsterdam, Delft, Eindhoven, Enschede, Groningen, Leiden, Maastricht, Nijmegen, Rotterdam, Stichtse Vecht, Tilburg, Utrecht, and Wageningen.
‡Students enrolled in higher education other than university.
§Incidence per 100,000 students. Total student numbers by category obtained from www.cbs.nl.

exposure among boys in a religious school facilitated the transmission of mumps virus, which overpowered the vaccine-induced protection (*11*,*12*). Similar to our findings, transmission in that outbreak shifted from adolescents to younger and older populations over time. The intense social crowding among students (e.g., large indoor social gatherings) partly explains why secondary vaccine failure occurred in the outbreak described in this study. A subgroup of students, including those living with many other students and members of university fraternities, may be at increased risk for infection (*6*,*7*). Crowding in nonstudent populations may not be as intense as among students, and mixing is usually with more heterogeneous age groups. In these circumstances, herd immunity is sufficient to prevent more widespread transmission. A lower rate of crowding may be one explanation for the relatively low numbers of cases among 4–12-year-olds, despite the generally lower IgG titers in this group than in adolescent students (*13*). Still, even though lower antibody levels do not automatically mean higher risk for mumps virus infection (*14*), a higher rate of illness would have been expected in the 4–12-year age group. An additional explanation for the lower apparent illness rate among these younger children might be a higher frequency of unapparent and subclinical infections, which would lead to many undiagnosed cases in this age group.

Most of the persons affected in the epidemic were male, a finding also observed in other studies (*15*,*16*). The reasons for male predominance are unclear, but significantly higher mumps antibody titers in female than in male persons have been demonstrated (*13*,*17*); this finding, in turn, may be linked to gender-associated genetic differences in immune response. Behavioral differences between sexes may also play a role.

Most cases occurred in persons who had received 2 doses of MMR, which suggests inadequate effectiveness of the vaccine. Recent studies indicate the effectiveness of MMR against mumps is moderate and lower than the clinical efficacy estimates (*1*,*18*). Postlicensure studies of 2 doses (Jeryl-Lynn strain) of MMR have provided a median VE estimate of 88% (range 79%–95%) (*2*). A recent

study of an outbreak of mumps at a student party in the Netherlands estimated a VE of 68% for 2 doses of MMR (*6*). This estimate is, however, uncertain because of the low number of unvaccinated case-patients. We attempted to provide VE estimates against clinical mumps applying the screening method; however, because this method is most vulnerable to error when proportions of the population and case-patients vaccinated are high (*19*), as in this study, the estimates became inaccurate and thus are not included in our results. The possible causes for lower than expected VE include secondary vaccine failure (waning immunity), intense exposure to high virus inoculum, and a possible mismatch between the vaccine genotype and circulating strains (*1*,*2*,*18*,*20*). However, because the level of antibodies correlating with protection remains unknown (*12*,*21*), we are unable to further elucidate the role of these factors.

Orchitis was the most common complication, consistent with previous outbreaks in a population with a similar age structure (*1*). However, orchitis occurred significantly more often among unvaccinated than vaccinated case-patients, and the vaccine was effective in preventing orchitis, which has previously been shown in a study based in part on the same study population (*22*) and in other studies (*11*,*23*). Vaccination also significantly reduced the risk for complications overall and for hospitalizations. A previous report described 3 cases of deafness (0.19% of all notified infections), 2 in unvaccinated persons (*24*). The frequency of 0.005% for unilateral deafness commonly cited in the literature (*25*) is considerably lower than that found in our study, but this difference is likely attributable to a different denominator population. A higher incidence of deafness has been reported from Japan using more appropriate denominators (*26*).

One limitation of our study was the short time span for assessing changes over time. Mumps cases have continued to occur after our study period, but the number of cases reported after September 2012 (180 as of August 31, 2013) is much lower than that reported during the previous years. Recent numbers indicate that a similar trend in changing patterns of age and geographic distribution is ongoing; most of the more recent cases have occurred in nonstudents and in age groups other than 18–25 years (data not shown). However, because of lower case numbers, this comparison must be interpreted with caution.

A further limitation of our study is that it is likely that many mumps cases are not notified because they are subclinical infections or because of reluctance to seek medical care; thus, these cases are not included in our analyses. Furthermore, complications that occurred after the notification date are not included; however, because vaccination status is probably not associated with the reporting of complications, we regard our VE estimates against complications as unbiased.

Although VE for mumps vaccination is not optimal for preventing clinical disease, our results support previous findings that vaccination limits the severity of disease. Because complications are the primary mumps-associated public health problem, these findings support the current vaccination recommendations. Still, this epidemic demonstrates that mumps virus can cause large outbreaks even in highly vaccinated populations. The observation that the incidence after the third season studied has been considerably lower than during previous seasons is consistent with the development of herd immunity among high-risk students resulting from the high rate of natural symptomatic and asymptomatic infections. However, the annual inflow of new susceptible students–unvaccinated and vaccinated–who start their studies could again lower overall immunity. A recent study suggested that use of a third MMR dose might be an effective control measure in certain outbreak situations (*27*). Introduction of a third MMR dose to the vaccination schedule has been considered in the Netherlands (*6*) but was not recommended because of relatively low overall illness rates associated with mumps and other factors, including an expected low vaccine uptake. Although the vaccine remains effective in most settings and significantly reduces the risk for complications, further research is needed to understand the limitations of MMR, and modeling is warranted to understand the dynamics of mumps virus transmission in future.

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Dr Sane is a fellow in the European Programme for Intervention Epidemiology Training, European Centre for Disease Prevention and Control, and based at National Institute for Public Health and the Environment in the Netherlands. His primary research interests include epidemiology of viral diseases, especially vaccine-preventable and vector-borne infections.

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Address for correspondence: Jussi Sane, Epidemiology and Surveillance Unit, RIVM/National Institute for Public Health and the Environment, PO Box 1, 3720 BA Bilthoven, the Netherlands; email: jussi.sane@rivm.nl

High Rates of Antimicrobial Drug Resistance Gene Acquisition after International Travel, the Netherlands

Christian J.H. von Wintersdorff, John Penders, Ellen E. Stobberingh, Astrid M.L. Oude Lashof, Christian J.P.A. Hoebe, Paul H.M. Savelkoul, and Petra F.G. Wolffs

We investigated the effect of international travel on the gut resistome of 122 healthy travelers from the Netherlands by using a targeted metagenomic approach. Our results confirm high acquisition rates of the extended-spectrum β-lactamase encoding gene bla_{CTX-M}, documenting a rise in prevalence from 9.0% before travel to 33.6% after travel (p<0.001). The prevalence of quinolone resistance encoding genes *qnrB* and *qnrS* increased from 6.6% and 8.2% before travel to 36.9% and 55.7% after travel, respectively (both p<0.001). Travel to Southeast Asia and the Indian subcontinent was associated with the highest acquisition rates of *qnrS* and both bla_{CTX-M} and *qnrS*, respectively. Investigation of the associations between the acquisitions of the bla_{CTX-M} and *qnr* genes showed that acquisition of a bla_{CTX-M} gene was not associated with that of a *qnrB* (p = 0.305) or *qnrS* (p = 0.080) gene. These findings support the increasing evidence that travelers contribute to the spread of antimicrobial drug resistance.

Antimicrobial drug resistance is a public health threat worldwide that limits clinical treatment options for bacterial infections. Most research on antimicrobial drug resistance has been focused on resistance in clinically relevant pathogenic bacteria. However, a vast and largely unexplored reservoir of resistance genes is present in nonpathogenic bacteria living in the environment or as commensal agents (*1*–*5*). Because of horizontal gene transfer (HGT) among microbes of diverse species and genera, antimicrobial drug resistance mechanisms in an organism, regardless of whether it is a pathogen, have the potential to emerge in clinically relevant pathogens (*6*). Several of such HGT interactions between clinically relevant pathogens and environmental species have been described; for example, the plasmid-mediated quinolone resistance encoding *qnrA* gene originated from the chromosomes of the aquatic bacterium *Shewanella algae* (*7*). Another well-known example is the extended-spectrum β-lactamase (ESBL) encoding bla_{CTX-M} gene, which originates from chromosomal genes of environmental *Kluyvera* species (*8*) and has emerged as the most prevalent cause of plasmid-mediated ESBL.

Resistance reservoirs have unpredictable and immense potential for rendering antimicrobial drugs ineffective. The human gut microbiota warrants special attention because of its high density of microorganisms and high accessibility (*9*). The gastrointestinal tract is constantly exposed to numerous bacteria from the environment, e.g., food, water, soil, other humans, or animals. These incoming bacteria often harbor antimicrobial drug resistance genes (*10*), which can be transferred to the indigenous microbial communities through HGT, where they may enrich the pool of available antimicrobial resistance elements in the gut microbiota.

Potential for intercontinental transfer of antimicrobial drug–resistant bacteria in the microbiota necessitates studies that focus on the antimicrobial resistance of the gut microbiome as a whole, the so-called "gut resistome," by using culture-independent metagenomic approaches (*9*). Metagenomic approaches avoid the bias that is introduced when selective culturing is applied because ≈80% of the gut microbiota is not cultivatable (*11*).

Travel to geographic areas in which rates of bacteria that are resistant to antimicrobial drugs are high has been indicated as a risk factor for the acquisition of such bacteria (*12*). Studies in Australia (*13*), Sweden (*14*,*15*), and the Netherlands (*16*) have shown that international

Author affiliations: Maastricht University Medical Center, Maastricht, the Netherlands (C.J.H. von Wintersdorff, J. Penders, E.E. Stobberingh, A.M.L. Oude Lashof, C.J.P.A. Hoebe, P. Savelkoul, P.H.M. Wolffs); and South Limburg Public Health Service, Geleen, the Netherlands (C.J.P.A. Hoebe)

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travel is a major risk factor for colonization with ESBL-producing *Enterobacteriaceae*. Likely, these resistant strains are acquired from the environment during travel, e.g., through food consumption (*17*). Because the human intestinal microbiome will come in contact with many different bacterial species from travel-related environments, the effect of international travel on antimicrobial drug resistance is most likely limited to neither opportunistic pathogens, such as *Escherichia coli*, nor to ESBL-encoding resistance genes.

In this study, we aimed to investigate the effect of international travel on the human gut resistome. By using a targeted (PCR-based) metagenomic approach, we compared the presence and relative abundance of specific resistance determinants in the entire human gut microbiome before and after international travel.

Materials and Methods

Population and Design

Healthy long-distance travelers were recruited during November 2010–August 2012 through travel clinics (EASE Travel Clinic & Health Support, www.ease-travelclinic.nl/en/) located in the southern part of the Netherlands. Travelers consenting to participate were asked to collect a fecal sample before and immediately after travel and to provide records of the duration and destination of their travel, illnesses or complaints during travel, drug use, and antimicrobial drug use within the 3 months preceding travel. The fecal samples were sent to clinics by regular mail on the same day of collection and were processed on the day of receipt. The study comprised 122 travelers.

The countries visited were categorized into geographic regions. These regions were Southeast Asia (Asia excluding the Indian subcontinent and the Middle East), the Indian subcontinent (Bangladesh, Bhutan, India, Nepal, Pakistan, and Sri Lanka), northern Africa (countries north of the equator), southern Africa (countries south of the equator), southern Europe, Central America, and South America.

Fecal Specimen Processing and DNA Extraction

Fecal samples were diluted 10-fold in peptone/water solution (Oxoid, Basingstoke, UK) containing 20% (vol/vol) glycerol (Merck, Darmstadt, Germany) and homogenized by vortexing. They were stored at –20°C until molecular analysis was performed.

For the extraction of metagenomic DNA, 200 µL of diluted feces was added to a 2-mL vial containing 0.5 g of 0.1 mm zirconia/silica beads (BioSpec, Bartlesville, OK, USA), 4 glass beads, 3.0–3.5 mm (BioSpec), and 1.2 µL of lysis buffer from the PSP Spin Stool Kit (Stratec Molecular, Berlin, Germany). Samples were disrupted in a Magna Lyser device (Roche, Basel, Switzerland) in 3 cycles of 1 min. at 5,500 rpm. Subsequently, metagenomic DNA was isolated from the samples by using the PSP Spin Stool Kit according to the manufacturer's instructions. DNA was eluted in 200 µL elution buffer and stored at –20°C until further analysis.

Real-time PCR

Real-time PCR was performed to detect and quantify the β-lactamase–encoding genes *cfxA*, $bla_{\text{CTX-M}}$, and bla_{NDM}; tetracycline resistance–encoding genes *tetM* and *tetQ*; macrolide resistance–encoding gene *ermB*; aminoglycoside resistance–encoding gene *aac(6')-aph(2'')*; and quinolone resistance encoding genes *qnrA*, *qnrB*, and *qnrS*. The 16S rDNA was amplified as a reference gene to normalize for the amount of bacterial DNA in the samples.

The 16S rDNA, *cfxA*, *tetM*, *tetQ*, *ermB*, and *aac(6')-aph(2'')* targets were amplified by using a MyiQ Single-Color Real-Time PCR Detection System (BioRad, Hercules, CA, USA) in 25-µL reactions containing 12.5 µL iQ SYBR Green Supermix (BioRad) and 5-µL template DNA. Melting curves were checked for each sample to confirm amplification of the correct product. For every target, amplified PCR products of 10 random positive samples were separated by agarose gel electrophoresis to control for purity and size of the amplicons. Finally, for all genes except the 16S rDNA (because of expected heterozygous amplicons), these products were sequenced by using the PCR primers and an ABI BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing data were obtained by using an ABI 3730 DNA Analyzer (Applied Biosystems) and were analyzed by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The $bla_{\text{CTX-M}}$, bla_{NDM}, *qnrA*, *qnrB*, and *qnrS* genes were amplified on a 7900HT Fast Real-Time PCR System (Applied Biosystems) in 25-µL reactions containing 12.5 µL ABsolute QPCR ROX Mix (Thermo Scientific, Waltham, MA, USA) and 10-µL template DNA. The $bla_{\text{CTX-M}}$ assay enables identification of the various phylogenetic groups by use of 4 probes. The probes to detect $bla_{\text{CTX-M}}$ groups 1 and 2 were combined in the first reaction, and the probe to detect $bla_{\text{CTX-M}}$ group 9 was combined with a probe to detect all groups except for the CTX-M-1 group in a second reaction. All primer and probe sequences and PCR conditions for each target are displayed in Table 1.

To determine the efficiency of the PCR, cycle thresholds obtained from a series of 5 template DNA dilutions of at least 3 different samples were graphed on the y-axis versus the log of the dilution on the x-axis. For bla_{NDM}, a clinical isolate was used because no positive fecal samples were available. The PCR efficiencies were 16S rDNA, 94.0%; *cfxA*, 99.0%; *tetM*, 97.6%; *tetQ*, 95.9%; *ermB*,

Table 1. PCR primer/probe sequences and additional PCR conditions to identify antimicrobial resistance genes in gut microbiota after international travel, the Netherlands, 2010–2012

Primer/probe	Sequence,* 5′→3′	Final conc., nM	Amplicon size, bp	Cycling conditions	Ref.
16S-rDNA_F	TGGAGAGTTTGATCCTGGCTCAG	500	526	95°C, 4 min	(*19*)
16S-rDNA_R	TACCGCGGCTGCTGGCAC	250		35 × 95°C, 15 s; 65°C, 60 s	
cfxA_F	TGACAGTGAGAGATTTGCTGC	300	150	95°C, 3 min	(*19*)
cfxA_R	GGTCAGCCGACATTTCCTCTT	300		40 × 95°C, 15s; 60°C, 15s; 72°C, 30s	
tetM_F	ACACGCCAGGACATATGGAT	300	126	95°C, 3 min	(*19*)
tetM_R	GGGAATCCCCATTTTCCTAA	300		40 × 95°C, 15s; 57°C, 15s; 72°C, 30s	
tetQ_F	CAAGGTGATATCCGCTCTGA	300	128	95°C, 3 min	(*19*)
tetQ_R	GGAAAATCGTTCTTCCAGCA	300		40 × 95°C, 15s; 57°C, 15s; 72°C, 30s	
ermB_F	AAGGGCATTTAACGACGAAACTG	300	438	95°C, 3 min	This study
ermB_R	ATTTATCTGGAACATCTGTGGTATG	300		40 × 95°C, 20s; 60°C, 30s; 72°C, 40s	
aac6-aph2_F	TTGGGAAGATGAAGTTTTTAGA	300	173	95°C, 3 min	(*20*)
aac6-aph2_R	CCTTTACTCCAATAATTTGGCT	300		40 × 95°C, 15s; 57°C, 20s; 72°C, 30s	
CTX-M_F	ATGTGCAGYACCAGTAARGTKATGGC	500	336	95°C, 15 min	(*21*)
CTX-M_R	ATCACKCGGRTCGCCNGGRAT	500		40 × 95°C, 15s; 58°C, 20s	
CTX-M-1	JOE-CCCGACAGCTGGGAGACGAAACGT-BHQ1	100		72°C, 30s	
CTX-M-2	6FAM-CAGGTGCTTATCGCTCTCGCTCTGTT-BHQ1	100			
CTX-M-9	JOE-CTGGATCGCACTGAACCTACGCTGA-BHQ1	100			
CTX-M-2-8-9-25	6FAM-CGACAATACYGCCATGAA-MGB-NFQ	100			
NDM_F	ATTAGCCGCTGCATTGAT	400	154	95°C, 15 min	(*21*)
NDM_R	CATGTCGAGATAGGAAGTG	400		42 × 95°C, 15s; 60°C, 60s	
NDM_probe	6FAM- CTG[+C]CA[+G]AC[+A]TT[+C]GGTGC-BHQ1	200			
qnrA_F	CAGTTTCGAGGATTGCAGTT	400	148	95°C, 15 min	(*23*)
qnrA_R	CCTGAACTCTATGCCAAAGC	400		45 × 95°C, 30s; 52°C, 30s;	
qnrA_probe	6FAM-AAGGGTGYCACTTCAGCTATGCC-BHQ1	100		72°C, 30s	
qnrB_F	CAGATTTYCGCGGCGCAAG	400	134	95°C, 15 min	(*23*)
qnrB_R	TTCCCACAGCTCRCAYTTTTC	400		45 × 95°C, 30s; 55°C, 30s;	
qnrB_probe	6FAM-CGCACCTGGTTTTGYAGYGCMTATATCAC-BHQ1	100		72°C, 30s	
qnrS_F	TCAAGTGAGTAATCGTATGTA	400	157	95°C, 15 min	(*23*)
qnrS_R	GTCTGACTCTTTCAGTGAT	400		45 × 95°C, 30s; 55°C, 30s	
qnrS_probe	6FAM-CCAGCGATTTTCAAACAACTCAC-BHQ1	100		72°C, 30s	

*Nucleic acids between brackets and preceded by + are locked nucleic acids; nM, nanomolar; conc., concentration; ref., reference.

95.5%; *aac(6')-aph(2")*, 97.0%; $bla_{\text{CTX-M-1+2}}$, 98.2%; $bla_{\text{CTX-M-9+2–8–9–25}}$, 96.7%; bla_{NDM}, 98.4%; *qnrA*, 97.4%; *qnrB*, 101.0%; and *qnrS*, 102.5%.

We determined PCR detection limits for $bla_{\text{CTX-M}}$, *qnrB*, and *qnrS*. Clinical isolates harboring these genes were suspended in a 0.5 McFarland solution, then diluted 10-fold in sterile saline solution. Quantification of CFU in the suspensions was achieved by inoculating blood agar plates (Oxoid) and counting the number of colonies after overnight incubation at 37°C. Next, 20 µL of the quantified suspensions was mixed with 180 µL of feces and submitted to DNA extraction as described above. Subsequently, quantitative PCR was performed on extracted DNA to generate standard curves for quantification. For $bla_{\text{CTX-M}}$, the detection limit was 12–40 CFU/PCR. For *qnrB* and *qnrS*, the detection limit was 1–5 CFU/PCR.

Statistical Analyses

We calculated differences in relative resistance gene abundances between samples from before and after travel for each traveler by using the ΔDCt method with a Pfaffl modification to correct for PCR efficiency (ratio: Etarget^ΔCTtarget/Ereference^ΔCTreference) (*24*), which is the standard method to measure the relative change in mRNA expression levels by using

real-time PCR. However, in this study, rather than measuring mRNA expression levels, the relative amount of target DNA present was measured by using this method. The 16S rDNA was used as the reference gene.

To better visualize increases and decreases in gene abundances in graphs, we converted abundance ratios to a fold change. To determine the overall abundance change of a resistance gene, ratios were log-transformed. A 2-tailed, 1-sample *t* test was used to test whether the mean log ratio significantly differed from 0.

The number of fecal samples positive for a resistance gene after travel was compared with the positive samples obtained before travel by using the McNemar test for paired samples. Multivariable logistic regression analyses were used to test for the association between age, sex, travel destination and duration, traveler's diarrhea, and antimicrobial drug use preceding travel (independent variables) and the acquisition of antimicrobial resistance genes (dependent variable). The association between acquisitions of multiple resistance genes was determined by a χ^2 test. All analyses were performed by using IBM SPSS Statistics version 20 (www-01.ibm.com/support/docview.wss?uid=swg24029274). Results were interpreted as statistically significant when p was <0.05.

Results

Study Population

The study comprised 122 travelers (71 women, 51 men) whose median age was 43 years (range 18–72 years). The median length of stay abroad was 21 days (range 5–240 days). Fourteen participants traveled for >60 days; 5 participants traveled for ≥120 days. Most participants visited 1 country; 22 visited >1 country. Six participants visited >1 of the defined geographic regions (Table 2); 7 participants did not provide information about their destination.

Prevalence of Resistance Genes in Fecal Samples

Figure 1 shows the prevalence of the antimicrobial drug resistance determinants in fecal samples from the 122 healthy volunteers before and after international travel. The *cfxA* gene was detected in 111 (91.0%) fecal samples before travel and in 115 (94.3%) samples after travel. The ESBL encoding $bla_{\text{CTX-M}}$ gene was prevalent in 11 (9.0%) before travel and in 41 (33.6%) samples after travel, which was a significant increase (p<0.001).

After travel, samples from 5 participants contained $bla_{\text{CTX-M}}$ genes of 2 different phylogenetic groups. Before travel, single CTX-M variant was detected for 2 of these persons, and $bla_{\text{CTX-M}}$ genes were not detected for the other 3 persons. After travel, the gene was not detected in the samples of 6 persons who were positive for the $bla_{\text{CTX-M}}$ gene before travel. The carbapenemase-encoding gene bla_{NDM} was not detected in any sample.

The prevalence of both *tetM* and *tetQ* was very high in the fecal samples. The *tetM* gene was present in all samples before travel and in 121 (99.2%) samples after travel, and *tetQ* was detected in all samples before and after travel. The prevalence of the *ermB* gene was also high in samples both before and after travel (99.2% for both). The prevalence of the *aac(6')-aph(2'');* gene was not altered by traveling; this gene was present in 79 (64.5%) of samples before travel and in 86 (70.5%) samples after travel.

Before travel, prevalence of the quinolone resistance genes *qnrA*, *qnrB*, and *qnrS* was relatively low: 0.8%, 6.6%, and 8.2%, respectively. After travel, each of the 3 genes increased: *qnrA*, *qnrB*, and *qnrS* were detected in 3.3%, 36.9%, and 55.7% of samples, respectively. *qnrB* and *qnrS* were significantly higher after than before travel (p<0.001).

Relative Gene Abundance Before and After Travel

Because the prevalence of the *cfxA*, *tetM*, *tetQ*, and *ermB* genes was very high before and after travel, we compared the relative abundance of the genes in both samples from each traveler to determine whether traveling influenced the gene abundance. For all 4 genes, the observed changes in gene abundance per traveler were distributed between increases and decreases (Figure 2). Determining the overall increase or decrease of the abundance of each gene showed that none of the investigated genes changed significantly (p>0.05 for all) in abundance after travel.

Table 2. Characteristics of 122 travelers observed for rates of antimicrobial resistance gene acquisition after international travel, the Netherlands, 2010–2012*

Characteristic	No. (%)
Sex	
M	51 (41.8)
F	71 (58.2)
Clinical finding	
Traveler's diarrhea	45 (36.9)
Antimicrobial drug use	15 (12.3)
Region visited	
Southeast Asia	28 (23.0)
Indian subcontinent	31 (25.4)
Northern Africa	16 (13.1)
Southern Africa	17 (13.9)
Southern Europe	6 (4.9)
Central America	4 (3.3)
South America	6 (4.9)
Other/multiple	7 (5.7)

*Median age, y (range) of travelers was 42.7 (18–72) and median travel duration, (range) was 21.0 (5–20) months. Countries in respective regions are as follows: Southeast Asia (Indonesia, Philippines, Malaysia, Myanmar, Cambodia, Thailand, Vietnam), Indian subcontinent (India, Nepal, Sri Lanka), northern Africa (Canary Islands, Egypt, Gambia, Ghana, Togo, Morocco, Senegal, Uganda), southern Africa (Namibia, Kenya, Tanzania, Zanzibar, Mauritius, South Africa), Central America (Panama, Costa Rica, Mexico), South America (Argentina, Bolivia, Brazil, Columbia, Peru, Suriname), southern Europe (Croatia, Spain, Turkey), other (Australia, Fiji, New Zealand, Oman).

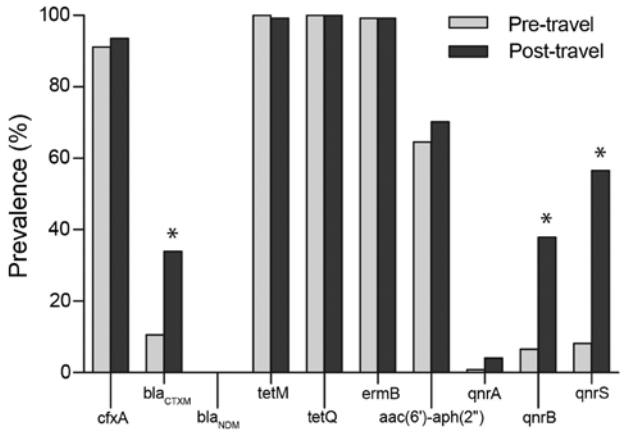

Figure 1. Prevalence (%) of antimicrobial drug resistance determinants in fecal samples from 122 healthy travelers from the Netherlands before and after travel, 2010–2012. Statistical significance of the prevalence between the 2 groups was calculated by using the McNemar test for paired samples and is indicated by * ($p<0.001$).

Effect of Travel Destination and Other Risk Factors on Gene Acquisition

The rate of acquisition of a $bla_{\text{CTX-M}}$ gene was highest for travelers visiting the Indian subcontinent (58.1%; $p<0.05$, OR 26.22, 95% CI 2.86–240.38) (Table 3). Travel to other regions was associated with a $bla_{\text{CTX-M}}$ acquisition rate of 17.9% for Southeast Asia and 31.3% and 29.4% for northern and southern Africa, respectively. In the combined category comprising southern Europe, Central America, and South America, 1 $bla_{\text{CTX-M}}$ acquisition (6.3%) was detected in a traveler who had been to southern Europe (Turkey).

The acquisition of the *qnrB* gene was not associated with travel to a specific region, whereas the acquisition of *qnrS* was highest for Southeast Asia (75.0%; $p = 0.001$, OR 15.7, 95% CI 3.1–79.2), and second highest for the Indian subcontinent (61.3%; $p<0.05$, OR 9.2, 95% CI 1.9–43.9). The acquisition rate was also elevated for northern Africa (43.8%) and southern Africa (35.3%) but not significantly so.

We also investigated associations between age, sex, travel destination and duration, traveler's diarrhea, and antimicrobial drug use preceding the travel and the acquisition of resistance genes. No associations were found (Table 3).

Phylogenetic Groups of $bla_{\text{CTX-M}}$ Genes and Association with *qnr* Genes

Of the 41 $bla_{\text{CTX-M}}$ genes acquired during travel, 24 belonged to the CTX-M-1 group, 2 belonged to the CTX-M-2 group, 6 were of the CTX-M-9 group, and 9 were positive for the CTX-M-2–8-9–25 probe but not for the CTX-M-2 or 9 probe, indicating that these genes were in groups 8 or 25. The CTX-M groups acquired per region are shown in Table 4. In contrast, 9/11 CTX-M types detected in the pre-travel samples belonged to the CTX-M-9 group and 2/11 to the CTX-M-1 group.

Associations between the acquisitions of the $bla_{\text{CTX-M}}$ and *qnr* genes were also investigated (Table 5). The acquisition of a $bla_{\text{CTX-M}}$ gene was not associated with that of a *qnrB* ($p = 0.305$) or *qnrS* gene ($p = 0.080$); neither was the gain of a $bla_{\text{CTX-M}}$ gene of the CTX-M-1 group, which was the dominant acquired type (58.5%) associated with the acquisition of either *qnrB* ($p = 0.631$) or *qnrS* ($p = 0.256$).

Discussion

We used a metagenomic approach to study effects of international travel on part of the resistome of the human gut microbiota. Our results provide insights into the prevalence of the investigated resistance genes in the human gut microbiota and demonstrate high rates of acquisition of the ESBL encoding gene $bla_{\text{CTX-M}}$ and quinolone resistance encoding genes *qnrB* and *qnrS* related to international travel. The prevalence of these genes increased from 9.0%, 6.6%, and 8.2% before travel to 33.6%, 36.9%, and 55.7% after travel, respectively.

Prospective cohort studies among travelers from Australia (*13*), the Netherlands (*16*), and Sweden (*14*,*15*) showed that international travel was a risk factor for colonization with ESBL-producing *Enterobacteriaceae* spp. and that travel to India or the Indian subcontinent was the highest risk factor. These findings agree with the rates of $bla_{\text{CTX-M}}$ acquisition found in our study, which were highest for travelers to the Indian subcontinent.

The phylogenetic types of the $bla_{\text{CTX-M}}$ gene that were acquired in our study group were clearly dominated by CTX-M group 1, especially in the Indian subcontinent. This geographical association corresponds to the aforementioned cohort studies (*13*–*16*), which showed that ESBL-producing *Enterobacteriaceae* identified in travelers to India or the Indian subcontinent mainly comprise CTX-M group 1. Although the statistical power of our study was insufficient to analyze the specific CTX-M groups, it was striking that genes of the CTX-M-2 group were detected twice and those of either group 8 or 25 were detected 9 times. In previous studies, these CTX-M groups were not detected at all (*13*,*14*) or were detected only sporadically (*15*,*16*). The difference in results could be caused by our use of a metagenomic approach, which might detect $bla_{\text{CTX-M}}$ in a much wider array of species than did studies investigating specific cultured *Enterobacteriaceae* spp. This difference in approach might furthermore explain that of the $bla_{\text{CTX-M}}$ genes detected before travel in the population in our study, most (9/11, 82%) were of the CTX-M-9 group, which contrasts studies that report that $bla_{\text{CTX-M-15}}$ (which belongs to the CTX-M-1 group) is predominant in

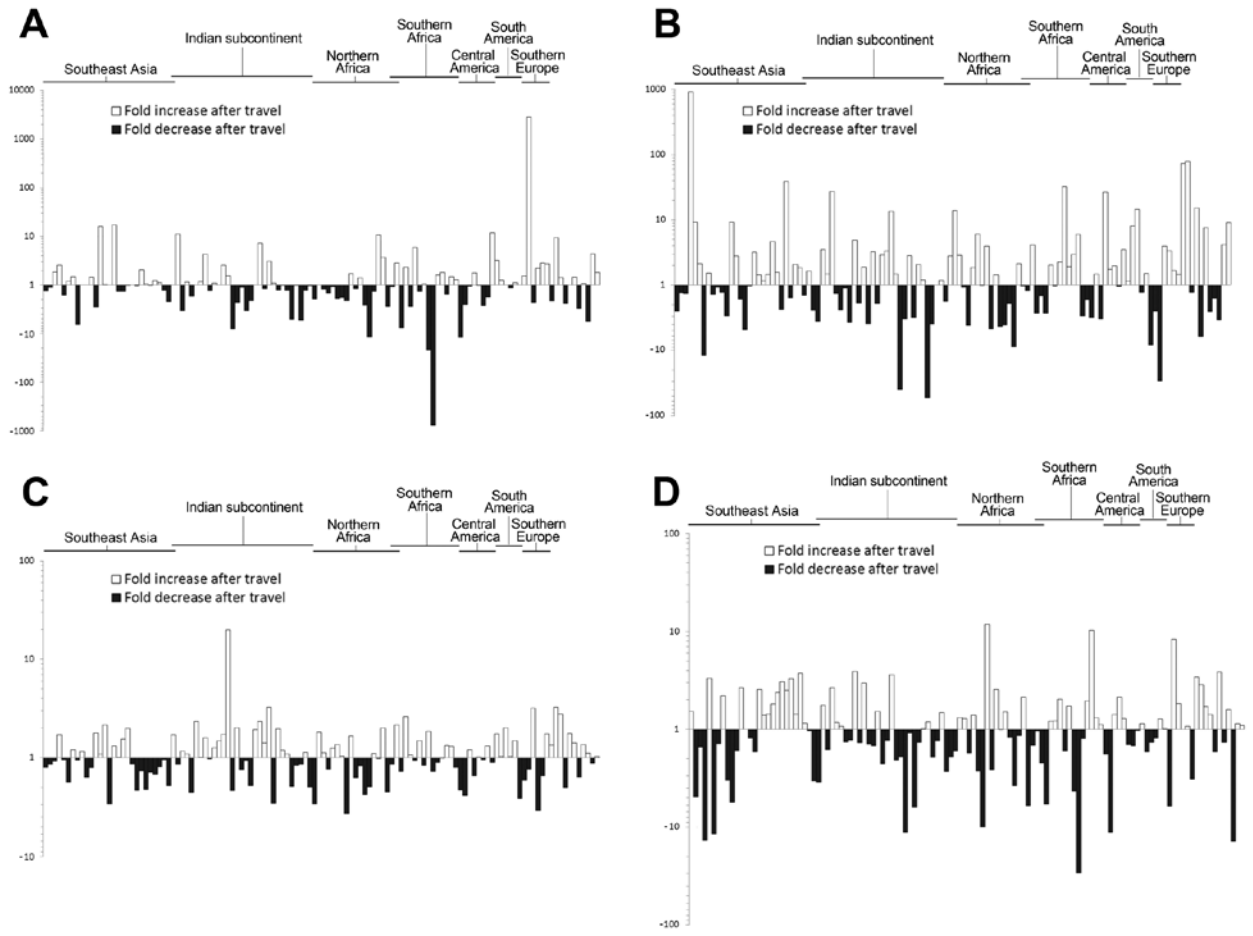

Figure 2. Relative changes in gene abundance before and after travel for each of 122 healthy travelers from the Netherlands during 2010–2012 for genes *cfxA* (A), *tetM* (B), *tetQ* (C), and *ermB* (D). Increases are shown with white bars on the positive *y*-axis; decreases are shown in dark gray bars on the negative *y*-axis. Each bar on the *x*-axis represents the change in a different study participant. The travel destination regions of the participants are indicated above the graph. No region is indicated for some travelers who either visited >1 of these regions or visited countries that were not in the defined regions (see Table 2).

ESBL-producing *Enterobacteriaceae* in the Netherlands (*16*,*25*,*26*). Aside from the different method used, the population sizes in these studies were larger than the cohort in our current study.

Plasmid-mediated quinolone resistance genes, such as the *qnr* variants, provide low-level quinolone resistance. However, these genes are relevant because they facilitate the emergence of higher-level resistance and thus can speed the development and spread of resistance to these antimicrobial agents (*27*). Although foreign travel has been associated with the acquisition of plasmid-mediated quinolone resistant–positive isolates (*28*–*30*), these genes have thus far not been focused on in prospective cohort studies investigating the effects of travel on antimicrobial resistance.

A study by Vien et al. that investigated the prevalence of the *qnr* genes in fecal swab samples from children in Vietnam who had acute respiratory tract infections (*23*) showed very high *qnrS* prevalence (74.5%). Travel to areas with such a high prevalence could be a major risk factor for acquisition of these genes. Five (83%) of 6 participants in our study who had traveled to Vietnam acquired a *qnrS* gene. In total, 11 volunteers had traveled to Cambodia, Thailand, Vietnam, or a combination of these geographically neighboring countries, and 9 (82%) acquired a *qnrS* gene. These data suggest that organisms carrying the *qnrS* gene are highly prevalent in these areas and that travelers visiting these areas have a high risk for exposure to those organisms.

Coexistence of *qnr* genes with various other resistance genes, such as $bla_{\text{CTX-M}}$, on the same plasmid is well known (*31*–*34*) and could be related to our finding that both types of genes were more prevalent in the study participants' samples after travel. However, we found no association between these genes in these samples. The *qnrS* gene

Table 3. Associations between travel-associated risk factors and rates of *bla*$_{CTX-M}$, *qnrB*, and *qnrS* acquisition among 122 healthy travelers from the Netherlands, 2010–2012*

Traveler characteristic	No. travelers	Antimicrobial drug resistance genes acquired by travelers					
		bla$_{CTX-M}$		*qnrB*		*qnrS*	
		No. (%)	OR (95% CI)‡	No. (%)	OR (95% CI)‡	No. (%)	OR (95% CI)‡
Region visited							
Europe and America§¶	16†	1 (6.3)	1.00	6 (37.5)	1.00	3 (18.8)	1.00
Southeast Asia	28†	5 (17.9)	3.34 (0.34–33.14)	7 (25.0)	0.47 (0.12–1.90)	**21 (75.0)**	**15.74 (3.13–79.24)**
Indian subcontinent	31†	**18 (58.1)**	**26.22 (2.86–240.18)**	10 (32.3)	0.71 (0.18–2.71)	**19 (61.3)**	**9.23 (1.94–43.87)**
Northern Africa	16†	5 (31.3)	7.28 (0.70–75.92)	5 (31.3)	0.64 (0.14–2.98)	7 (43.8)	2.90 (0.54–15.57)
Southern Africa	17†	5 (29.4)	5.57 (0.56–55.77)	5 (29.4)	0.65 (0.15–2.84)	6 (35.3)	2.41 (0.46–12.66)
Sex							
F§	71	25 (35.2)	1.00	25 (35.2)	1.00	41 (57.7)	1.00
M	51	13 (25.5)	0.62 (0.23–1.67)	15 (29.4)	1.06 (0.44–2.57)	21 (41.2)	0.39 (0.15–1.00)
Antimicrobial drug use							
No§	107	32 (29.9)	1.00	35 (32.7)	1.00	52 (48.6)	1.00
Yes	15	6 (40.0)	1.44 (0.40–5.25)	5 (33.3)	1.28 (0.36–4.51)	10 (66.7)	1.64 (0.43–6.22)
Traveler's diarrhea							
No§	77	20 (26.0)	1.00	25 (32.5)	1.00	40 (51.9)	1.00
Yes	45	18 (40.0)	1.84 (0.70–4.82)	15 (33.3)	0.97 (0.40–2.37)	22 (48.9)	0.65 (0.26–1.63)

*OR, odds ratio. Boldface indicates statistical significance (p<0.05).
†Numbers do not total 122 because the 14 travelers who visited multiple or unknown regions were added to a remainder category not included in this table.
‡ORs and 95% CIs of the associations between risk factor and acquisition of resistance gene (negative before travel and positive after travel) by multivariable logistic regression analysis. Models included the following variables: travel destination, age, travel duration, sex, and antimicrobial drug use within 3 mo. preceding the travel, and traveler's diarrhea.
§Reference category.
¶Southern Europe, Central and South America, previously reported non–high-risk regions, were pooled to establish an adequately sized reference category.

was most often acquired by travelers who visited Southeast Asia and, to a lesser extent, the Indian subcontinent, whereas the acquisition rate for *bla*$_{CTX-M}$ was clearly highest for travelers to the Indian subcontinent but was not higher for travelers to Southeast Asia than for travelers to other regions. These findings indicate that although travel to the Indian subcontinent is a high-risk factor for acquiring both of these genes, these risk factors are not necessarily related.

Compared with culturing methods, a metagenomic approach has the advantage of being able to detect resistance in a much wider array of species; however, a limitation is that it is not yet known in which organisms the acquired resistance genes detected in our study are present, nor if they are being expressed. Another limitation of our study is that the study population was not large enough for us to conduct a more extensive risk analysis. Future studies that conduct more extensive analyses for risk factors, such as antimicrobial drug use, travel destination, and duration of travel, would benefit from larger populations. Furthermore, in future studies inclusion of a follow-up sampling of travelers would be highly relevant for investigating the period in which these acquired resistance genes remain in the resistome and if the perseverance or even HGT of these genes in the resistome is promoted by factors such as selective pressure introduced by antimicrobial drug use. Little is known about the duration of travel-acquired resistant organisms in the human microbiota, although their continued viability plays a key role in the ability to further spread these organisms or resistance elements.

During our investigation of several targeted resistance genes, it became evident that resistance genes from foreign environments are being introduced into the gut resistome at high rates related to international travel. Although the consequences of these changes in the resistome are difficult to predict, the introduction of these genes into the genetic pool of resistance elements may create opportunities for the horizontal transfer to other organisms in the gut microbiota.

Table 4. CTX-M groups of the acquired genes during travels by 122 travelers from the Netherlands, 2010–2012

Region	CTX-M group			
	1	2	9	8/25
Southeast Asia	2	1	3	0
Indian subcontinent	15	0	2	3
Northern Africa	3	0	1	1
Southern Africa	2	0	0	3
Southern Europe	0	1	0	0
Other	2	0	0	2
Total	24	2	6	9

Table 5. Associations between acquisition of *bla*$_{CTX-M}$ and *qnrB* or *qnrS* during travels by 122 travelers from the Netherlands, 2010–2012

bla$_{CTX-M}$ acquisition	*qnrB* acquisition, no. (%)		*qnrS* acquisition, no. (%)	
	No	Yes	No	Yes
No	59 (70.2)	25 (29.8)	46 (54.8)	38 (45.2)
Yes	23 (60.5)	15 (39.5)	14 (39.8)	24 (63.2)

Our study data demonstrated an increasing prevalence of bla_{CTX-M}, *qnrB*, and *qnrS* genes in the feces of healthy volunteers from the Netherlands immediately after they returned from international travel. These findings contribute to the increasing evidence that travelers contribute to the spread of antimicrobial drug resistance.

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Mr von Wintersdorff is a PhD student at the Department of Medical Microbiology at the Maastricht University Medical Center, the Netherlands. His research interests include the microbial resistome and the horizontal gene transfer of antimicrobial resistance.

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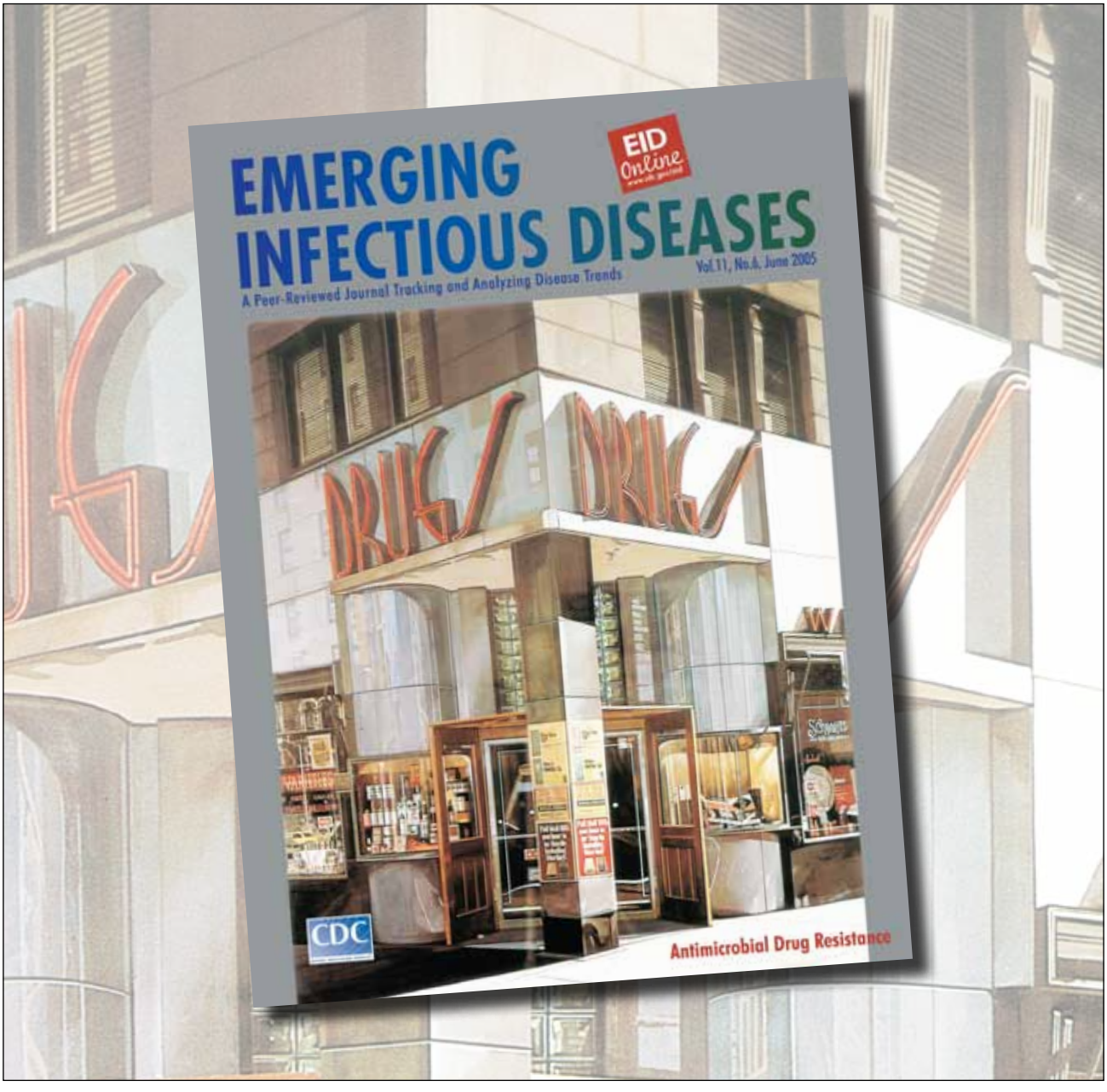
Address for correspondence: Petra F.G. Wolffs, Department of Medical Microbiology, Maastricht University Medical Center, PO Box 5800, 6202 AZ, Maastricht, the Netherlands; email: p.wolffs@mumc.nl



Characteristics of Patients Infected with Norovirus GII.4 Sydney 2012, Hong Kong, China

Martin C.W. Chan, Ting F. Leung, Angela K. Kwok, Nelson Lee, and Paul K.S. Chan

Norovirus GII.4 Sydney 2012 has spread globally since late 2012. We report hospitalization of patients infected with this strain skewed toward infants and young children among 174 cases during August 2012–July 2013 in Hong Kong, China. This group had higher fecal viral load (≈10-fold) than did older children and adults.

Norovirus infection is a leading cause of acute gastroenteritis in all age groups in industrialized and developing regions (*1*–*3*). In late 2012, a new norovirus strain of genogroup II, genotype 4 originating in Sydney, Australia (GII.4 Sydney 2012), became the predominant norovirus strain and caused a severe norovirus season globally (*4*–*6*). In Hong Kong, China, this strain caused an off-season communitywide surge in acute gastroenteritis during summer (July–October) 2012 (*6*,*7*). We report hospital admission of persons with this novel strain, which was skewed toward infants and young children for whom fecal viral load of this strain were higher than for patients in other age groups.

The Study

This 1-year prospective study was conducted during August 2012–July 2013. The study site, Prince of Wales Hospital, is a 1,400-bed acute care and general teaching hospital that serves a population of ≈600,000 (9% of the Hong Kong population). Fecal specimens were routinely collected on the day of admission from patients who had acute gastroenteritis and were suspected of having norovirus infections, but laboratory testing for norovirus was performed in weekly batches. Patients whose test results were positive on Monday of each week were enrolled in this study. We measured concentration of viral RNA in fecal specimens using quantitative reverse transcription PCR (qRT-PCR) (SuperScript III One-Step RT-PCR System with Platinum Taq, Life Technologies, Grand Island, NY, USA) and using primers and TaqMan probe targeting open reading frame 1/2 junction as described (*8*). Cycle threshold (C_t) was used as a proxy measure of fecal viral load. A positive control for which C_t value was known was included in each test run to check for batch-to-batch variation. We performed genotyping by generating an ≈500-bp amplicon that contained a partial RNA-dependent RNA polymerase and a partial major capsid gene. We used G1FF/G1SKR and G2FB/G2SKR primers as appropriate (*8*), then performed sequencing and phylogenetic analysis using the norovirus genotyping tool (www.rivm.nl/mpf/norovirus/typingtool). We extracted statistics on the catchment population of the hospital from the Hong Kong 2011 Population Census (www.census2011.gov.hk/en/district-profiles.html). We used the nonparametric Mann-Whitney U test for univariate comparison of continuous variables and Fisher exact test for categorical variables. We performed statistical analyses using Prism 5.04 (GraphPad). Two-tailed p values <0.05 were considered statistically significant. Ethics approval was obtained from the institutional clinical research ethics committee (reference number CRE-2013.330).

We analyzed specimens of 174 patients admitted with laboratory-confirmed norovirus infection (Table). The number of cases peaked in September 2012, then declined to a low level for the remaining study period. Of the 174 norovirus isolates collected, genotyping was successful in 140 (80.5%). Failure in genotyping was caused by low viral load; no new strains were identified. GII.4 Sydney 2012 strain accounted for most (125 [89.3%]) of the typed cases, followed by GII.3 (4 [2.9%]), and GII.6 (3 [2.1%]) (Table). The previous predominant strain, GII.4 New Orleans 2009, was not detected, and the GII.4 2006b strain was identified in 2 cases. Half of the GII.4 Sydney 2012 cases were selected for RNA-dependent RNA polymerase sequencing; no evidence of a recombinant GII.4 Sydney 2012 strain, which was reported from Denmark and Italy, was observed (*9*,*10*). Non-GII.4 strains were observed more frequently after the epidemic (November 2012–July 2013) than during the epidemic (August–October 2012) (23.5% vs 1.1%; p<0.0001).

The age distribution of persons with GII.4 Sydney 2012 and the catchment population of this study are shown in Figure1, panels A and B, respectively. The ages of study patients with GII.4 Sydney 2012 spanned all age groups from infants to persons >90 years of age. The median age was 3 years (interquartile range [IQR] 1–74 years). Median age of patients with GII.4 Sydney 2012 and all study patients did not differ significantly (p = 0.96). The D'Agostino-Pearson normality test indicated that the age distribution did not follow a Gaussian distribution (p<0.0001) but showed a strong positive skew toward infants and children <5 years of age. Among the 125 GII.4 Sydney 2012 cases, 66 (52.8%) were infants or children <5 years of age; the next largest group comprised adults >65 years of age (39 [31.2%]). A similar

Author affiliation: The Chinese University of Hong Kong, Hong Kong, China

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Table. Monthly distribution of norovirus cases, Hong Kong, China, August 2012–July 2013*

Year, month	All	No. typed	GII.4 Sydney 2012, no. (%)	Other GII.4 strains, no. (strain)	Other genotypes, no. (type)
2012					
Aug	33	32	32 (100)	0	0
Sept	46	40	39 (98)	1 (2006b)	0
Oct	26	17	15 (88)	1 (2006b)	1 (GII.6)
Nov	11	9	9 (100)	0	0
Dec	6	2	2 (100)	0	0
2013					
Jan	12	9	6 (67)	0	1 (GII.6), 1 (GII.8), 1 (GII.13)
Feb	7	6	5 (83)	0	1 (GI.8)
Mar	5	3	3 (100)	0	0
Apr	11	10	4 (40)	0	1 (GI.4), 1 (GII.6), 3 (GII.3), 1 (GII.13)
May	6	4	3 (75)	0	1 (GII.3)
Jun	5	4	3 (75)	0	1 (GII.13 and GII.17 coinfection)
Jul	6	4	4 (100)	0	0

*GII, genogroup II; subsequent number is genotype.

age distribution was observed when all study cases were analyzed. The GII.4 Sydney 2012 fecal viral load on initial examination is shown in Figure 2. Higher viral load was observed among infants and children <5 years of age and adults >65 years of age. The median viral load, as reflected by C_t, was highest for infants and children <5 years, next highest for adults >65 years of age, and lowest for the remaining group, respectively (16.7 [IQR 15.7–19.1] vs 19.1 [16.1–20.9] vs

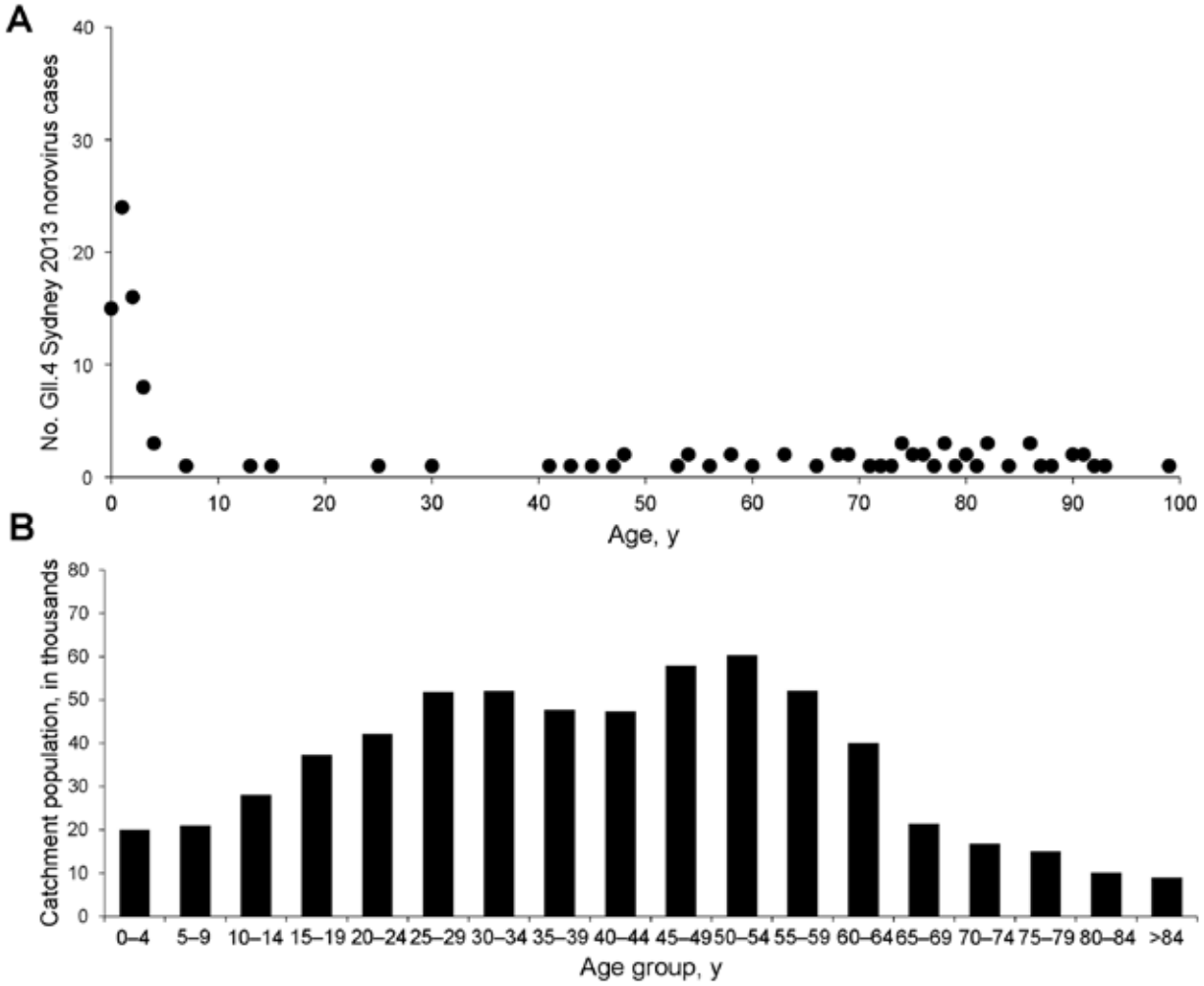

Figure 1. Age distribution of patients with A) norovirus strain of genogroup II, genotype 4 (GII.4 Sydney 2012) and B) study catchment population for the Prince of Wales Hospital area, Hong Kong, China, August 2012–July 2013.

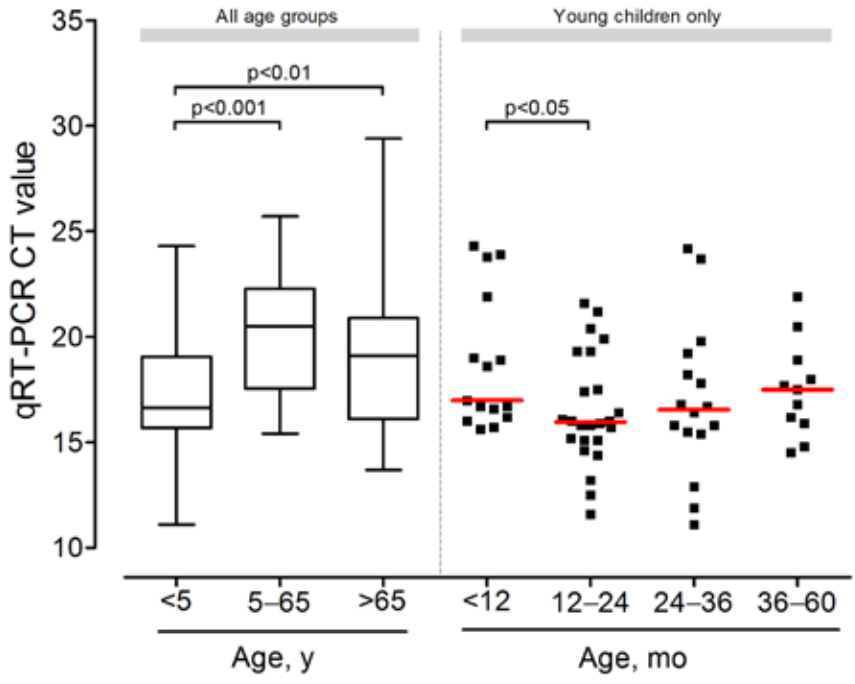

Figure 2. Box-plot of fecal viral load of patients with norovirus strain of genogroup II, genotype 4 (GII.4 Sydney 2012) at hospital admission, Hong Kong, China, August 2012–July 2013. Horizontal lines indicate median values. C_t, cycle threshold; GII.4, genogroup II genotype 4; qRT-PCR, quantitative reverse transcription PCR.

20.5 [17.6–22.3]). The median viral load of infants and children <5 years was 5.5-fold (p<0.001) and 14.4-fold (p<0.01) higher than that of adults >65 years of age and the remaining age group, respectively. Among infants and young children, the highest median viral load was observed in those 12–24 months of age, which was the group with the highest number of admissions (Figures 1 and 2). When all study cases were included, the viral load distribution was similar.

Conclusions

Noroviruses cause acute gastroenteritis in all age groups. However, most epidemiologic studies have focused either on community outbreaks or on a specific age group such as children; a recent meta-analysis concluded that studies involving all age groups in hospitals are limited and few (*3*). Our study design element of selecting no specific age group enabled us to determine that infants and young children represented approximately half of the hospitalized case-patients with norovirus gastroenteritis during the 1-year study period, during which the newly emerged GII.4 Sydney 2012 strain predominated. Our findings agree with evidence that infants and young children are likely to have the highest rate of infections in health care and community settings (*11–13*). In our study, infants and young children had the highest fecal viral load, compared with other age groups. Similarly lower C_t in children was reported in a historical cohort of norovirus gastroenteritis in the United Kingdom (1993–1996) (*14*). The higher viral load may relate to delayed viral clearance related to immune naivety. Higher fecal viral shedding also supports a recent mathematical model suggesting that children aged <5 years are more infectious than older children and adults (*15*).

Our study has limitations. Because only patients with diagnoses of norovirus made on a specific weekday each week were enrolled, a crude number of hospitalized patients was used instead of population-based incidence to estimate disease incidence. This limitation does not jeopardize our conclusions because our catchment population pyramid is constrictive, showing fewer younger persons. Second, because information was not complete about specimen collection times after each patient's illness onset, we could not adjust viral load data for specimen collection date; therefore, we cannot rule out a possible bias that might have been introduced if younger children were brought to the hospital earlier during their illness than older patients. Nevertheless, our finding that the most prevalent age group of hospitalized norovirus-infected patients showed the highest viral load implies that plans should be made and implemented for nosocomial infection control of this norovirus strain. Finally, whether our findings apply to other norovirus strains remains unanswered.

We showed that infants and children aged <5 years represent most patients hospitalized for norovirus (GII.4 Sydney 2012) gastroenteritis, and they might have higher viral load than infected persons in other age groups. Our findings may provide public health insights into understanding norovirus transmission in the community.

M.C.W.C. and P.K.S.C. designed and supervised the study. TFL and NL provided patient care and coordinated specimen

collection. M.C.W.C. and A.K.K. did laboratory testing. M.C.W.C. analyzed data and drafted the manuscript. All authors critically reviewed the manuscript and approved the final version. M.C.W.C. has access to all data and is responsible for the integrity of the study.

Dr Martin Chan is a research assistant professor in department of microbiology at the Chinese University of Hong Kong. His research focuses on epidemiology of gastrointestinal and respiratory viral infections, especially those associated with norovirus and influenza viruses.

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Address for correspondence: Paul K.S. Chan, Department of Microbiology, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong, China; email: paulkschan@cuhk.edu.hk

Pathology of US Porcine Epidemic Diarrhea Virus Strain PC21A in Gnotobiotic Pigs

Kwonil Jung, Qiuhong Wang,[1] Kelly A. Scheuer, Zhongyan Lu, Yan Zhang, and Linda J. Saif[1]

To understand the progression of porcine epidemic diarrhea virus infection, we inoculated gnotobiotic pigs with a newly emerged US strain, PC21A, of the virus. At 24–48 hours postinoculation, the pigs exhibited severe diarrhea and vomiting, fecal shedding, viremia, and severe atrophic enteritis. These findings confirm that strain PC21A is highly enteropathogenic.

A highly contagious coronavirus that causes porcine epidemic diarrhea (PED) was first reported in the United States in May 2013 in Iowa. Since then, the virus—porcine epidemic diarrhea virus (PEDV)—has spread rapidly nationwide (*1*,*2*). PEDV (family *Coronaviridae,* genus *Alphacoronavirus*) was previously reported only in Europe and Asia. The first US outbreaks caused a high number of deaths among suckling pigs and, as a consequence, substantial economic losses (*1*,*2*).

Results of PEDV pathogenesis studies using the prototype European PEDV strain, CV777, were reported in the 1980s (*3*,*4*). Strain CV777 infections caused intestinal villous atrophy with substantially reduced ratios of villous height to crypt depth (VH:CD) (*3*,*4*). Pathogenic features of CV777 are similar to those observed for Asian PEDV strains that circulated in the 1990s (*4*–*6*). To understand the progression of PEDV infection, we studied the pathogenesis of the newly emerged US strain, PC21A.

The Study

In June 2013, intestinal contents were obtained from a 1-day-old pig with diarrhea on a farm in Ohio, USA. PEDV strain PC21A was detected in the sample by reverse transcription PCR (RT-PCR) selective for the nucleocapsid gene (229–557 nt). The partial nucleocapsid gene sequence of PC21A was identical to that of 2 US PEDV outbreak strains from Colorado, USA: USA/Colorado/2013 (GenBank accession no. KF272920) and 13-019349 (GenBank accession no. KF267450). Only coronavirus-like particles were observed in the fecal sample by electron microscopy (Figure 1). The sample was negative for rotavirus groups A and C and for transmissible gastroenteritis virus/porcine respiratory coronavirus by RT-PCR (*7*,*8*).

The sample was bacteriologically sterilized by using 0.22-μm syringe filters and then prepared as inoculum. Near-term gnotobiotic pigs were delivered aseptically by hysterectomy from a specific pathogen–free sow (*9*). Six 10- to 35-day-old pigs were randomly assigned to a PEDV-infected group (pigs 1–5) or a negative control group (pig 6). Information about inoculation and inocula pig-passage number is described in Table 1. Pigs 1–3 and 5 were inoculated orally and/or intranasally with 6.3–9.0 $\log_{10}$ genomic equivalents (GE) of PEDV strain PC21A; pig 4 was exposed to the virus by indirect contact with inoculated pig 3. For each sample, the quantity of PEDV RNA GE was $\approx 10^6$ times higher than plaque assay results for a cell-adapted PEDV strain, PC22A. Clinical signs were monitored hourly. Pig 4 was monitored for longer-term clinical signs and virus shedding. Pigs were euthanized for pathologic examination at 3 stages of infection: acute, mid, and later stages (<24 h, 24–48 h, and >48 h, respectively, after onset of clinical signs). The Ohio State University Institutional Animal Care and Use Committee approved all animal-related experimental protocols.

Fecal or rectal swab samples were prepared as described (*9*). Virus RNA was extracted by using the MagMAX Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Titers of virus shed in feces were determined by TaqMan real-time RT-PCR using the OneStep RT-PCR Kit (QIAGEN, Valencia, CA, USA) as reported (*10*), with modifications in the forward primer and probe to provide a 100% match to the US strains: forward 5′-CGCAAAGACTGAACCCACTAAC-3′ and probe FAM-TGYYACCAYYACCACGACTCCTGC-BHQ. A standard curve was generated by using the PCR amplicon (PEDN 229/557) of strain PC21A. The detection limit was 10 GE per reaction, corresponding to 4.8 $\log_{10}$ and 3.8 $\log_{10}$ GE/mL of fecal and serum samples, respectively.

Small and large intestine tissues, lung, liver, heart, kidney, spleen, and mesenteric lymph node were examined grossly and histologically. Mean jejunal VH:CD was measured by using PAX-it software (PAXcam, Villa Park, IL, USA) as described (*11*). The frozen tissues were prepared and tested by immunofluorescence staining, as described (*12*), for the detection of PEDV antigen, using monoclonal antibody 6C8-1 against the spike protein of PEDV strain DR13 (provided by Daesub Song, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea).

Author affiliations: The Ohio State University, Wooster, Ohio, USA (K. Jung, Q. Wang, K.A. Scheuer, Z. Lu, L.J. Saif); and Ohio Department of Agriculture, Reynoldsburg, Ohio, USA (Y. Zhang)

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[1]These authors were co-principal investigators.

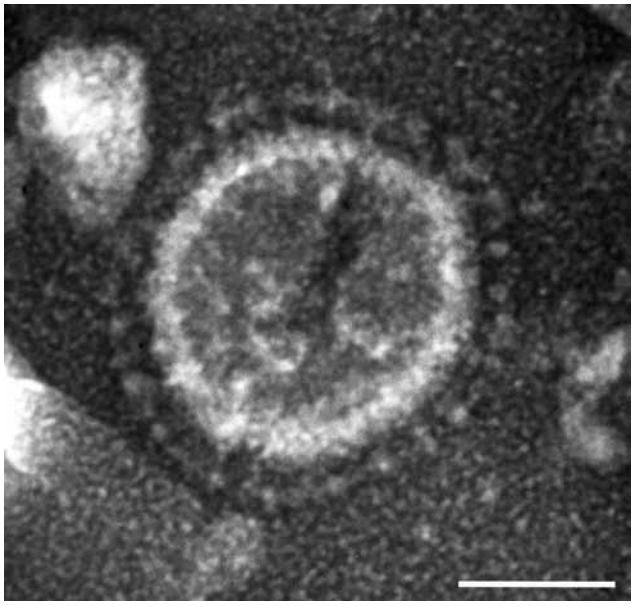

Figure 1. Electron micrograph of a US porcine epidemic diarrhea virus (PEDV) particle detected in a field fecal sample collected during a 2013 outbreak of PED on a farm in Ohio, USA; the fecal sample from which PEDV strain PC21A in this study was obtained was from a pig on the same farm during the same outbreak. The sample was negatively stained with 3% phosphotungstic acid. Scale bar = 50 nm.

Acute, severe watery diarrhea and vomiting developed in all inoculated pigs. Clinical signs developed 24–48 h after inoculation, regardless of the inoculum dose or number of inoculum pig passages (Table 1). Pig 4, which was followed longer, also exhibited dehydration, loss of bodyweight, and lethargy, but it consumed most of the milk that was offered. However, ≈120 h after onset of clinical signs, pig 4 collapsed after showing signs of disorientation and emaciation.

Immune electron microscopy, using a gnotobiotic pig hyperimmune serum to PEDV, showed only PEDV particles in the intestinal contents. For the pig-passaged PC21A strain, RT-PCR/PCR results were negative for transmissible gastroenteritis virus/porcine respiratory coronavirus (*7*), rotavirus groups A–C (*8*), caliciviruses (*13*,*14*), astroviruses (*15*), circoviruses, enterovirus, kobuvirus, and bocavirus. For pigs 1 and 2, the detection of fecal virus shedding 24–48 h after inoculation coincided with the onset of clinical signs; for pigs 3 and 4, fecal shedding occurred before the onset of clinical signs (Table 1).

By macroscopic examination, all infected pigs exhibited typical PEDV-like lesions, characterized by thin and transparent intestinal walls (duodenum to colon) and accumulation of large amounts of yellowish fluid in the intestinal lumen (Figure 2, panel A). The stomach was filled with curdled milk, possibly due to reduced intestinal peristalsis. The other internal organs appeared normal. Histologic lesions included acute diffuse, severe atrophic jejunitis (Figure 2, panel B) and mild vacuolation of superficial epithelial cells and subepithelial edema in cecum and colon (Figure 2, panel C). These findings were similar to those in conventional pigs naturally infected with Asian or US strains of PEDV and in caesarean-derived, colostrum-deprived pigs experimentally infected with CV777 (*2*,*3*,*5*,*6*). The mean jejunal VH:CD of the 5 infected pigs ranged from 1.2 to 3.4, probably depending on the stage of infection (Table 1), and that of the negative control pig was 6.3 (±0.2). VH:CD for pig 4, which was euthanized at a later stage of infection, was 1.5 (±0.2), a ratio indicative of continued cellular necrosis. Neither clinical signs nor lesions developed in the negative control pig during the experiment.

Immunofluorescence-stained cells were observed mainly in the epithelium of atrophied villi of small (duodenum to ileum) and large intestines (Table 2; Figure 2, panels D–F), as reported in other studies (*2*,*3*,*5*). The immunofluorescence was confined to the villous epithelial cells (Figure 2, panels D–F). A few immunofluorescence-stained cells were detected infrequently in the Peyer patches of pig 4. Lung tissues of the infected pigs did not show immunofluorescence staining, indicating that PEDV does not infect

Table 1. Design and results of a study of the pathology of US PEDV strain PC21A in gnotobiotic pigs, 2013*

Pig status, pig no.; age, d, at inoculation	Inoculum passage no.	Intranasal/oral inoculum, log_{10} GE	Fecal shedding, log_{10} GE/mL, by PIH					Onset of clinical signs, PIH	Viral RNA in serum sample at euthanasia, log_{10} GE/mL†
			0	24	48	72	96		
PEDV-inoculated									
1; 10	1	7.3/7.7	<4.8†	10.7‡	–	–	–	25–26	7.6
2; 18	2	6.3/6.8	<4.8	<4.8	11.0	11.2‡	–	44–46	6.3
3; 24	3	8.3/8.8	<4.8	10.2	12.3‡	–	–	44–46	5.7
4; 24§	4	Indirect contact	<4.8	10.9	9.6	10.6	ND	2–4§	7.6
5; 35	4	0/9.0	<4.8	ND	ND	–	–	26–30	4.8
Negative control									
6; 26	.	.	<4.8	<4.8	<4.8	<4.8	<4.8	.	<3.8†

*PEDV, porcine epidemic diarrhea virus; GE, genome equivalents; PIH, postinoculation hour; –, no result (pig euthanized); ND, not determined.
†Detected by real-time reverse transcription PCR with a detection limit of 4.8 log_{10} GE/mL for fecal samples and 3.8 log_{10} GE/mL for serum samples.
‡Euthanized.
§At 24 days of age, noninoculated pig 4 was exposed by indirect contact to pig 3 (at PIH 0) through small holes drilled into the stainless steel divider panel located between the 2 pigs in the shared pig tub isolator unit. Clinical signs and virus shedding were monitored after indirect contact. Diarrhea and vomiting developed in pig 4 approximately 2–4 h after clinical signs developed in pig 3 (i.e., in pig 4, signs developed 46–50 h after indirect contact with inoculated pig 3); pig 4 was euthanized ≈120 h after the onset of clinical signs.

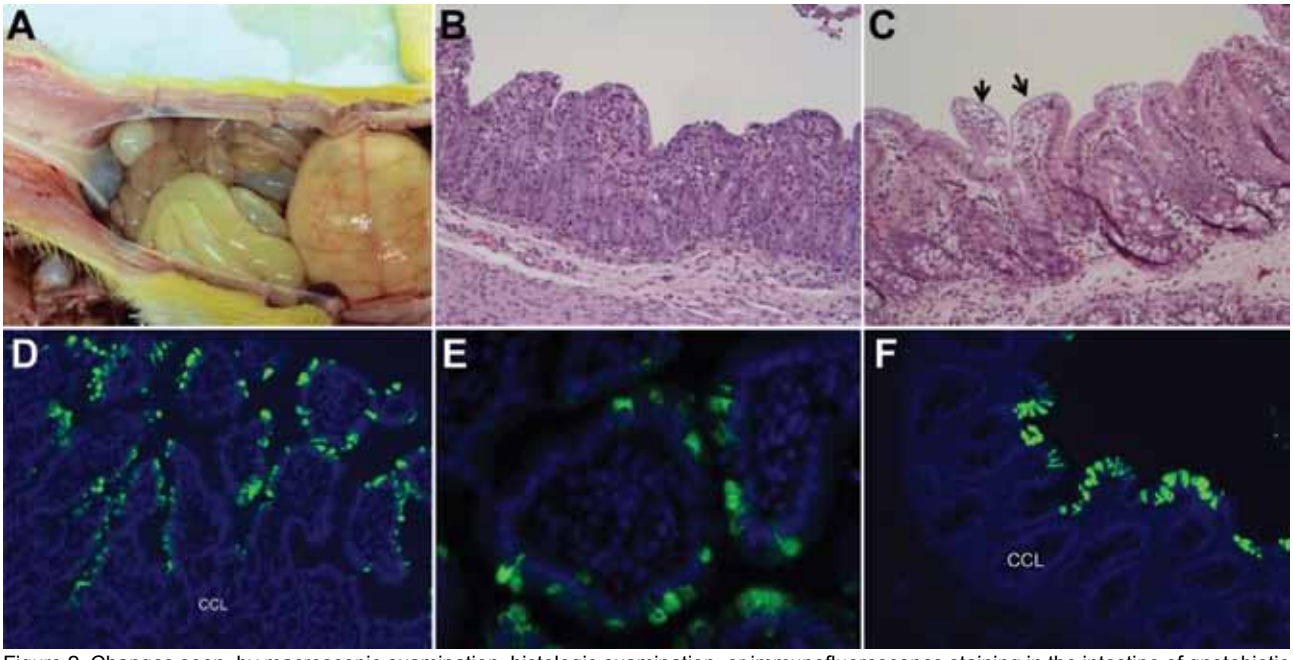

Figure 2. Changes seen, by macroscopic examination, histologic examination, or immunofluorescence staining in the intestine of gnotobiotic pigs inoculated with porcine epidemic diarrhea virus (PEDV; US strain PC21A). A) Intestine of pig 1 at postinoculation hour (PIH) 30 (4–5 h after onset of clinical signs), showing thin and transparent intestinal walls (duodenum to colon) and extended stomach filled with curdled milk. B) Hematoxylin and eosin (H&E)–stained jejunum of pig 3 at PIH 46 (at onset of clinical signs), showing acute diffuse, severe atrophic jejunitis. Original magnification ×200. C) H&E-stained cecum of noninoculated pig 4 (which was exposed to inoculated pig 3 at PIH 0) at 120 h after onset of clinical signs. Acute diffuse, mild vacuolation of superficial epithelial cells (arrows) and subepithelial edema are seen. Original magnification ×200. D) Immunofluorescence staining of jejunum of pig 5 at PIH 67 (37–41 h after onset of clinical signs), indicating that the epithelial cells lining atrophied villi are positive for PEDV. Original magnification ×200. E) Immunofluorescence staining of jejunum of pig 3 at PIH 46 (at onset of clinical signs), showing localization of PEDV antigens in the cytoplasm of enterocytes. Original magnification ×600. F) Immunofluorescence staining of colon of pig 2 at PIH 72 (26–28 h after onset of clinical signs), showing large numbers of PEDV-positive cells. Original magnification ×200. CCL, crypt cell layer. Nuclei were stained with blue-fluorescent 4′, 6-diamidino-2-phenylindole, dihydrochloride.

lung tissues under the conditions tested. Although PC21A strain replicated in cecum and colon epithelial cells, cellular necrosis and villous atrophy were not evident. Whether PEDV infection of the large intestine contributes to the severity of PED is unclear.

All infected pigs tested at acute or later stages of infection had viral RNA titers of 4.8–7.6 $\log_{10}$ GE/mL in serum samples (Table 1). These titers were similar to those for field samples tested by real-time RT-PCR; 11 (55%) of 20 acute-phase serum samples collected from 13- to 20-week-old pigs with diarrhea from Ohio had viral RNA titers of 4.0–6.3 GE/mL. The early, severe diarrhea and vomiting and the PEDV fecal shedding at high titers may be accompanied by viremia. No infected pigs had detectable viral RNA in serum samples obtained before inoculation, and no negative control pig had detectable viral RNA during the experiment.

Table 2. Histopathologic findings in a study of the pathology of US PEDV strain PC21A in gnotobiotic pigs, 2013*

Pig status, no.	PIH at euthanasia (infection stage)	VH:CD, mean (±SD)	Antigen detection in frozen tissues†				
			Duodenum	Jejunum	Ileum	Cecum/colon	Lung
PEDV-inoculated							
1	30 (acute)	3.4 (1.7)	++	+++	+++	+++	–
2	72 (mid)	1.8 (0.3)	++	+++	+++	+++	–
3	46 (acute)	1.2 (0.3)	+	++	++	++	–
4‡	120 (later)‡	1.5 (0.2)	+	+/++	+/++	++/+++	–
5	67 (mid)	2.2 (0.4)	+	+++	+++	+	–
Negative control							
6	.	6.3 (0.7)	–	–	–	–	–

*PEDV, porcine epidemic diarrhea virus; PIH, postinoculation hour; VH:CD, ratio of villous height to crypt depth.
†Detected by immunofluorescence staining. +, 1%–29% of epithelial cells showed staining; ++, 30%–59% of epithelial cells showed staining; +++, 60%–100% of epithelial cells showed staining, as described (*4*); –, no cells showed staining.
‡At 24 days of age, noninoculated pig 4 was exposed by indirect contact to pig 3 (at PIH 0) through small holes drilled into the stainless steel divider panel located between the 2 pigs in the shared pig tub isolator unit. Clinical signs and virus shedding were monitored after indirect contact. Diarrhea and vomiting developed in pig 4 approximately 2–4 h after clinical signs developed in pig 3 (i.e., in pig 4, signs developed 46–50 h after indirect contact with inoculated pig 3); pig 4 was euthanized ≈120 h after the onset of clinical signs.

Conclusion

In 2013, the first US outbreaks of the rapidly spreading porcine virus, PEDV, caused a high number of pig deaths and substantial economic losses (*1,2*); however, little was known about progression of the disease. Our data confirm that US PEDV PC21A is highly enteropathogenic and acutely infects the entire intestine, but the jejunum and ileum are the primary sites of infection. PC21A infection causes severe atrophic enteritis accompanied by viremia that leads to severe diarrhea and vomiting.

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Dr Jung is a veterinary pathologist at The Ohio State University. His major research interests include diagnostic molecular pathology, pathogenesis, and immune responses to enteric viral infections, using germ-free animal models.

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Address for correspondence: Qiuhong Wang, Food Animal Health Research Program, Ohio Agricultural Research and Development Center, Department of Veterinary Preventive Medicine, The Ohio State University, 1680 Madison Ave, Wooster, Ohio 44691, USA; email: wang.655@osu.edu

Cetacean Morbillivirus in Coastal Indo-Pacific Bottlenose Dolphins, Western Australia

Nahiid Stephens, Pádraig J. Duignan, Jianning Wang, John Bingham, Hugh Finn, Lars Bejder, I. Anthony P. Patterson, and Carly Holyoake

Cetacean morbillivirus (CeMV) has caused several epizootics in multiple species of cetaceans globally and is an emerging disease among cetaceans in Australia. We detected CeMV in 2 stranded coastal Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) in Western Australia. Preliminary phylogenetic data suggest that this virus variant is divergent from known strains.

Cetacean morbillivirus (CeMV; family *Paramyxoviridae*) has caused several epizootics globally during the past 25 years. Three strains of CeMV—porpoise, dolphin, and pilot whale morbillivirus—are classified as 1 species (*1*). CeMV is more closely related to ruminant morbilliviruses and human measles virus than to canine and phocine distemper viruses (*1*,*2*).

Recently, a morbillivirus with phylogenetic similarity to dolphin morbillivirus (DMV) caused the death of a common bottlenose dolphin (*Tursiops truncatus*) in Queensland, northeastern Australia (*1*). Retrospective serologic testing from eastern Australia has confirmed seropositivity to morbillivirus in several species; these temporal data suggest circulation of a morbillivirus among cetaceans in this region as long ago as 1985 (*3*). However, whether DMV or variant viruses are circulating in the southwestern Pacific is unknown (*4*). The Queensland reports corroborate studies from 1997 that demonstrated high DMV seroprevalence in long-finned pilot whales (*Globicephala melas*) from northern New Zealand and seropositivity in 1 *T. truncatus* dolphin from Tasmania, Australia (*2*). Seroprevalence of morbillivirus among *G. melas* whales and melon-headed whales (*Peponocephala electra*) from the Tasman Sea (*2*,*3*) is similar to that found among North Atlantic pilot whales, a species in which infection is thought to be endemic (*5*).

Little is known about the prevalence and pathogenicity of CeMV in the Indian Ocean. A low DMV antibody titer by indirect ELISA was reported from a common dolphin (*Delphinus delphis*) from eastern South Africa (CeMV-related pathology was not seen), but no comparable data from Western Australia exist (*2*). We report the deaths of 2 Indo-Pacific bottlenose dolphins (*T. aduncus*) in Western Australia from opportunistic infections secondary to chronic morbillivirus-induced immunosuppression.

The Study

During June 2009 (midwinter), 3 deaths occurred among a population of ≈20–25 *T. aduncus* dolphins in the Swan River in Western Australia (32°04′S, 115°48′E) (*6*). By comparison, only 6 deaths were recorded during 2002–2008 (D. Coughran, pers. comm.; *7*). Necropsies and histopathologic, bacteriologic, and mycologic testing were performed on 2 of the dolphins (Table); the remains of dolphin 1 were too decomposed for detailed examination. Age class was estimated from morphometrics and reproductive development, supported by dental analysis. The API 20E test kit (bioMérieux, Inc., Boston, MA, USA) was used to identify isolated bacteria.

Both dolphins showed systemic lymphoid depletion. The cause of death for dolphin 2, a juvenile male (Table), was severe, focally extensive cerebral necrosis, secondary to vasculitis and thrombosis associated with abundant fungal hyphae characteristic of *Aspergillus* spp. (Figure 1, panel A); however, we could not culture the organism collected. Dolphin 3, a subadult female, died as a result of multifocal pyogranulomatous bronchopneumonia affecting ≈30% of the lungs; intralesional hyphae characteristic of *Aspergillus* spp. were found (Figure 1, panels B and C). At the periphery of the mycotic lesions were foci of type II pneumocyte hyperplasia, septal fibroplasia, and mononuclear infiltration. Occasional macrophages exhibited chromatin margination and intranuclear eosinophilic inclusions; syncytia were not seen (Figure 1, panel D). Multifocal bilateral renal necrosis associated with bacteria affected ≈30%–40% of the parenchyma. Using the API 20E test kit, we isolated *Staphylococcus aureus* and a gram-negative bacillus similar to *Mannheimia haemolytica* (67%) or *Morganella morganii* (26%). *Penicillium* spp., a presumptive contaminant, was the only fungus isolated.

Immunohistochemical (IHC) testing was conducted by using a monoclonal antibody for canine distemper virus

Author affiliations: Murdoch University, Perth, Western Australia, Australia (N. Stephens, H. Finn, L. Bejder, C. Holyoake); University of Calgary, Calgary, Alberta, Canada (P.J. Duignan); CSIRO, East Geelong, Victoria, Australia (J. Wang, J. Bingham); and Agri-Food and Biosciences Institute, Belfast, Northern Ireland, UK (I.A.P. Patterson)

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Table. Summary of findings for 3 bottlenose dolphins recovered from the Swan River, Western Australia, Australia*

Dolphin no.	Date recovered	Description	Pathologic findings	IHC results	RT-PCR results
1	2009 Jun 5	Juvenile male in good body condition; TL 210 cm	Too decomposed for necropsy	ND	ND
2	2009 Jun 8	Juvenile male in good body condition; TL 210 cm; postmortem interval ≈72 h	1. Encephalitis: severe, focally extensive, suppurative, and necrotizing, with multifocal vasculitis and thrombus formation, with intralesional fungal hyphae† 2. Splenic and mesenteric node lymphoid depletion, severe 3. Segmental jejunal submucosal hemorrhage with thrombosis 4. Pulmonary (*Halocercus* sp.) and gastric (*Anisakis* sp.) nematodes; trematode (*Campanula* sp.) infestation of the biliary and pancreatic ducts	Widespread positive staining of lymphocytes, mesenteric lymph node‡	Positive for morbillivirus N and P genes§
3	2009 Jun 21	Subadult female in good body condition; TL 222.5 cm; postmortem interval ≈72 h	1. Bronchointerstitial pneumonia: multifocal, pyogranulomatous, associated with intralesional fungal hyphae¶ 2. Nephritis: multifocal, necrotizing, severe, acute, with intralesional bacteria 3. Severe systemic lymphoid depletion 4. Fishing line entanglement, right fluke: chronic and proliferative, with granulation tissue formation, dermatitis, and hyperkeratosis 5. Pulmonary (*Halocercus* sp.) and gastric (*Anisakis* sp.) nematodes; trematode (*Campanula* sp.) infestation of the biliary and pancreatic ducts	Widespread staining of lymphocytes, mesenteric lymph nodes; weak staining of vascular endothelium, mesenteric lymph node; widespread staining in hepatic Kupffer cells, sinusoidal endothelial cells, and biliary epithelium#	Positive for morbillivirus N and P genes**

*Located at 32°04′S, 115°48′E. TL, total length in centimeters; IHC, immunohistochemical; RT-PCR, reverse transcription PCR; ND, not done; N, nucleoprotein; P, phosphoprotein.
†See Figure 1, panel A.
‡See Figure 1, panel E.
§See Figure 2, Swan River 1.
¶See Figure 1, panels B–D.
#See Figure 1, panel F.
**See Figure 2, Swan River 2.

nucleoprotein (VMRD, Inc., Pullman, WA, USA) diluted at 1:100, according to published protocols (*8*). Specific positive staining was found in multiple tissues from dolphins 2 and 3, including lymphocytes within lymph nodes, hepatic sinusoidal endothelial cells and Kupffer cells, biliary epithelium, and tunica media myocytes of blood vessels within the liver and mesenteric lymph nodes (Figure 1, panels E and F).

Reverse transcription PCR (RT-PCR) was performed as described (*1*). Amplified products (≈238 bp from the nucleoprotein [N] gene and ≈425 bp from the phosphoprotein [P] gene) were sequenced and compared with sequences of DMV and other morbilliviruses by using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Identical sequences of the highly conserved N and P genes were obtained from dolphins 2 and 3; these genes showed 79%–83% (dolphin 2) and 75%–79% (dolphin 3) nucleotide identity to sequences of CeMV strains from GenBank. Nucleotide comparison between the N and P gene sequences from the dolphins from Western Australia and the dolphin from Queensland (*1*) revealed only 83% and 79% similarity, respectively. Partial N and P gene sequencing and analysis using MEGA5 software (*1*) indicated that the Western Australia variant differed from other cetacean morbilliviruses and represents a distinct lineage (Figure 2).

Conclusions

We found CeMV in dolphins that died in the Indian Ocean in 2009; this finding thus predates reports of the virus in animals on the eastern coast of Australia (*1*,*3*) and recent confirmation of the virus in South Australia (C. Kemper and I. Tomo, pers. comm.). The virus we found is phylogenetically distinct from that isolated from cetaceans in eastern Australia. Complete sequencing of viruses from all Australian regions is needed, but preliminary data suggest that the variant from Queensland clusters with viruses isolated from cetaceans in the Northern Hemisphere, whereas the variant from Western Australia is distinct from other morbilliviruses—closely related to, but divergent from, other cetacean morbilliviruses. Our data also suggest that the variant from Western Australia is the least divergent of all cetacean morbilliviruses from the terrestrial viruses rinderpest, peste des petit ruminants, and measles.

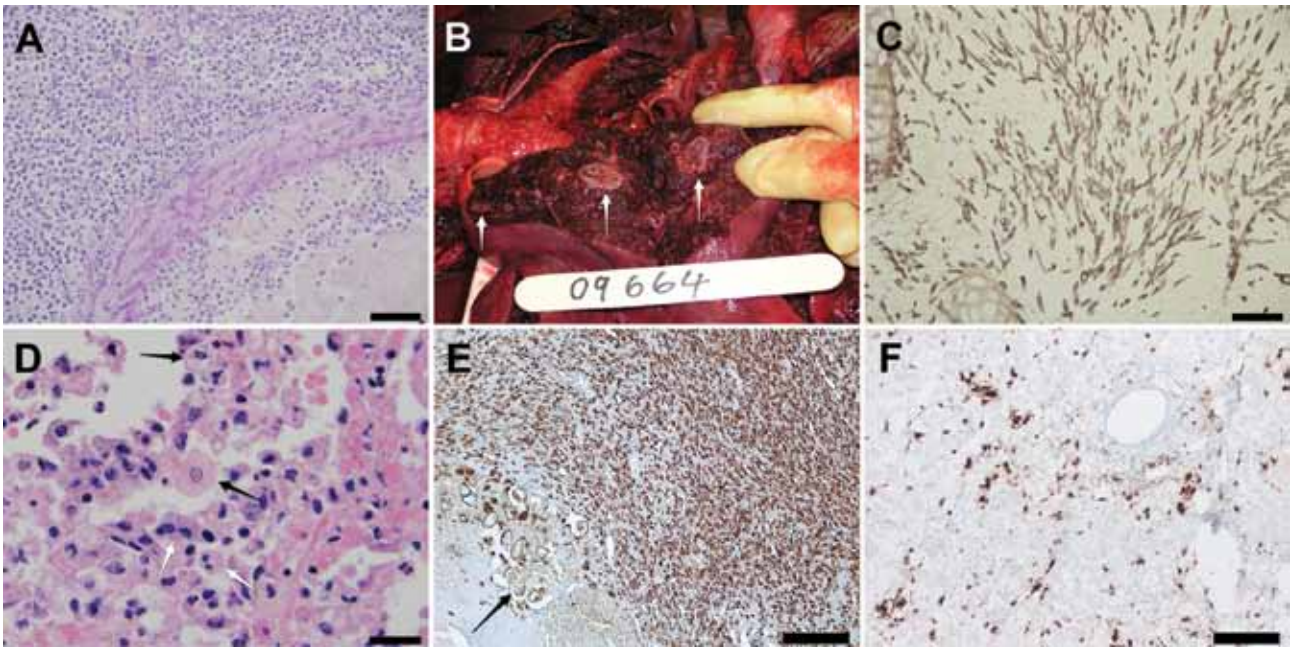

Figure 1. Images of tissue samples from 2 stranded coastal Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) from Western Australia, Australia. A) Brain of dolphin 2 showing cerebral hemisphere with focally extensive suppurative and necrotizing encephalitis surrounding an arteriole. There are intramural and perivascular septate branching hyphae. Hematoxylin and eosin stain. Scale bar = 50 μm. B) Lung of dolphin 3 showing a transected lobar surface exhibiting multifocal pyogranulomas (white arrows). C) Lung of dolphin 3 showing bronchointerstitial pneumonia with branching septate hyphae within a bronchiolar lumen and surrounding the bronchiolar cartilage. Grocott hexamine silver. Scale bar = 50 μm. D) Lung of dolphin 3 showing alveolar lumens filled with desquamated pneumocytes, macrophages, and neutrophils. Enlarged macrophages are occasionally binucleate (white arrows) and rarely exhibit eosinophilic intracytoplasmic inclusions or margination of chromatin and eosinophilic intranuclear inclusions (black arrows). Hematoxylin and eosin stain. Scale bar = 20 μm. E) Mesenteric lymph node of dolphin 2 showing intense staining of morbilliviral antigen in lymphocytes within the cortex. Thick-walled structures (arrow) are trematode eggs. DAB and hematoxylin stain. Scale bar = 200 μm. F) Liver of dolphin 3 showing morbillivirus antigen in Kupffer cells and sinusoidal endothelial cells. DAB and hematoxylin stain. Scale bar = 100 μm.

Rinderpest is considered the archetypal genus member (*9*); our data suggest that the Western Australia variant is the most closely related cetacean morbillivirus to the terrestrial members of the genus.

Lesions in the dolphins we examined were not those typically seen in classic acute morbillivirus infections, and diagnosis required IHC testing and RT-PCR. The signs we saw may be more common for infections that occur in temperate/tropical waters, wherein infected animals survive the viral infection but succumb to opportunistic infections facilitated by virus-induced chronic immunosuppression following lymphoid depletion. During mass mortality events involving various species that were ultimately attributed to CeMV on the US Atlantic coast (1982, 1987–1988) and Gulf Coast (1993–1994) (*10*,*13*) and in the Mediterranean Sea (1990–1992 and 2006–2007) (*10*), opportunistic infections were common (*1*,*10*). In 1 study, antigen was detectable by IHC testing in just 53% of cases (*11*); RT-PCR increased detection rates to >90% in autolysed tissues or for cases in which the acute phase had passed and few to no pathognomonic lesions remained (*11*).

Diagnosis of CeMV is a challenge in areas in which epidemics have not been recorded, in CeMV-endemic areas in which clinical cases are rare, or in new hosts. Subclinical infection may play a more critical epidemiologic role than previously thought, further complicating assessment of deaths (*12*). Archived serum samples from the western Atlantic documented a pattern of recurrent mortality events in bottlenose dolphins dating back to 1982 (*13*); investigations have also shown that CeMV may persist between outbreaks without causing clinical disease (*12*). Infection may thus become endemic, and periodic incursions into immunologically naive populations may cause deaths at epidemic rates (*12*). A similar scenario of virus circulation without clinical disease has been proposed for common dolphins (*D. delphis*) in the eastern North Pacific (*14*) and for Mediterranean striped dolphins (*Stenella coeruleoalba*) and pilot whales (*G. melas*) (*10*,*15*). A 30-year study of strandings in Western Australia (1981–2010) showed a pattern of mortality peaks and troughs in bottlenose dolphins similar to that seen along the US Atlantic coast (*7*,*13*). A peak in 2009 coincided

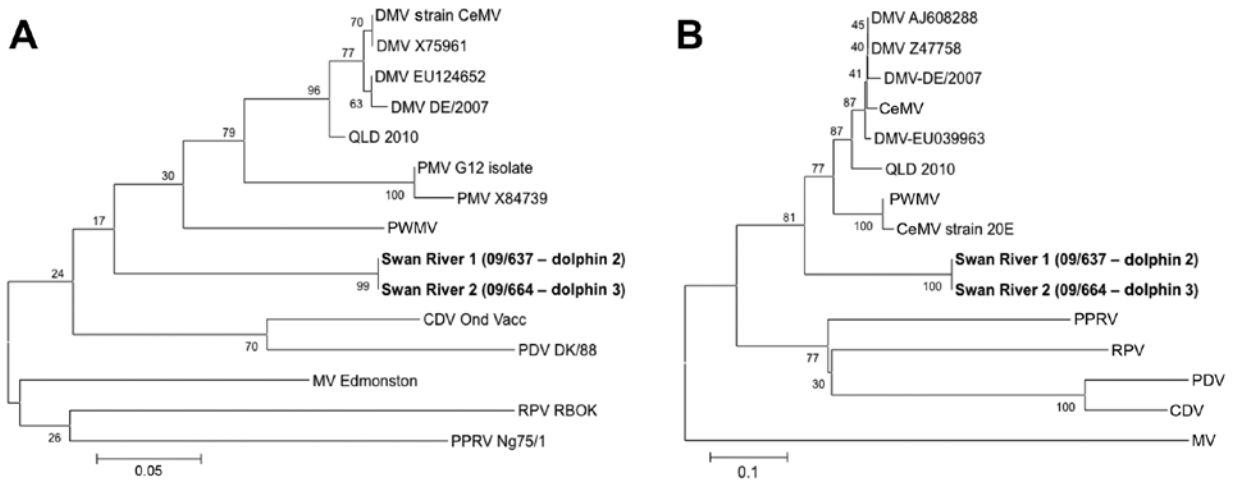

Figure 2. Phylogenetic trees showing partial sequences of morbillivirus nucleoprotein (A) and phosphoprotein (B) genes of cetacean morbillivirus (CeMV) isolates found in 2 stranded coastal Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) from Western Australia, Australia (boldface), and those of other known morbilliviruses. Trees were generated by the neighbor-joining method; bootstrap (1,000 replicates) values of >50 are indicated at the internal nodes. The length of each pair of branches represents the distance between the sequence pairs. Scale bars indicate percentage of nucleotide differences. DMV, dolphin morbillivirus; QLD, Queensland (Australia); PMV, porpoise morbillivirus; PWMV, pilot whale morbillivirus; CDV, canine distemper virus; OND vacc, Onderstepoort strain (used for vaccination); PDV, phocine distemper virus; MV, measles virus; RPV, rinderpest virus; PPRV, peste des petits ruminants virus.

with the cases we report. Further study of CeMV circulation and spread is needed.

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Dr Stephens is a lecturer in veterinary pathology and a PhD candidate at Murdoch University, Perth, Western Australia. Her primary research interests are anatomic pathology and disease ecology, particularly diseases of marine mammals and other wildlife.

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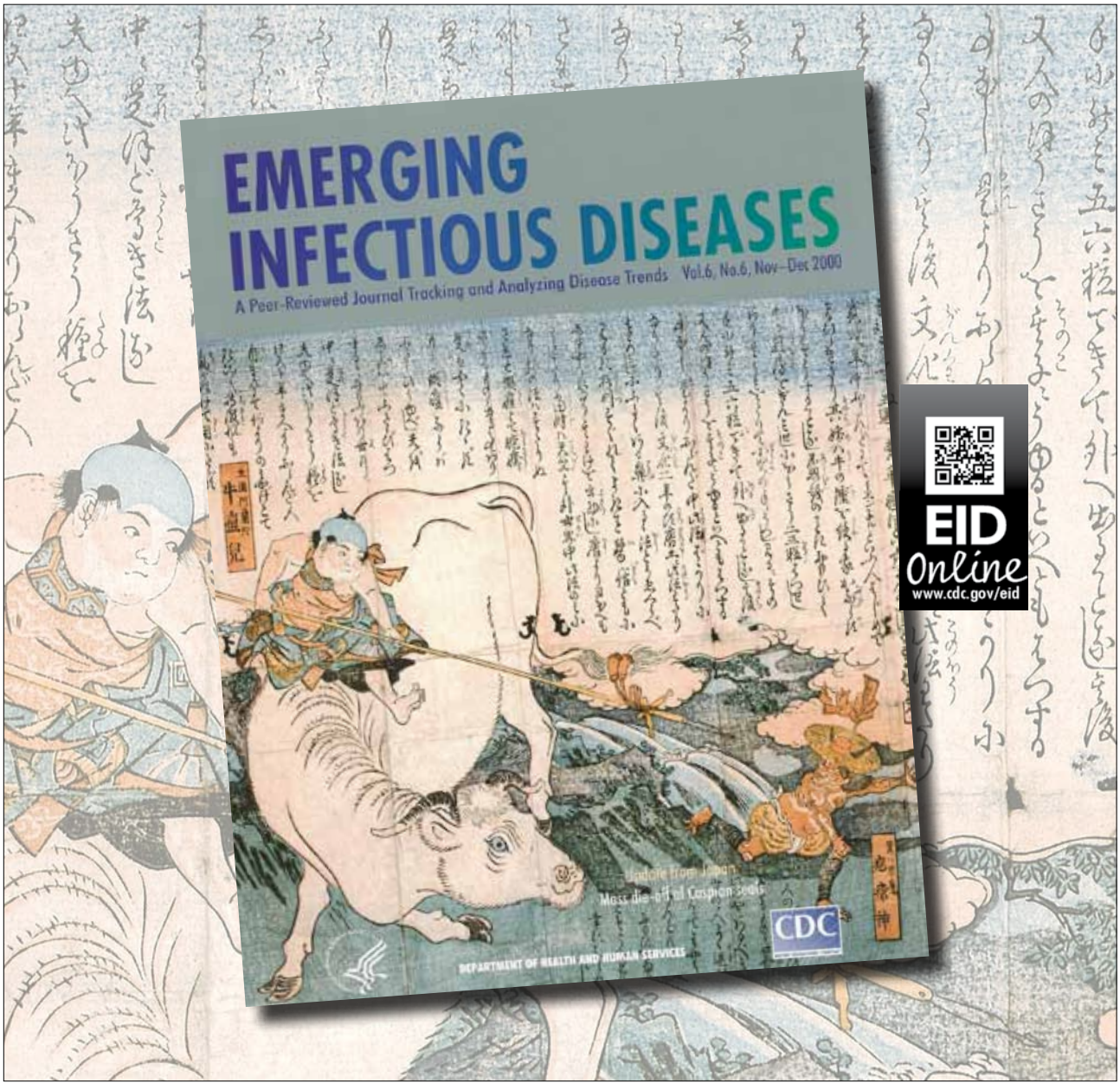
Address for correspondence: Nahiid Stephens, School of Veterinary and Life Sciences, Murdoch University, 90 South Street, Murdoch, Perth, WA 6150, Australia; email: N.Stephens@murdoch.edu.au



Genetic Characterization of Clade 2.3.2.1 Avian Influenza A(H5N1) Viruses, Indonesia, 2012

Ni Luh Putu Indi Dharmayanti, Risza Hartawan, Pudjiatmoko, Hendra Wibawa, Hardiman, Amanda Balish, Ruben Donis, C. Todd Davis, and Gina Samaan

After reports of unusually high mortality rates among ducks on farms in Java Island, Indonesia, in September 2012, influenza A(H5N1) viruses were detected and characterized. Sequence analyses revealed all genes clustered with contemporary clade 2.3.2.1 viruses, rather than enzootic clade 2.1.3 viruses, indicating the introduction of an exotic H5N1 clade into Indonesia.

Highly pathogenic avian influenza A(H5N1) virus has circulated in poultry in Indonesia since 2003 (*1*,*2*). The phylogeny of A(H5N1) viruses detected during 2003–2011 indicated all genes descended from 1 ancestral virus with a clade 2.1 hemagglutinin (HA) introduced into Indonesia before 2003 (*3*). These viruses became enzootic and evolved into second-, third-, and fourth-order HA clades, leading to the recent dominance of clade 2.1.3.2 viruses (*4*). Outbreaks in poultry typically caused high mortality rates among gallinaceous birds, especially layer, broiler, and native chickens. The virus seemed less pathogenic in aquatic birds (*5*). However, reports of duck deaths and a higher than usual mortality rate (100% in some outbreaks) in backyard farms in Central Java, Jogjakarta, and East Java Provinces, Indonesia, in September 2012 triggered a joint outbreak investigation by animal and public health authorities (*6*). We describe the genetic characteristics of viruses isolated from A(H5N1) infection outbreaks in these 3 provinces on Java Island, where a previously unrecognized clade was detected.

Author affiliations: Indonesian Research Center for Veterinary Science, Bogor, Indonesia (N.L.P.I. Dharmayanti, R. Hartawan, Hardiman); Ministry of Agriculture, Jakarta, Indonesia (Pudjiatmoko); Disease Investigation Center Wates, Jogjakarta, Indonesia (H. Wibawa); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (A. Balish, R. Donis, C.T. Davis); and Centers for Disease Control and Prevention, Jakarta (G. Samaan)

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The Study

We investigated 9 small-holding duck farms that reported bird deaths during September 12–November 5, 2012 (*6*). Cloacal swab samples were collected from sick birds, placed in 1,000 μL of viral transport medium, and sent for testing at laboratories of the regional Ministry of Agriculture Disease Investigation Center, Jogjakarta. Seventeen A(H5N1)–positive samples were forwarded to the National Animal Health Laboratory, Indonesian Research Center for Veterinary Science (IRCVS), for virus isolation and genome sequencing.

In addition, IRCVS collected 122 cloacal swab samples from birds and 58 environmental swab samples (from defeathering machines) at 5 live-bird markets (LBMs) in East Java Province during November 5–8, 2012. RNA extracted from farm and LBM specimens was tested for influenza A matrix gene to identify presumptive A(H5N1)-positive samples (*7*). Select positive samples were inoculated in 9–11-day-old embryonated, specific pathogen–free eggs. Allantoic fluid was harvested 36 h postinfection and tested for HA with chicken erythrocytes to confirm virus isolation (*8*).

Samples showing suspected A(H5N1) infection were propagated in a Biosafety Level-3 laboratory at IRCVS in compliance with biosafety regulations. Ten virus isolates (7 from duck farms, 3 from LBMs) were chosen for full-length HA gene sequencing (GenBank accession nos. KC417271–KC417277, KC757643); 4 were selected for genome sequencing. Results of reverse transcription PCR and sequencing primers are available on request. Sequencing and consensus sequence generation were conducted as described (*9*). Phylogenetic trees were generated by using MEGA4 (*10*) (Figure; online Technical Appendix 1, wwwnc.cdc.gov/EID/article/20/4/13-0517-Techapp1.pdf).

Phylogenetic analysis revealed that A(H5N1) isolates from samples collected from duck farm outbreaks and an LBM were not related to isolates in long-established Indonesian clade 2.1; rather, the HA genes closely resembled those of clade 2.3.2.1 viruses recently found in Vietnam, China, and Hong Kong (Figure). Full-length HA genes showed 97%–98%-nt identity with recent viruses from Vietnam and clustered in a larger group containing viruses from many Asian regions during 2009–2012. The environmental sample from an East Java LBM shared >99% nt similarity with viruses from samples at duck farms, indicating spread of this A(H5N1) clade into the marketing chain. A poultry sample from the same district as the 2.3.2.1 virus was identified as clade 2.1.3.2 (Figure), indicating likely cocirculation.

The 8 clade 2.3.2.1 HA genes analyzed possessed a multibasic amino acid cleavage site (Table 1). The cleavage site sequence of the clade 2.3.2.1 viruses from Indonesia (PQRE^{del}RRRKR↓G) differed from recent clade 2.1.3.2

Figure. Partial phylogenetic tree of influenza A(H5N1) hemagglutinin (HA) gene sequences. The phylogenetic tree was generated in MEGA version 4 (www.megasoftware.net), using neighbor-joining analysis with 1,000 bootstrap replicates and the Kimura 2-parameter model. Viruses characterized in this study are indicated with a dot. The HA tree was rooted to A/goose/Guangdong/1/1996. The full figure is available online (wwwnc.cdc.gov/EID/article/20/4/13-0517-F1.htm).

viruses (PQRE**S**RR**K**KR↓G) by a Ser deletion at position 325 and a K328R substitution. Like other serotype H5N1 HA proteins, all isolates possessed a conserved glutamine at position 222 (equivalent to H3 position 226) and glycine at position 224 (H3 position 228), indicating no substantial changes in avian receptor-binding specificity (Table 1) (*11*). The clade 2.3.2.1 viruses from Indonesia possessed 6 or 7 potential N-linked glycosylation sites (7 in clade 2.1.3.2 viruses), but unlike 2.1.3.2 viruses, all 2.3.2.1 viruses lacked the potential glycosylation site at position 154.

Up to 29 conserved amino acid changes occurred in the mature HA1 protein between clade 2.3.2.1 and clade 2.1.3.2 viruses found recently in Indonesia, indicating these A(H5N1) virus subgroups probably diverged substantially

Table 1. Genetic characteristics of influenza A(H5N1) clade 2.3.2.1 viruses found in Indonesia, 2012*†

	PB2		PB1-F2		HA‡			NA	M2		NS sequence	
Strain name	aa 627	aa 701	PB1-F2 truncation	aa 66	aa 222	aa 224	Cleavage site	aa 203	aa 27	aa 31	aa 80–84 del	PDZ binding ligand
A/Hubei/1/2010	E	D	90 aa	N	Q	G	PQRERRRKR↓G	I	I	S	Yes	ESEV
A/Hong Kong/6841/2010	E	D	90 aa	N	Q	G	PQRERRRKR↓G	I	I	S	Yes	ESEV
A/env/East Java/LBM-LM13/2012	E	D	57 aa	N	Q	G	PQRERRRKR↓G	V	I	S	Yes	ESEV
A/duck/Sukoharjo/BBVW-1428–9/2012	E	D	57 aa	N	Q	G	PQRERRRKR↓G	I	I	S	Yes	ESEV
A/duck/Bantul/BBVW-1443-9/2012	E	D	57 aa	N	Q	G	PQRERRRKR↓G	I	I	S	Yes	ESEV
A/duck/Sleman/BBVW-1463–10/2012	E	D	57 aa	N	Q	G	PQRERRRKR↓G	ND	I	S	Yes	ESEV
A/md/Tegal/BBVW-1732-11/2012	ND	ND	ND	ND	Q	G	PQRERRRKR↓G	ND	ND	ND	ND	ND
A/dk/Blitar/BBVW-1731-11/2012	ND	ND	ND	ND	Q	G	PQRERRRKR↓G	ND	ND	ND	ND	ND
A/dk/Tegal/BBVW-1727-11/2012	ND	ND	ND	ND	Q	G	PQRERRRKR↓G	ND	ND	ND	ND	ND
A/dk/Wonogiri/BBVW-1730-11/2012	ND	ND	ND	ND	Q	G	PQRERRRKR↓G	ND	ND	ND	ND	ND

*HA, hemagluttinen; NA, neuraminidase; M2, matrix 2; NS, nonstructural; ND, not determined.
†Numbering of the first and last nucleotide position of the gene that was sequenced is as follows: PB2, 1618–2192; PB1, 130–632; PA, 34–429; HA, 1–1710; NP, 268–755; NA, 55–1314; M, 55–919; NS, 25–690.
‡Glycosylation motif at 154 was absent for all strains.

Table 2. Hemagglutination-inhibition assay of clade 2.3.2.1 highly pathogenic avian influenza A(H5N1) virus introduced into Indonesia, 2012*

		Reference ferret antiserum								
		1	2.2.1	2.3.4	2.1.3.2	2.3.2.1	2.3.2.1	2.3.2.1	2.3.2.1	2.3.2.1
Antigen†	Clade	VN/1203	EG/321	ANH/1	IND/ 12379	CH/1 RG30	BS/HK/ 1161	BHG/MG/ X53	HK/ 6841	DK/VN/ 1584
Reference strains										
VN/1203	1	**320***	20	40	<10	10	<10	10	10	10
EG/321	2.2.1	80	**1,280**	80	20	40	10	80	80	40
ANH/1	2.3.4	160	80	**640**	80	<10	<10	<10	40	10
IND/12379	2.1.3.2	10	10	40	**1,280**	<10	<10	<10	40	40
CH/1 RG30	2.3.2.1	40	80	20	20	**640**	40	160	640	160
BS/HK/1161	2.3.2.1	<10	40	10	<10	320	**80**	160	640	80
BHG/MG/X53	2.3.2.1	10	80	20	20	320	40	**320**	640	320
HK/6841	2.3.2.1	10	20	10	20	160	20	320	**640**	160
DK/VN/1584	2.3.2.1	<10	40	10	20	320	20	320	320	**160**
Test strain										
A/environment/East Java/LBM-LM13/2012	2.3.2.1	<10	40	10	10	160	40	320	320	160

*Homologous titers of reference antigen to ferret antiserum are indicated in boldface.
†Strains: VN/1203, A/Vietnam/1203/2004; EG/321, A/Egypt/2321-NAMRU3/2007; ANH/1, A/Anhui/1/2005; IND/12379, A/Indonesia/NIHRD12379/2012; CH/1 RG30, A/Hubei/1/2010 IDCDC-RG30; BS/HK/1161, A/barn swallow/Hong Kong/1161/2010; BHG/MG/X53, A/barheaded goose/Mongolia/X53/2009; HK/6841, A/Hong Kong/6841/2010; DK/VN/1584, A/duck/Vietnam/NCVD-1584/2012.

in antigenicity. In contrast, the HA1 of the new viruses collected in Indonesia differed by 8–10 aa from A/Hubei/1/2010, the most closely related clade 2.3.2.1 A(H5N1) candidate vaccine virus recommended by the World Health Organization (online Technical Appendix 2, wwwnc.cdc.gov/EID/article/20/4/13-0517-Techapp2.pdf) (*12*).

To test the antigenic relationship of the clade 2.3.2.1 virus to the endemic clade 2.1.3.2 virus, we conducted a hemagglutination-inhibition test with ferret antiserum raised against viruses from these and other H5N1 clades (Table 2) (*8*). As the HA1 protein sequence differences suggest, clade 2.1.3.2 antiserum did not inhibit hemagglutination by a representative clade 2.3.2.1 virus from Indonesia, A/environment/East Java/LBM-LM13/2012. In contrast, this virus cross-reacted with antiserum to clade 2.3.2.1 viruses from other countries at heterologous titers generally within 2-fold of or equivalent to the homologous virus titer. The Indonesian clade 2.3.2.1 virus was most closely related antigenically to viruses that clustered genetically into the A/Hong Kong/6841/2010-like group of clade 2.3.2.1 (Table 2).

All 4 isolates exhibited the typical 20-aa deletion in the stalk region (residue 48–68) of the neuraminidase gene (NA). Although 1 sample had an Ile203Val substitution in the NA, which has been associated with reduced susceptibility to oseltamivir, no other markers of resistance in the NA or M2 were identified (Table 1). All 4 viruses had NS1 protein sequences with the typical deletion at position 80–84 and an intact H5N1 consensus PDZ binding motif (ESEV). A truncated form (57 aa) of the PB1-F2 protein was found in all viruses characterized. Although the functional consequences of this truncation are unknown, this represents a change from the typical full-length 90-aa protein found in most A(H5N1) viruses (*13*). All other amino acid residues and motifs of interest in the internal genes of the 4 viruses sequenced in this study represented avian consensus sequences.

Phylogenetic comparison of the NA and internal gene segments revealed ancestral origins of the new viruses similar to those of the HA gene (online Technical Appendix 1). Although partial nucleotide sequences from some genes were available for analysis (Table 1), sequence identities and phylogenetic comparisons to other clade 2.3.2.1 genomes in GenBank and Global Initiative on Sharing Avian Influenza Data databases confirmed their relatedness to viruses circulating recently in China, Vietnam, and Hong Kong. Individual gene sequence analysis did not show reassortment between these clade 2.3.2.1 viruses and the previously identified clade 2.1.3.2 genotype virus in Indonesia.

Conclusions

Detection of a novel clade of A(H5N1) virus in Indonesia marks a potential turning point in the molecular epidemiology of this virus. Indonesia has the highest number of human A(H5N1) infections because of ongoing outbreaks in poultry (*14*,*15*). Whether this new virus will become entrenched, as did clade 2.1.3 viruses over the past decade, remains to be seen, as do its effects on the incidence of human infection. Potential cocirculation of subtypes of 2 different clades warrants review of diagnostic methods and vaccination strategy to maximize effectiveness of disease control interventions. The lack of antigenic relatedness between the clade 2.3.2.1 and 2.1.3.2 viruses must be considered when evaluating A(H5N1) serologic diagnostic reagents used in Indonesia. This change also may have implications in selecting prepandemic candidate vaccine virus for the region. Furthermore,

poultry vaccines may need to be matched antigenically to circulating virus if clade 2.3.2.1 virus continues to circulate in Indonesia. Introduction of this virus is a stark reminder of the value of control measures to reduce the spread of subtype H5N1 and the need for enhanced surveillance of humans and poultry to monitor changes in its genetic and immunologic features.

Dr Dharmayanti is a researcher in the Virology Department at the Indonesian Research Center for Veterinary Science, Ministry of Agriculture. Her primary research interest is avian influenza.

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Address for correspondence: Ni Luh Putu Indi Dharmayanti, Virology Department, Indonesian Research Center for Veterinary Science, Jalan RE Martadinata 30, Bogor 16114, Indonesia; email: nlpdharmayanti@yahoo.com

Clinical Malaria along the China–Myanmar Border, Yunnan Province, China, January 2011–August 2012

Guofa Zhou,[1] Ling Sun,[1] Rongji Xia, Yizhong Duan, Jianwei Xu, Henglin Yang, Ying Wang, Ming-chieh Lee, Zheng Xiang, Guiyun Yan, Liwang Cui, and Zhaoqing Yang

Passive surveillance for malaria cases was conducted in Yunnan Province, China, along the China–Myanmar border. Infection with *Plasmodium vivax* and *P. falciparum* protozoa accounted for 69% and 28% of the cases, respectively. Most patients were adult men. Cross-border travel into Myanmar was a key risk factor for *P. falciparum* malaria in China.

Increased global efforts to control and eliminate malaria are leading to substantial declines in malaria-related illness and death (*1*). *Plasmodium vivax* is the predominant malaria-causing species in China, followed by *P. falciparum*. Cross-border migration from Myanmar is suspected to be the major source for the introduction of *P. falciparum* malaria in southwestern China. During the past decade, the incidence of malaria in China has declined tremendously; the reduction in Myanmar has been less dramatic (*1–5*). To identify risk factors for clinical malaria and, in turn, to inform the ongoing malaria elimination programs in China, we conducted passive surveillance for malaria at health facilities along the China–Myanmar border in Yunnan Province, China, during January 2011–August 2012.

The Study

The Southeast Asia Malaria Research Center (www.niaid.nih.gov/LabsAndResources/resources/icemr/centers/Pages/southeastasia.aspx), an International Center of Excellence for Malaria Research, in collaboration with the Chinese Center for Disease Control and Prevention, conducted passive malaria case detection along the China–Myanmar border. Surveillance was conducted at 60 hospitals and health care centers in Tengchong, Yingjiang, Longchuan, and Ruili Counties in Yunnan Province, China. According to the Sixth National Population Census of the People's Republic of China conducted in 2010 (http://chinadatacenter.org/Announcement/AnnouncementContent.aspx?id=470), the population of the 4 counties totaled ≈1.5 million. During 2010, Ruili and Yingjiang Counties reported the highest incidence of malaria in China (*2*).

Persons who sought care for febrile illnesses at 1 of the 60 surveillance site hospitals or health care centers were screened for clinical signs and symptoms of malaria. Case report forms were used to collect the following information from patients: demographic characteristics, occupation, education level, clinical symptoms, history of malaria in the preceding 12 months, history of travel within the 2 weeks preceding the clinic visit, history of fever, and use of measures to prevent malaria. For each suspected case-patient, thick and thin blood smears were prepared and examined by 3 experienced microscopists to provide a final diagnosis and parasite densities. Patients were considered to have clinical malaria if they had signs and symptoms consistent with malaria and a plasmodium-positive blood smear; severe malaria was defined according to World Health Organization criteria (*6*).

During January 2011–August 2012, a total of 8,296 Chinese and Myanmarese persons sought care for fever at the surveillance sites; 656 (7.9%) of the patients had other signs and symptoms consistent with malaria. Blood smear examination by microscope confirmed malaria infection in 303 (46.1%) of the 656 patients (Table 1). Protozoa of all 4 *Plasmodium* spp. that cause malaria in humans were detected; however, *P. vivax* and *P. falciparum* accounted for 69.0% and 27.7%, respectively, of the cases. Transmission peaked during April–July; cases of *P. falciparum* infection were detected primarily during the peak season (Figure). Asexual parasite densities were 1,285 and 2,515 parasites/mL, for *P. vivax* and *P. falciparum*, respectively. Chinese patients had fever for a median of 3.0 days, and Myanmarese patients (>90% of whom lived in China) had fever for a median of 2.5 days (range 1–10 days; $p>0.05$) before seeking care at a surveillance site. A total of 4 (1.9%) patients with *P. vivax* malaria and 13 (15.5%) patients with *P. falciparum* malaria had severe symptoms at the first clinical visit and were treated as inpatients.

A total of 84.4% of suspected and confirmed malaria case-patients in our passive case surveillance were Chinese.

Author affiliations: Kunming Medical University, Kunming City, China (G. Zhou, L. Sun, Z. Xiang, Z. Yang); University of California, Irvine, Irvine, California, USA (G. Zhou, M.-C. Lee, G. Yan); Longchuan Center for Disease Control and Prevention, Longchuan, China (R. Xia); Tengchong Center for Disease Control and Prevention, Techong, China (Y. Duan); Yunnan Institute of Parasitic Diseases, Pu'er, China (J. Xu, H. Yang); Third Military Medical University, Chongqing, China (Y. Wang); and Pennsylvania State University, University Park, Pennsylvania, USA (L. Cui)

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[1]These authors contributed equally to this article.

Table 1. Demographic characteristics for participants in a study of clinical malaria along the China–Myanmar border, Yunnan Province, China, January 2011–August 2012*

Characteristic	No. (%) febrile case-patients, n = 8,296	No. (%) suspected cases, n = 656	No. (%) confirmed cases, n = 303	Odds ratio (95% CI)
Nationality				
Chinese	6,002 (83)	586 (89)	257 (85)	1
Myanmarese	1,232 (17)	70 (11)	46 (15)	2.5 (1.5–4.1)†
Sex				
F	3,648 (44)	88 (13)	27 (9)	1
M	4,629 (56)	568 (87)	276 (91)	2.1 (1.3–3.5)‡
Age, y				
<18	1,864 (23)	66 (10)	16 (5)	1
≥18	6,359 (77)	590 (90)	287 (95)	3.0 (1.6–5.3)†
Occupation				
Indoor worker§	NC	64 (10)	10 (3)	1
Farmer	NC	433 (66)	203 (67)	4.8 (2.4–9.6)†
Business person	NC	78 (12)	41 (14)	6.0 (2.7–13.4)†
Mobile worker¶	NC	78 (12)	49 (16)	9.1 (4.0–20.6)†
Use of preventive measures#				
No	NC	257 (39)	209 (69)	1
Yes	NC	399 (61)	94 (31)	0.07 (0.05–0.10)†

*For some case reports, information was missing for nationality, sex, age, or occupation. NC, not calculated because information was missing for a considerable number of febrile cases.
†p<p 0.001.
‡P<0.01.
§Students, preschool children, office workers, and housewives were categorized as "indoor work."
¶Mobile workers included truck drivers, construction workers and casual workers who worked in plantation farms.
#Indicates use of bed net, indoor residual spray, and repellents.

However, among patients with suspected malaria, Myanmarese patients were 2.5 times more likely than Chinese patients to have malaria (odds ratio [OR] 2.5, 95% CI 1.5%–4.1%; p<0.0001) (Table 1). Male patients were more likely than female patients to have malaria (OR 2.1, 95% CI 1.3%–3.5%; p<0.01), and most malaria case-patients were 18–60 years of age (OR 3.0, 95% CI 1.6%–5.3%; p<0.0001) (Table 1). Compared with persons who worked indoors (e.g., students, office workers, and housewives), persons who worked outdoors (e.g., construction workers, traders, truck drivers who traveled frequently, and farmers) were at higher risk for malaria (Table 1). Patients who reported using measures to prevent malaria (e.g., insecticide-treated nets and repellents) had a 14-fold lower odds of getting malaria than did patients who did not report using any preventive measures (OR 0.07, 95% CI 0.05%–0.10%; p<0.0001).

Among the 110 suspected malaria case-patients who reported travel during the 2 weeks before seeking care at a surveillance site, 54 were confirmed by blood-smear examination to have clinical malaria: 31 patients had *P. vivax* infections, 21 had *P. falciparum* infections, and 2 had mixed infections. After we adjusted for the confounding effects of age and sex, patients reporting travel across the border, >1 km into Myanmar, were 15 times more likely than nontravelers to have *P. falciparum* malaria (adjusted OR 15.0, 95% CI 2.9%–175.0%; p<0.001); however, travel into Myanmar was not significantly

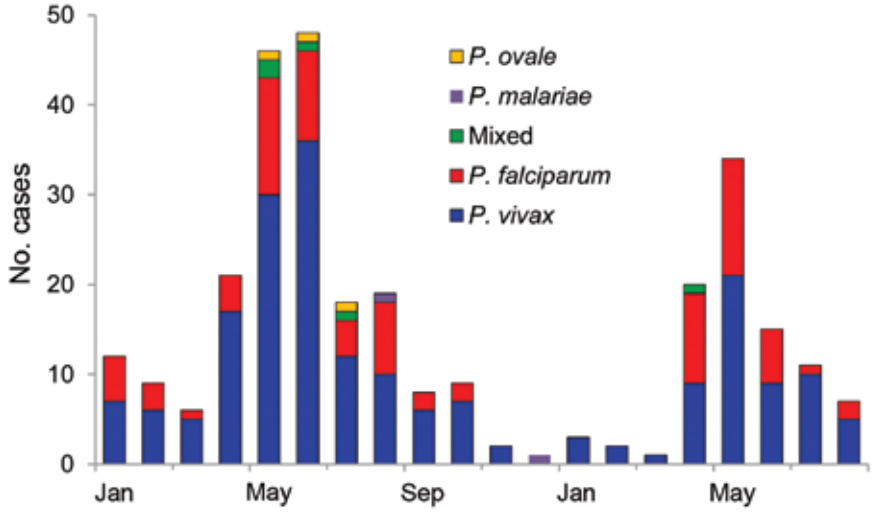

Figure. Number of confirmed malaria cases caused by various *Plasmodium* spp. protozoa in 4 counties of Yunnan Province, China, along the China–Myanmar border, January 2011–August 2012. Mixed, *P. vivax*/*P. falciparum* infection.

Table 2. Association between travel history and malaria for participants in a study of clinical malaria along the China–Myanmar border, Yunnan Province, China, January 2011–August 2012*

		Plasmodium vivax			*P. falciparum*		
Travel history	No malaria	No. cases	Odds ratio (95% CI)	Adjusted odds ratio (95% CI)†	No. cases	Odds ratio (95% CI)	Adjusted odds ratio (95% CI)†
None	297	175	1	1	63	1	1
Local‡	32	14	0.7 (0.4%–1.4%)	0.9 (0.5%–1.8%)	1	0.1 (0.0%–1.1%)	0.8 (0.2%–2.0%)
In Myanmar§	24	19	1.3 (0.7%–2.5%)	1.9 (0.8%–4.9%)	20	3.9 (2.0%– 7.5%)	15.0 (2.9%–175.0%)

*Travel within 2 weeks before study participants sought care at a surveillance site health center or hospital. Twelve case-patients with missing travel histories were excluded from the analysis.
†Adjusted odds ratios were adjusted for age and sex obtained from logistic regression.
‡Included local travel within China and to border towns in Myanmar (<1 km inside Myanmar).
§Travel to areas within Myanmar (>1 km), excluding border towns.

associated with *P. vivax* malaria (adjusted OR 1.9, 95% CI 0.8%–4.9%) (Table 2).

Conclusions

Most previous studies of malaria in China have analyzed case reports collected and reported by counties as a part of their routine health reporting system (*3–5*,*7–10*). Such information is prone to reporting bias and to underreporting (*11*,*12*). Furthermore, most publications implicating cross-border activity as a risk for malaria have not adequately delineated how migration and travel data were collected or how these variables were defined (*2–4*,*8*,*10*).

Our study has 2 major strengths: data were collected prospectively and the association with travel to Myanmar was determined on the basis of travel histories within the 2 weeks before study participants sought care at a surveillance site hospital or health center. Our observation that 44% of the febrile case-patients were female, although female patients comprised only 9% of the malaria case-patients, supports the association between occupation and cross-border travel and risk for malaria.

Despite recent reductions in the number of malaria cases in the border counties, our findings suggest that *P. vivax* malaria persists in areas of Yunnan Province along the China–Myanmar border, whereas cases of *P. falciparum* malaria are probably imported from Myanmar (*8*,*13*). Cross-border trade, logging, quarry and plantation activities, and construction in Myanmar may reintroduce *P. falciparum* parasite to Yunnan Province. Whether the findings from this surveillance system, which focused on the China–Myanmar border areas, can be extrapolated to a larger geographic region needs further validation. Future elimination efforts should focus on the effects of cross-border activities on malaria parasite transmission, and elimination efforts should include more intensive surveillance so that prevention and control activities can be directed at hot-spot regions along the China–Myanmar border.

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Dr Zhou is an associate scientist in the Program of Public Health at the University of California, Irvine, Irvine, California, USA. His research focuses on ecological epidemiology of infectious disease and vector ecology.

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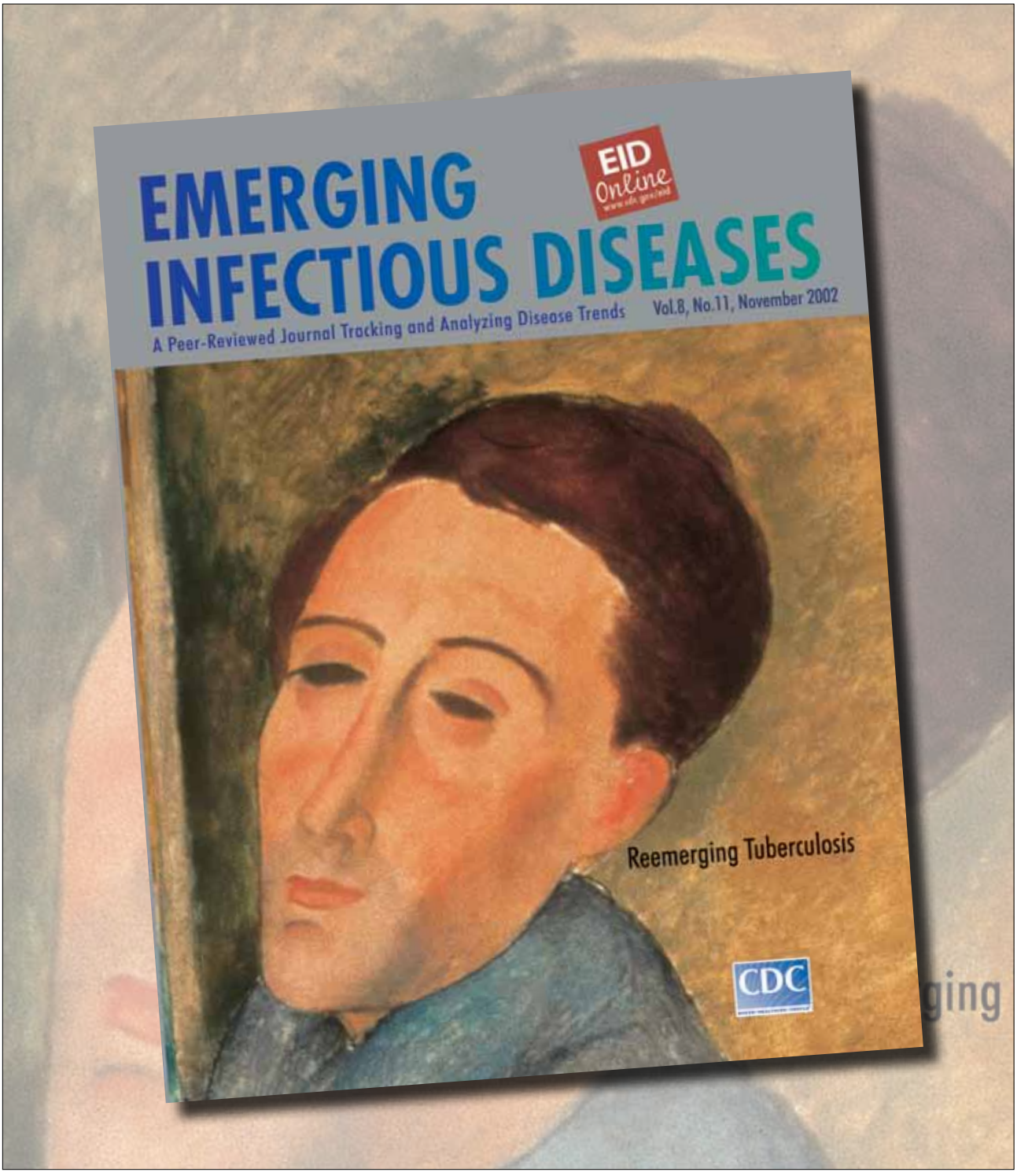
Address for correspondence: Guiyun Yan, Program in Public Health, University of California, Irvine, Irvine, CA 92697, USA; email: guiyuny@uci.edu, luc2@psu.edu, or zhaoqingy92@hotmail.com



Spread of Virulent Group A *Streptococcus* Type *emm59* from Montana to Wyoming, USA

Christopher C. Brown,[1] Randall J. Olsen, Nahuel Fittipaldi, Monica L. Morman, Peter L. Fort, Robert Neuwirth, Mohammed Majeed, William B. Woodward, and James M. Musser

Full-genome sequencing showed that a recently emerged and hypervirulent clone of group A *Streptococcus* type *emm59* active in Canada and parts of the United States has now caused severe invasive infections in rural northeastern Wyoming. Phylogenetic analysis of genome data indicated that the strain was likely introduced from Montana.

Strains of group A *Streptococcus* (GAS) type *emm59* historically have not been commonly associated with invasive infections. However, a striking increase in the frequency and severity of invasive infections caused by type *emm59* strains recently occurred in Canada (*1*).

Four of the authors (M.L.M., P.L.F., R.N., and M.M.) cared for 4 patients whose cases were temporally clustered and who had severe invasive infections caused by GAS in northeastern Wyoming, USA. Of the 4 case-patients, 2 were directly linked. Case-patient 4 contracted invasive infection from a close-contact family member (case-patient 2). Case-patient 3 was a physician who had cared for case-patients 1 and 2. These infections occurred in an area close to where a cluster of 6 invasive *emm59* infections occurred in Montana in 2010 (*2*). This fact led us to determine whether genetically related *emm59* strains were responsible. Full-genome sequencing confirmed this hypothesis (Figure).

Author affiliations: Campbell County Memorial Hospital, Gillette, Wyoming, USA (C.C. Brown, M.L. Morman, P. Fort, R. Neuwirth, M. Majeed, W.B. Woodward); Houston Methodist Research Institute, Houston, Texas, USA (R.J. Olsen, J.M. Musser); Houston Methodist Hospital System, Houston (R.J. Olsen, J.M. Musser); Public Health Ontario, Toronto, Ontario, Canada (N. Fittipaldi); and University of Toronto, Toronto (N. Fittipaldi)

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The Cases

Case-patient 1

A 19-year-old otherwise healthy man had generalized body aches, mild confusion, and cough and was treated symptomatically as an outpatient. The next day, he was found unconscious at his home. In the emergency department, he had hypoxemia, metabolic acidosis, renal failure, leukopenia with 34% bands, and severe unilateral pneumonia. Coagulopathy and sepsis-induced cardiomyopathy with global hypokinesia and reduced ejection fraction developed. He was discharged after a complicated hospital stay. GAS isolated from his throat and blood were discarded but a convalescent-phase serum sample was strongly reactive in a GAS-specific ELISA.

Case-patient 2

On day 13 after disease onset in case-patient 1, a 78-year-old man with multiple medical problems was hospitalized with cellulitis of the left upper arm. An intravenous line had been inserted into the arm during a cardiac-related hospital admission 4 days earlier. Blood cultures grew GAS. Intravenous vancomycin was administered, and he was discharged and followed up as an outpatient. The following morning, he was found obtunded at home. He had hypoxemia, hypotension, new acute renal failure, and new full-thickness skin necrosis with bullae, mottling, and ecchymoses of the affected arm. He was hospitalized, given broad-spectrum antimicrobial drugs, and provided fluid and pressor support. Imaging studies showed evidence of necrotizing fasciitis. The affected arm was amputated and necrotizing fasciitis was confirmed. He died within 48 hours after hospitalization.

Case-patient 3

Nineteen days after case-patient 1 was treated and 2 days after case-patient 2 died, generalized chills, profound fatigue, and fever of 102°F developed in a 46-year-old man (physician) who cared for case-patients 1 and 2. Within 24 hours, he had tender cervical lymphadenopathy. At hospitalization, he had normal vital signs tender, left-sided lymphadenopathy, and a leukocyte count of 12,000 cells/mL with 26% bands. He was given broad-spectrum, intravenous, antimicrobial drugs. Imaging studies showed major cervical lymphadenopathy. Blood cultures were negative for bacteria, and a throat culture was not obtained. His recovery was slow and protracted. Convalescent-phase serum analyzed by ELISA was positive for GAS-specific antibodies.

[1]Current affiliation: Memorial Hospital of Sweetwater County, Rock Springs, Wyoming, USA.

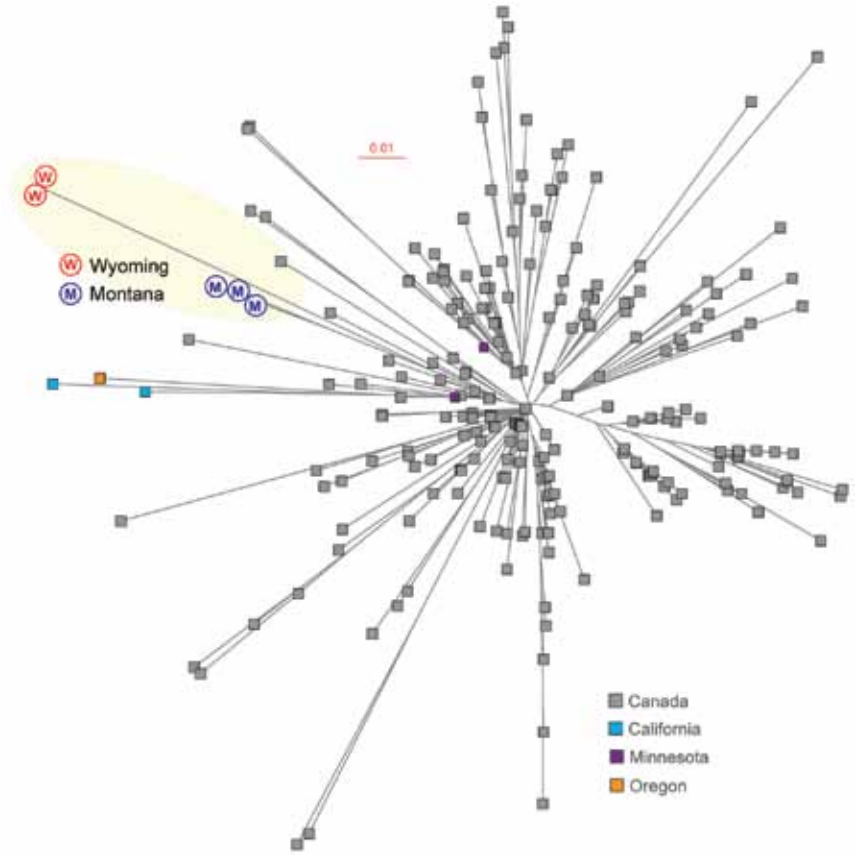

Figure. Inferred genetic relationships among group A *Streptococcus emm59* strains on the basis of 773 concatenated single nucleotide polymorphisms identified by genome sequencing. Strains from Montana (M) and Wyoming (W), USA, are shown in blue and red, respectively. Strains from Canada, and from California, Minnesota, and Oregon, USA, are shown for reference. Scale bar indicates nucleotide substitutions per site.

Case-patient 4

Twenty-five days after the onset of illness in case-patient 1, an inflamed papule developed on the right ring finger of a 46-year-old woman who lived with case-patient 2. The papule showed improvement after treatment with oral antimicrobial drugs. Five days later, malaise, runny nose, anorexia, exertional dyspnea, and pain in the proximal region of the left lower leg developed without associated physical findings. Her symptoms worsened over several days. A 10-cm area of ecchymosis on the thigh and major pain on palpation throughout the leg developed that was out of proportion to visual findings. In the emergency department, she had tachypnea, tachycardia, hypotension, and a normal body temperature. Laboratory studies showed acute renal failure and a leukocyte count of 20,000 cells/mL with neutrophilia but no bands. The patient was transferred to a tertiary care facility because of a presumptive diagnosis of necrotizing fasciitis. She had a complicated hospital course that included amputation of the lower left leg and 2 subsequent surgical extensions. Results of histopathologic analysis of the amputated leg were consistent with those for necrotizing fasciitis. The patient survived.

Strains from case-patients 2 and 4 were sent to Houston Methodist Hospital for genome sequencing to a 65-fold depth of coverage by using a MiSeq Personal Sequencer Instrument (Illumina, San Diego, CA, USA). The 2 *emm59* case strains differed from each by only a 1-nt deletion. Genome data for the 2 sequenced strains have been deposited in the short-read archive of the National Center for Biotechnology Information (Bethesda, MD, USA) under accession nos. SAMN01991041 and SAMN01991042.

Comparison of the genomes of the strains from Wyoming with hundreds of *emm59* genomes (*2*–*4*), determined that the case organisms were most closely related to strains that caused a cluster of 6 invasive infections in rural south central Montana in 2010 (*2*). The *emm59* strains from Wyoming and Montana differed from each another by only 15 single-nucleotide polymorphisms, including 1 in the *covS* gene, which encodes the sensor kinase partner of a key 2-component signal-transduction system (*5*). Structural changes or inactivation of *covR* or *covS* genes can result in up-regulation of up to ≈15% of the GAS transcriptome and increased virulence (*6*–*9*).

Because these cases in Wyoming were temporally and geographically clustered and were caused by closely related genetic variants of the unusually virulent *emm59* clone, an epidemiologic survey was conducted in an effort to identify a reservoir of the case clone. For the first part of the study, 579 cultures were obtained from in-hospital and out-of-hospital contacts, staff, environmental surfaces, and

community throat and wound infections. These cultures were obtained from 168 hospital and clinic employees, 343 hospitalized and ambulatory patients and community contacts of the GAS-infected patients, and 68 hospital and community environmental surfaces. These cultures yielded 39 GAS strains, none of which were *emm59*.

The second part of the study included a convenience sample of 17 GAS strains obtained from patients with pharyngitis or skin infections in the geographic area in and around the community where the invasive infections occurred. None of these 17 strains were *emm59*. Many of the *emm* types commonly causing infections in the United States (*10*) (www.cdc.gov/abcs/pathogens/pathogen-links.html) were identified in this sample of 56 GAS strains, including *emm1*, *emm2*, *emm3*, *emm4*, *emm12*, and *emm28*. This result is consistent with the striking lack of *emm59* strains among strains isolated from patients who had pharyngitis during a large survey in Canada (*11*).

Conclusions

One of the authors (J.M.M.) suggested that the recent and remarkable change in the epidemiologic and virulence behavior of *emm59* GAS strains in Canada and the United States warranted increased attention by public health authorities and infectious diseases practioners (*2*). We document that an unusually virulent *emm59* clone has now emerged to cause severe infections and 1 death in rural northeastern Wyoming. The *emm59* strains have recently emerged as the dominant cause of invasive GAS cases in Minnesota (www.cdc.gov/abcs/reports-findings/survreports/gas12.html), which further illustrates the ability of this clone to successfully spread to other geographic areas and cause abundant infections.

One limitation of our study is that we had isolates available from only 2 of the 4 case-patients. However, extensive review of hospital records identified only 1 case of GAS necrotizing fasciitis in the past 5 years, which underscores the otherwise rarity of these episodes in northeastern Wyoming. Thus, we believe we can reasonably hypothesize that all 4 case-patients were infected with clonally related *emm59* GAS strains.

Given the speed and modest cost of full-genome sequencing and its role in human health, we remain interested in studying the spread of this clone. Persons responding to clinical situations that might warrant strain genome sequencing should contact the corresponding author.

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Dr Brown is director of the hospitalist program and an infectious disease consultant at Memorial Hospital of Sweetwater County, Rock Springs, Wyoming. His research interests are the pathophysiology of sepsis syndromes.

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Address for correspondence: James M. Musser, Department of Pathology and Genomic Medicine, Houston Methodist Hospital System, 6565 Fannin St, B490, Houston, TX 77030, USA; email: jmmusser@houstonmethodist.org

Burkholderia pseudomallei Type G in Western Hemisphere

Jay E. Gee, Christopher J. Allender, Apichai Tuanyok, Mindy G. Elrod, and Alex R. Hoffmaster

Burkholderia pseudomallei isolates from the Western Hemisphere are difficult to differentiate from those from regions in which melioidosis is traditionally endemic. We used internal transcribed spacer typing to determine that *B. pseudomallei* isolates from the Western Hemisphere are consistently type G. Knowledge of this relationship might be useful for epidemiologic investigations.

Burkholderia pseudomallei is the causative agent of the disease melioidosis. Melioidosis is considered endemic to Southeast Asia and northern Australia. However, sporadic cases do occur elsewhere in the world, especially in tropical areas (*1*).

The predominant method of molecular subtyping of *B. pseudomallei* is multilocus sequence typing (MLST), which is based on a comparison of the alleles of 7 housekeeping genes to generate a sequence type (ST) (*2*). These data can then be analyzed by using tools such as eBURST, which is used to infer phylogenetic patterns (*3*).

As of May 30, 2013, a total of 3,028 *B. pseudomallei* isolates were listed in the MLST database (www.mlst.net); these isolates are predominantly from Southeast Asia (1,036 isolates) and Australia (1,776). Some entries are from other Pacific areas (e.g., New Caledonia [9 isolates] and Hong Kong [39]) and other parts of the world (e.g., Africa [8], Europe [15], and the Western Hemisphere [30]) or of unknown origin (32).

For a population study, Pearson et al. analyzed the STs in the *B. pseudomallei* MLST database along with other data, such as single-nucleotide polymorphisms from whole-genome sequencing data (*4*). Their study indicated some population structures associated with geographic origin, in particular, clades associated with isolates from Southeast Asia or northern Australia. The data were used to support a hypothesis that *B. pseudomallei* originated on the Australian continent, spread to Southeast Asia, and from there spread throughout the world (*4*).

For an isolate of unknown origin, however, MLST alone may not provide information about geographic origin. For example, isolates from the Western Hemisphere have yielded STs unique to that hemisphere, but when these isolates were analyzed by eBURST or other methods, no distinct clade was found. In the clusters in which these STs appear, STs are intermixed with those from regions in which melioidosis is traditionally endemic, such as Southeast Asia (*2*,*4*). Therefore, a method for determining whether an isolate of unknown origin is from the Western Hemisphere is desirable.

Recently, Ligouri et al. developed a typing scheme by measuring the length polymorphisms in the 16S–23S internal transcribed spacer (ITS) of *Burkholderia* spp. (*5*). The typing scheme consists of 10 types: A, B, C, D, E, F, G, CE, GE, and GC. Ligouri et al. found that some types are unique to a given *Burkholderia* species (i.e., A = *B. thailandensis*, B = *B. humptydooensis*, D = *B. oklahomensis*, and *F = B. cepacia*). They also determined that type C could be found in *B. mallei* and in *B. pseudomallei*. The remaining 5 types were exclusive to *B. pseudomallei* (*5*). Ligouri et al. determined that types C, E, GE, and CE were the predominant types for isolates from northern Australia and Southeast Asia and that type G was rare in Australia (4 isolates) and Southeast Asia (3). They noted, on the basis of a limited number of strains from these regions, that type G was overrepresented in isolates from other parts of the world: Madagascar (1 isolate), Ecuador (2), Puerto Rico (2), Venezuela (1), and Kenya (1). They hypothesized that a genetic bottleneck occurred during the dispersal of type G to regions outside of Southeast Asia and Australia (*5*).

ITS typing of *B. pseudomallei* might be a powerful tool for linking cases of melioidosis to regions outside of those in which melioidosis in highly endemic, such as Southeast Asia and northern Australia. To further investigate this trend, we assessed the ITS types of *B. pseudomallei* from the Western Hemisphere.

The Study

All tested Western Hemisphere isolates from our collection were ITS type G (Table). In addition to those isolates tested, we performed in silico analysis of whole-genome sequencing data from other *B. pseudomallei* isolates in our collection or publicly available data with origins in the Western Hemisphere and found them to also be ITS type G (Table). As expected, eBURST analysis of the STs from the type G strains that originated in the Western Hemisphere did not yield a discrete clade. These STs are interspersed with other STs that are predominantly from Southeast Asia (Figure). Our results support the findings of

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (J.E. Gee, M.G. Elrod, A.R. Hoffmaster); and Northern Arizona University, Flagstaff, Arizona, USA (C.J. Allender, A. Tuanyok)

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Table. Analysis results for *Burkholderia pseudomallei* isolates from Western Hemisphere*

Identification no.	ITS type	YLF gene	MLST	Source
Swiss2010	G	+	92	Switzerland ex Martinique 2010
PR1998	G†	+†	92	Puerto Rico 1998 (GenBank accession no. pending)
4900CF	G†	+†	92	Brazil: cystic fibrosis patient 2007 (GenBank accession no. ARZE000000
CA2009	G	+	95	USA (California) ex Mexico 2009
FL2009	G	+	297	USA (Florida) ex Puerto Rico 2009
FL2012	G	+	297	USA (Florida) ex Trinidad 2012
PR2012	G	+	297	Puerto Rico 2012
MX2013	G	ND	297	Mexico 2013
PB08298010	G†	+	426	USA (Arizona) locally acquired 2008 (GenBank accession no. ARZO00000
CA2012d	G	+	436	USA (California) ex Guatemala 2012
2002734728	G	+	518	USA (California) Iguana 2007
PB 1007001	G	+	518	USA (Arizona) ex Costa Rica 2010
CA2013a	G	ND	518	USA (California) Iguana 2013
NY2010	G	+	698	USA (New York) ex Aruba 2010
724644	G	+	698	USA (Massachusetts) ex Aruba 2010
BCC215	G†	+†	ND‡	Brazil (Ceara) 2003 (GenBank accession no. ABBR00000000)

*ITS, internal transcribed spacer; YLF, *Yersinia*-like fimbrial; MLST, multilocus sequence type; ex, diagnosis made in first location but infection acquired in s location; ND, not determined.
†Determined by analysis of whole-genome sequence.
‡Allele for gltB not available.

Ligouri et al. (*5*). The predominance of type G in isolates in our panel of isolates from the Western Hemisphere is consistent with a hypothesis that these isolates are derived from a bottleneck that occurred during dispersal to the rest of the world from Southeast Asia.

Other supporting evidence is provided by testing for the *Yersinia*-like fimbrial (YLF) gene by YLF PCR. The presence of the YLF gene is associated with *B. pseudomallei* isolates from Southeast Asia (*6*). For those strains tested, all were positive for YLF or the YLF gene was present

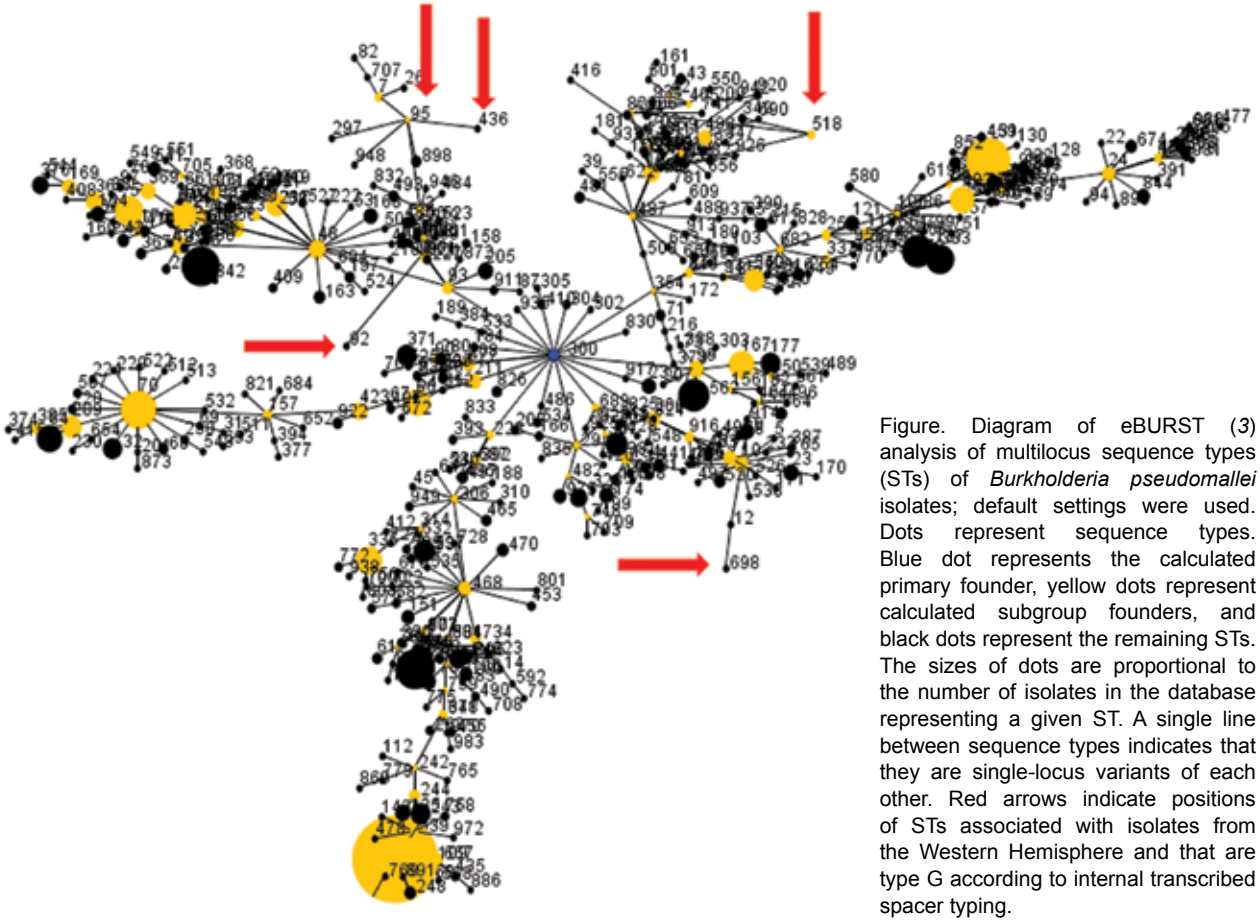

Figure. Diagram of eBURST (*3*) analysis of multilocus sequence types (STs) of *Burkholderia pseudomallei* isolates; default settings were used. Dots represent sequence types. Blue dot represents the calculated primary founder, yellow dots represent calculated subgroup founders, and black dots represent the remaining STs. The sizes of dots are proportional to the number of isolates in the database representing a given ST. A single line between sequence types indicates that they are single-locus variants of each other. Red arrows indicate positions of STs associated with isolates from the Western Hemisphere and that are type G according to internal transcribed spacer typing.

according to in silico analysis of whole-genome sequencing data when available (Table).

Conclusions

In melioidosis cases for which the origin is unclear, such as for patients with unknown exposure histories or histories of travel to multiple regions to which melioidosis is endemic, ITS typing along with other molecular epidemiologic tools might be useful for assessing the *B. pseudomallei* origins. More insight into the relationship of Western Hemisphere isolates to isolates from regions to which melioidosis is highly endemic might come from whole-genome sequencing. As more isolates are analyzed, such studies might enable higher confidence in the geographic origin of isolates.

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Dr Gee is a research biologist in the Bacterial Special Pathogens Branch, Division of High-Consequence Pathogens and Pathology, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention. His research interests focus on the molecular epidemiology of *B. pseudomallei* and *B. mallei* and on other emerging bacterial pathogens.

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Address for correspondence: Jay E. Gee, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop G34, Atlanta, GA 30333, USA; email: xzg4@cdc.gov

Vibrio parahaemolyticus, Southern Coastal Region of China, 2007–2012

Yinghui Li, Xu Xie, Xiaolu Shi, Yiman Lin, Yaqun Qiu, Jin Mou, Qiongcheng Chen, Yan Lu, Li Zhou, Min Jiang, Honghu Sun, Hanwu Ma, Jinquan Cheng, and Qinghua Hu

We analyzed the prevalence and characteristics of *Vibrio parahaemolyticus* among patients with acute infectious diarrhea in the southern coastal region of China. *V. parahaemolyticus* was the leading cause of bacterial infectious diarrhea in this region during 2007–2012. Serotype O3:K6 strains were most common, followed by serotypes O4:K8 and O3:K29.

Vibrio parahaemolyticus, a halophilic bacterium, is recognized as a major cause of acute gastroenteritis worldwide, often associated with the consumption of raw or undercooked shellfish. *V. parahaemolyticus* infections are caused by diverse serotypes; however, serotype O3:K6 has been reported to be dominant and has been a widespread serotype since 1997 (*1*).

V. parahaemolyticus has been the leading cause of foodborne outbreaks and bacterial infectious diarrhea in China since the 1990s, especially in coastal regions (*2*,*3*). Serotype O3:K6 was documented as the dominant serotype in Zhejiang Province, China, in 2002 and was proven to be a pandemic clone in 2008 (*4*). However, long-term fluctuation in the frequency of infections with the pandemic strains of *V. parahaemolyticus* remains unknown.

In 2007, laboratory-based surveillance for acute infectious diarrhea at 11 sentinel hospitals was established in Shenzhen City in the southern coastal region of China with *V. parahaemolyticus* as one of the target pathogens. To characterize *V. parahaemolyticus* infections and clarify its prevalence in this region, we analyzed all *V. parahaemolyticus* cases captured by this surveillance during 2007–2012.

Author affiliations: Shenzhen Major Infectious Disease Control Key Laboratory, Shenzhen Center for Disease Control and Prevention, Shenzhen, China (Y. Li, X. Xie, X. Shi, Y. Lin, Y. Qiu, J. Mou, Q. Chen, Y. Lu, L. Zhou, M. Jiang, H. Ma, J. Cheng, Q. Hu); Sichuan University, Chengdu, China (H. Sun); and Shenzhen University, Shenzhen (Q. Hu)

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The Study

Surveillance was conducted among outpatients who had ≥3 loose or liquid stools during a 24-hour period but lasting ≤14 days. A total of 1,488 *V. parahaemolyticus* infections were identified from 24,696 enrolled outpatients (6.0% of outpatients). More than half of the patients (835; 56.1%) were male. Patients ranged in age from 4 months to 84 years (median 27 years); 1,383 (92.9%) patients were 15–39 years of age. Most (914; 61.4%) patients were part of the large transient population living in Shenzhen. Of all patients with *V. parahemoliticus* infection, 1,150 (77.3%) had watery diarrhea, 1,176 (79.0%) had abdominal pain, 730 (49.1%) had vomiting, 206 (13.8%) had fever, and 4 (0.3%) had blood in stools.

Obvious monthly peaks of *V. parahaemolyticus* infections were found during the warmer months (June–October). Up to 30% of diarrhea cases covered by the surveillance could be attributed to *V. paraheamolyticus* infections during this period.

All 1,488 *V. parahaemolyticus*isolates were serotyped by slide agglutination by using a commercial serum (Denka-Seiken Ltd., Tokyo, Japan); 47 serotypes were detected. The O3:K6 serotype was dominant throughout the surveillance years (996 isolates; 66.9%), followed by O4:K8 (156 isolates; 10.5%) and O3:K29 (51 isolates; 3.4%). However, O3:K29 appeared more frequently during 2009 and 2010. Four other serotypes occurred as clusters in different years: O1:KUT in 2008, O1:K56 in 2010, O4:K68 in 2010, and O5:K68 in 2009 (Table 1).

Representative strains were selected for PCR of the virulence genes thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) (*5*,*6*). A total of 833 isolates covering all 47 serotypes (1–560 isolates for each serotype) were screened. Most (788; 94.6%) strains were tdh^+trh^-; 28 isolates representing 5 serotypes were tdh^+trh^+ (O1:KUT, n = 18; O4:K12, n = 3; O1:K69, n = 4; O10:K52, n = 2; O4:K55, n = 1), and 17 isolates of 10 serotypes were tdh^-trh^- (O1:KUT, n = 5; O3:K6, n = 4; O1:K1, O1:KUT, O3:K1, O3:KUT, O4:K63, O10:KUT, OUT:K19, and OUT:K55, n = 1 each).

A total of 196 tdh^+trh^- isolates, representing the leading 10 serotypes (6–75 isolates for each serotype), were selected for group-specific PCR (GS-PCR) of *toxRS*/new sequence (*7*). Results demonstrated that pandemic genotype strains (110; 56.12%) prevailed among the leading 10 serotypes in Shenzhen. Most (68; 90.7%) O3:K6 isolates; all O1:K36, O4:K68, O5:K68, and O1:K25 isolates; and 6 (28.6%) O1:KUT isolates gave positive results by GS-PCR, whereas results were negative for the other 4 serotypes.

We analyzed 127 isolates by using the *V. parahaemolyticus*multilocus sequence typing (MLST) scheme (http://pubmlst.org/vparahaemolyticus) (Figure). Clonal complex

Table 1. Serotype composition of 1,488 *Vibrio parahaemolyticus* isolates from patients with acute diarrhea, southern coastal region of China, 2007–2012

Serotype	No. isolates						
	2007	2008	2009	2010	2011	2012	Total
O3:K6	153	94	180	210	198	161	996
O4:K8	12	10	49	42	25	18	156
O3:K29	2	1	24	18	5	1	51
O1:KUT	1	20	4	4	10	8	47
O1:K56	0	0	0	28	0	1	29
O1:K36	5	4	3	9	2	1	24
O4:K9	1	5	6	1	3	7	23
O4:K68	2	4	2	10	2	0	20
O5:K68	2	0	10	3	1	0	16
O1:K25	5	1	3	3	2	1	15
O11:K36	0	0	5	7	1	1	14
O2:K3	0	3	3	3	0	4	13
O3:KUT	0	4	3	1	0	1	9
O4:K11	2	0	2	1	0	1	6
O4:K55	0	1	0	2	2	1	6
O1:K41	1	0	1	0	1	2	5
O4:K13	0	1	4	0	0	0	5
O4:KUT	0	1	1	0	0	3	5
O8:K41	1	1	0	0	0	3	5
OUT:KUT	1	1	1	0	0	1	4
O6:K18	0	1	1	0	0	1	3
O8:K21	0	0	2	0	0	1	3
OUT:K68	3	0	0	0	0	0	3
O1:K1	1	1	0	0	0	0	2
O1:K68	0	0	0	2	0	0	2
O1:K69	0	0	0	0	2	0	2
O10:K52	0	0	0	0	0	2	2
O3:K59	0	0	0	1	0	1	2
O4:K12	0	0	0	0	0	2	2
O1:K5	0	0	0	0	0	1	1
O10:KUT	0	0	0	0	1	0	1
O11:K19	1	0	0	0	0	0	1
O11:KUT	0	0	0	0	1	0	1
O2:KUT	0	0	0	1	0	0	1
O3:K1	0	0	1	0	0	0	1
O3:K2	0	0	0	1	0	0	1
O3:K28	0	0	0	1	0	0	1
O3:K41	0	0	0	1	0	0	1
O3:K45	0	1	0	0	0	0	1
O3:K54	1	0	0	0	0	0	1
O3:K56	0	0	1	0	0	0	1
O3:K7	0	0	0	0	0	1	1
O4:K63	0	0	0	1	0	0	1
O8:KUT	0	0	0	0	0	1	1
OUT:K19	1	0	0	0	0	0	1
OUT:K44	0	1	0	0	0	0	1
OUT:K55	0	0	0	0	1	0	1

3 (CC3) predominated (93 isolates; 73.2%). A new clonal complex, CC120, and sequence type (ST), 265, a presumed new ancestor of CC345, were identified (Table 2).

Conclusions

During 2007–2012, *V. parahaemolyticus* was the dominant bacterial cause of acute diarrhea in the southern coastal region of China, surpassing *Salmonella* spp., diarrheagenic *Escherichia coli*, and *Shigella* spp. (data not shown). These findings differ from those for central and northern regions of China (*8*,*9*). Most case-patients in this surveillance were 15–39 years of age. The distribution of the 47 serotypes detected revealed the diversity of *V. parahaemolyticus*, which might explain the continuing epidemic of *V. parahaemolyticus* infections in this region.

V. parahaemolyticus serotype O3:K6, which emerged worldwide in 1997 as a pandemic clone and spread throughout Asia and to the Americas, Europe, and Africa (*1*), has been dominant in Shenzhen Province. Most (68; 90.7%) O3:K6 isolates tested were new clones, defined by *toxRS*-targeted GS-PCR, providing evidence that pandemic O3:K6 has spread to China. Most (63; 98.5%) serotype O3:K6 strains were identified as CC3 by MLST; serotype O1:KUT, O1:K36, O4:K68, O5:K68, and O1:K25 strains were positive by GS-PCR in our study and mostly

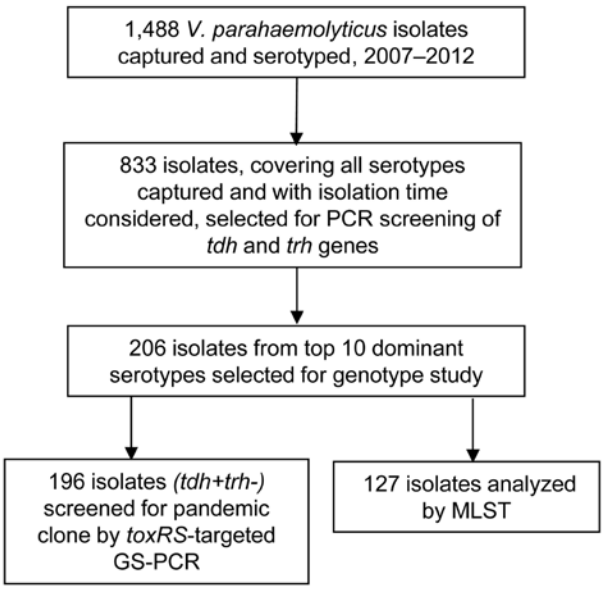

Figure. Study design and eligibility for serotype and genotype analysis of Vibrio parahaemolyticus isolates from patients with acute diarrhea, southern coastal region of China, 2007–2012. GS-PCR, group-specific PCR; MLST, multilocus sequence typing.

documented as O3:K6 serovariants (*1*). These results indicate the long-term evolution of O3:K6 in Asia.

V. parahaemolyticus serotype O4:K8 has been an epidemic strain in Asia (*10*,*11*) and reported in Peru (*12*) but has been rarely seen in North America, Africa, and Europe. Serotype O4:K8 isolates from this study expressed a nonpandemic genotype. We presume that the evolution of O4:K8 was affected by local mutation and recombination rather than by a global pandemic, similar to a finding reported in Japan in 2007 (*13*). Notably, ST265 was predominant among strains with serotype O4:K8, whereas ST345, the previously considered founder of CC345, was not found (Table 2). Although ST265 might be a potential branch from ST345 in other regions, our findings strongly suggest that ST265 should be considered the epidemic clonal founder of CC345 in China. Overall, serotypes O3:K6 and O4:K8, stable subpopulations of the diverse *V. parahaemolyticus* population in our surveillance, have clearly been epidemic in China.

V. parahaemolyticus serotypes O3:K29 and O1:K56 were mainly reported in Asia, with nonpandemic groups identified in Japan (*10*,*14*). Our study showed that prevalence of serotype O3:K29 *V. parahaemolyticus* suddenly fluctuated during 2009 and 2010 and prevalence of O1:K56 fluctuated in 2010, but no focal outbreaks were confirmed; this finding indicates that sporadic outbreaks might have occurred. In addition, serotype O3:K29 isolates were identified as ST120 and the newly determined ST480, both belonging to CC120. Limited information could be obtained from the MLST database about the O1:K56 strains, and the isolates we tested were classified as ST8.

Further, our study found *V. parahaemolyticus* serotype O1:KUT might contain ≥1 character K antigens; however, 18 isolates harbored both *tdh* and *trh* genes, a combination that is not found frequently. Therefore, an O1:KUT epidemic clone might be prevalent in this region.

Whereas *V. parahaemolyticus* often is associated with the consumption of raw or undercooked shellfish, data from this surveillance program showed that most patients were transient residents who lived in rural areas and seldom ate seafood. However, epidemiologic data showed that *V. parahaemolyticus* infection was associated with eating outdoors and consumption of salad vegetables. Cross-contamination in food processing might be the source of infection; further epidemiologic investigation is under way.

In summary, *V. parahaemolyticus* has been prevalent for a long time in the southern coastal region of China, and diverse serotypes and multiple clones of the bacterium are circulating. On the basis of successful efforts to reduce prevalence of *V. parahaemolyticus* infections in Japan (*15*),

Table 2. Results of GS-PCR and MLST analysis of the 10 mostly commonly found serotypes of *Vibrio parahaemolyticus* isolates from patients with acute diarrhea, southern coastal region of China, 2007–2012*

	GS-PCR, n = 196		MLST, n = 127		
Serotype	No. positive, n = 110	No. negative, n = 86	No. isolates tested	ST (no. isolates)	CC
O3:K6	68	7	64	ST3 (60), ST487 (1), ST489 (1), ST526 (1)	CC3
				ST497 (1)	Singleton
O4:K8	0	30	16	ST265 (14), ST189 (1), ST438 (1)	CC345
O3:K29	0	14	13	ST120 (11), ST480 (2)	CC120
O1:KUT	6	15	3	ST3 (3)	CC3
O1:K56	0	10	8	ST8 (8)	CC8
O1:K36	9	0	7	ST3 (7)	CC3
O4:K9	0	10	3	ST332 (3)	Singleton
O4:K68	10	0	5	ST3 (5)	CC3
O5:K68	6	0	3	ST3 (3)	CC3
O1:K25	11	0	5	ST3 (4)	CC3
				ST481 (1)	Singleton

*GS-PCR, group-specific PCR; MLST, multilocus sequence typing; ST, sequence type; CC, clonal complex.

we suggest holistic approaches involving regulations and guidance on fishery products and food hygiene to decrease the incidence of these infections in China.

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Ms Li is a senior researcher in the Shenzhen Major Infectious Disease Control Key Laboratory, Shenzhen Center for Disease Control and Prevention. Her research interests include laboratory-based surveillance of foodborne pathogens and antimicrobial drug–resistant bacteria, particularly *V. parahaemolyticus* and diarrheagenic *E. coli*.

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Address for correspondence: Qinghua Hu, Shenzhen Major Infectious Disease Control Key Laboratory, Shenzhen Center for Disease Control and Prevention, 8 Longyuan Rd, Nanshan District, Shenzhen, Guangdong Province, 518055, People's Republic of China; email: huqinghua03@163.com or cjinquan@sohu.com

Diagnostic Methods for and Clinical Pictures of Polyomavirus Primary Infections in Children, Finland

Tingting Chen, Laura Tanner, Ville Simell, Lea Hedman, Marjaana Mäkinen, Mohammadreza Sadeghi, Riitta Veijola, Heikki Hyöty, Jorma Ilonen, Mikael Knip, Jorma Toppari, Olli Simell, Maria Söderlund-Venermo, and Klaus Hedman

We used comprehensive serodiagnostic methods (IgM, IgG, and IgG avidity) and PCR to study Merkel cell polyomavirus and trichodysplasia spinulosa-associated polyomavirus infections in children observed from infancy to adolescence. Comparing seroconversion intervals with previous and subsequent intervals, we found that primary infections with these 2 viruses were asymptomatic in childhood.

Two novel human polyomaviruses can cause skin diseases that are predominant in immunosuppressed persons. Merkel cell polyomavirus (MCPyV) is associated with Merkel cell carcinoma, which is an uncommon aggressive skin cancer, and trichodysplasia spinulosa-associated polyomavirus (TSPyV) is associated with trichodysplasia spinulosa, which is a rare skin disorder (*1*–*3*). In contrast with the rarity of the diseases, serologic studies have shown that both of these polyomaviruses infect humans of all ages; their seroprevalence has been assessed at 60%–80% among adults (*4*–*6*). We have reported high rates of MCPyV and TSPyV seroconversion among young children, indicating that primary exposures to these viruses occur extensively in early life (*5*,*7*). We observed children from infancy to 13 years of age by using comprehensive diagnostic methods for MCPyV and TSPyV and investigated pediatric primary infections with these 2 viruses for clinical correlates.

Author affiliations: University of Helsinki, Helsinki, Finland (T. Chen, L. Hedman, M. Sadeghi, M. Knip, M. Söderlund-Venermo, K. Hedman); Haartman Institute, Helsinki (T. Chen, L. Hedman, M. Sadeghi, M. Söderlund-Venermo, K. Hedman); University of Turku, Turku, Finland (L. Tanner, V. Simell, M. Mäkinen, J. Ilonen, J.Toppari, O. Simell); Hospital District of Southwest Finland, Turku (V. Simell, M. Mäkinen); Helsinki University Central Hospital, Helsinki (L. Hedman, M. Knip, K. Hedman); University of Oulu, Oulu, Finland (R. Veijola); University of Tampere, Tampere, Finland (H. Hyöty); Fimlab laboratories, Tampere (H. Hyöty); University of Eastern Finland, Kuopio, Finland (J. Ilonen); Tampere University Hospital, Tampere (M. Knip); and Folkhälsan Research Center, Helsinki (M. Knip)

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The Study

This retrospective study was conducted during January 2011–July 2013 on a subset of a prospective study in which children were enrolled at birth and observed until young adolescence (*8*). We observed 144 children born during 1995–2004, from whom final samples were obtained during 2004–2008.

On average, 13 serum samples per child were obtained during the study period (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/20/4/13-1015-Techapp1.pdf). At each follow-up visit, clinical symptoms or illnesses since the previous visit were recorded (*8*). The ethics committee of the Hospital District of Southwest Finland (www.vsshp.fi/en/) approved the study.

IgG enzyme immunoassays (EIA) for MCPyV and TSPyV were conducted as described, except that we omitted subtraction of antigen-free background in the MCPyV assay (*5*,*7*). The respective lower and higher EIA cutoff values for IgG absence and presence were 0.120 and 0.210 for MCPyV and 0.100 and 0.240 for TSPyV (*5*,*7*). IgM EIAs for these 2 human polyomaviruses were developed as for human bocavirus 1(HBoV1) (*8*). The cutoff values were 0.207 and 0.260 for MCPyV and 0.194 and 0.240 for TSPyV. The IgG avidity assays were conducted as for HBoV1 (method A [*9*]). The respective cutoff values for low and high avidity were 15% and 25%.

Serum samples obtained during the final examination of each child were screened for MCPyV IgG and TSPyV IgG. Previous samples had not been tested. The children whose final samples lacked virus IgG were considered to be IgG negative; those whose final samples showed virus IgG were considered to have seroconverted. Each previous serum sample for each child who seroconverted was analyzed for IgG and IgM to identify the time period of seroconversion. Serum samples collected immediately before, at, and after the IgG seroconversion were examined for viral DNA; the seroconversion sample, the subsequent sample, and the final sample were examined for IgG avidity.

Of the 144 children, 45 (31%) showed IgG seroconversion for MCPyV and 39 (27%) for TSPyV. Before they were 1 year of age, 4 children showed IgG seroconversion for MCPyV at 0.68–0.94 year of age, 1 child showed seroconversion for TSPyV at 0.80 year, and another child showed MCPyV IgG, IgM, and low avidity of IgG in the first sample, which was collected at 0.63 year (online Technical Appendix Table 2). None of these children who

seroconverted early in life showed maternal IgG to the corresponding virus. Comparing participants at 0–1 year of age with those at 9–13 years, the seroprevalence for MCPyV caused by acquired infections rose from 3.4% to 65% and for TSPyV, from 0.7% to 53%. Seroconversions for each virus continued throughout the study (Figure).

Of the 45 children who seroconverted for MCPyV, 28 (62%) showed additional markers of primary infection at the time of IgG seroconversion: IgM was present in 15 (33%), and low avidity of IgG was detected in 23 (51%). Of the 39 TSPyV–seroconverted children, 32 (82%) showed corresponding markers: IgM in 30 (67%) and low avidity of IgG in 13 (29%). Samples did not show MCPyV viremia at or flanking the seroconversion, and TSPyV viremia was observed at low quantity ($<10^4$ copies/mL) in the samples of 2 who seroconverted. Except 4 seroconverters for MCPyV and 1 for TSPyV, all showed long-term maturation of IgG avidity to the corresponding virus.

Maternal IgG showed in 10 (22%) of the 45 who seroconverted for MCPyV and in 12 (31%) of the 39 for TSPyV at sampling ages of 0.23–0.62 year; these maternal IgGs were no longer discernable at 0.49–1.07 year. After the first year of life, the age at seroconversion with either virus did not appear to correlate with the presence or absence of maternal antibodies.

To determine clinical correlates of MCPyV and TSPyV primary infections, all infection-related symptoms and illnesses during the seroconversion interval were compared with those during the previous or subsequent interval for each patient who seroconverted (Table). Infection-related symptoms during the seroconversion interval were reported for 73% of children who seroconverted for MCPyV and for 82% of those who seroconverted for TSPyV. The occurrences of symptoms, however, were not notably different from those during the previous or subsequent intervals. Exanthema was reported in 7 (15.9%) children at the MCPyV seroconversion interval and in 1 child in each of the adjacent periods (2.2% before, 2.3% after). However, the differences were not statistically significant (p = 0.0703).

Conclusions

The seroprevalence of MCPyV and TSPyV among children ≤13 years of age increased because of acquired infections, a finding consistent with reports that primary infections with these 2 viruses are ubiquitous in childhood (*5*,*7*). The seroprevalence of these viruses had not reached a plateau by the end of study; thus, some infections are expected to occur later than 13 years of age. We expect that some children have become infected with these viruses after exiting the study.

For both viruses, prevalence of maternal antibodies during infancy was high, supporting recent findings by Martel-Jantin et al. (*10*). Although the age of the child at seroconversion did not appear to correlate with the presence of maternal antibodies, these antibodies were absent from children who showed viral infection during infancy, raising the possibility that maternal immunity may protect infants from infection by these viruses. However, the prevalence of MCPyV infection among infants was higher than that of TSPyV, possibly caused by MCPyV shedding from parental skin (*10*–*12*).

We did not observe any symptom associated with the time of seroconversion for these 2 viruses, which implies that primary exposures to these viruses during childhood cause no symptoms. When studying MCPyV infections in adult men who seroconverted, Tolstov et al. also found no clinical associations (*13*). Their observations and ours in the current study of MCPyV and TSPyV support

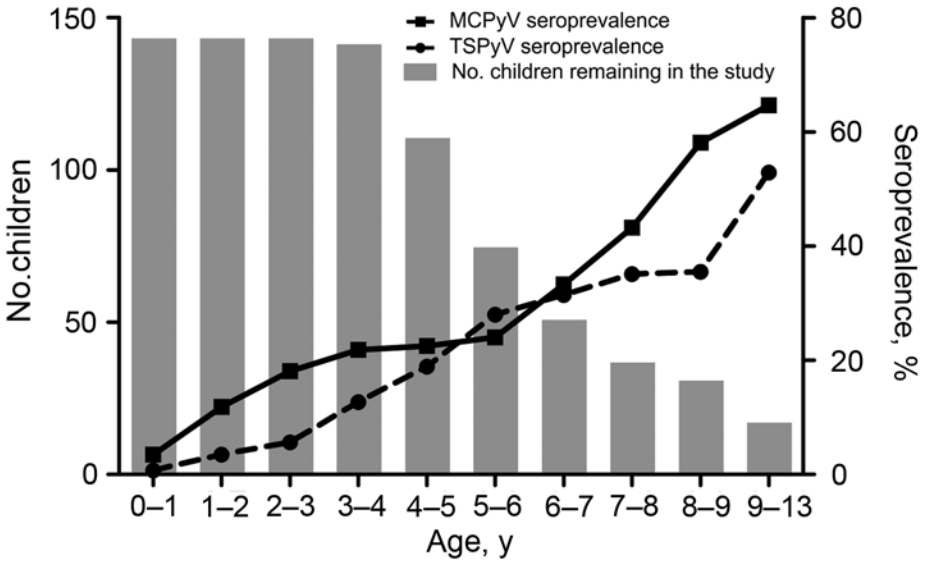

Figure. Seroprevalence related to polyomavirus primary infections in children in Finland during follow-up, January 2011–July 2013. Seroprevalence was calculated by the formula: Seroprevalence = (no. seropositive children remaining in the study at each age category) × 100.

Table. Infection-related signs and symptoms during MCPyV and TSPyV seroconversions compared with previous and subsequent intervals for asymptomatic polyomavirus infections in children, Finland, January 2011–July 2013*

Virus sign/symptom	Interval, no. (%)	Previous interval		Subsequent interval	
		No. (%)	p value	No. (%)	p value
MCPyV seroconversion, n = 45					
URTI	14 (31.8)	10 (22.2)	0.4545	18 (41.9)	0.3018
LRTI	3 (6.8)	2 (4.4)	1.0000	3 (7.0)	1.0000
Fever without RTI	2 (6.8)	2 (4.4)	1.0000	3 (7.0)	0.6250
Gastroenteritis	9 (20.5)	9 (20.0)	1.0000	11 (25.6)	0.5488
Acute tonsillitis	1 (2.3)	0	ND	1 (2.3)	1.0000
Acute otitis media	10 (22.7)	11 (24.4)	1.0000	13 (30.2)	0.6291
Acute conjunctivitis	2 (4.5)	1 (2.2)	1.0000	1 (2.3)	1.0000
Exanthema	7 (15.9)	1 (2.2)	0.0703	1 (2.3)	0.0703
Acute sinusitis	2 (4.5)	0	ND	1 (2.3)	1.0000
Total	32 (72.7)	26 (57.8)	0.2632	31 (72.1)	1.0000
No data	1	0	NA	2	NA
TSPyV seroconversion, n = 39					
URTI	14 (35.9)	9 (23.1)	0.3593	13 (35.1)	1.0000
LRTI	3 (7.7)	3 (7.7)	1.0000	2 (5.4)	1.0000
Fever without RTI	2 (5.1)	5 (12.8)	0.3750	4 (10.8)	0.6875
Gastroenteritis	10 (25.6)	12 (30.8)	0.7744	5 (13.5)	0.3877
Acute tonsillitis	1 (2.6)	1 (2.6)	1.0000	2 (5.4)	1.0000
Acute otitis media	8 (20.5)	12 (30.8)	0.3877	8 (21.6)	1.0000
Acute conjunctivitis	4 (10.3)	5 (12.8)	1.0000	3 (8.1)	1.0000
Exanthema	2 (5.1)	3 (7.7)	1.0000	1 (2.7)	1.0000
Acute sinusitis	3 (7.7)	2 (5.1)	1.0000	2 (5.4)	ND
Total	32 (82.1)	30 (76.9)	0.7266	30 (81.1)	1.0000
No data	0	0	NA	2	NA

*Liddell exact test was used; p<0.05 was considered significant. MCPyV, Merkel cell polyomavirus; TSPyV, trichodysplasia spinulosa-associated polyomavirus; LRTI, lower respiratory tract infection; URTI, upper respiratory tract infection; ND, no data; NA, not applicable.

the common notion that the prototypic human PyVs, BKPyV and JCPyV, also establish persistence without initial signs (*14*,*15*).

Exanthema, a common sign of skin infection, did not appear infrequently during MCPyV seroconversion; however, the rate did not reach statistical significance (p = 0.0703), which might be related to our limited cohort size. We cannot rule out that data for some transient skin-related signs may not have been captured during periodic patient interviews. Last, we do not believe that our study represents polyomavirus infections during childhood worldwide. Larger studies of more geographically diverse populations are needed to determine whether primary infection with MCPyV or TSPyV is always asymptomatic.

Although IgM and low-avidity IgG were observed in more than half of the seroconversion samples, the frequency of viremia was extremely low, indicating serodiagnostics as the strategy of choice in diagnosing primary infections with MCPyV and TSPyV. Our study suggests that these ubiquitous polyomaviruses circulate silently among children.

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Ms Chen was a PhD student at the University of Helsinki when conducting this study, and is now a postdoctoral researcher at Albert Einstein College of Medicine, Bronx, New York, USA. Her research interests are development of comprehensive diagnostics for the emerging human DNA viruses, including polyomavirus and anellovirus.

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Address for correspondence: Tingting Chen, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx, NY, USA 10461; email: tingting.chen@einstein.yu.edu

Hepatitis E Antibodies in Laboratory Rabbits from 2 US Vendors

Leslie Birke, Stephania A. Cormier, Dahui You, Rhett W. Stout, Christian Clement, Merlin Johnson, and Hilary Thompson

We tested laboratory rabbits from 2 US vendors for antibodies against hepatitis E virus (HEV); Seroprevalences were 40% and 50%. Retrospective analysis of an ocular herpes simplex 1 experiment demonstrated that HEV seropositivity had no effect on experiment outcome. HEV probably is widespread in research rabbits, but effects on research remain unknown.

Hepatitis E virus (HEV) is a single-stranded RNA virus in the family *Hepeviridae* (*1*). HEV is transmitted through the fecal–oral and bloodborne routes and typically causes transient hepatitis; however, such infections can be fatal, especially in pregnant women (20% mortality rate) and in immunocompromised persons (*2*,*3*).

HEV has 4 genotypes, and genotypes 3 and 4 are found in humans and other animals (*1*). The genomic sequence of US rabbit HEV (GenBank accession no. JX565469) has been analyzed and the sequence places rabbit HEV as a distant member of the zoonotic HEV genotype 3 (*4*). A study in France found HEV in farmed and wild rabbits and characterized a closely related human strain of HEV (*5*). Although HEV has been detected in rabbits from Asian and European counties, researchers previously thought that HEV was not a major presence in rabbits in the United States (*5*–*7*). A recent study identified a 36% prevalence of HEV antibodies in animals on 2 rabbit farms in Virginia (*1*).

Because HEV may be a confounding factor in research and is a potential zoonotic pathogen (*5*), we tested rabbits from 2 US suppliers for HEV seroprevalence. Supplier A was a local conventional rabbit farm, and supplier B was a commercial vendor of specific pathogen free (SPF) research rabbits. Our hypothesis was 3-fold: 1)HEV is a possibly undiagnosed pathogen in laboratory rabbits, 2) HEV seroprevalence is unlikely in rabbits from supplier B (a SPF supplier), and 3) HEV seroprevalence might confound research. To investigate the latter hypothesis, we retrospectively analyzed records from a subset of animals used in a herpes simplex 1 virus (HSV-1) research project.

Author affiliations: Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA (L. Birke, C. Clement, M. Johnson, H. Thompson); University of Tennessee Health Science Center, Memphis, Tennessee, USA (S.A. Cornier, D. You); and Louisiana State University, Baton Rouge, Louisiana, USA (R.W. Stout)

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The Study

Animals

Two shipments of New Zealand White rabbits (70 rabbits shipment 1 [rabbit nos. AN1–AN20 and AH1–AH30] in June 2012 to Louisiana State University Health Sciences Center [LSUHSC]; shipment 2 [rabbit nos. AH31–AH51] in October 2012) were obtained from supplier A at LSUHSC. Supplier A also provides rabbits to the meat production industry. A third shipment comprising 10 Dutch Belted SPF rabbits (nos. DB1–DB10) were obtained from supplier B in August 2012 at LSUHSC. Supplier B rabbits were SPF for *Pasturella multocida*, *Pasturella pneumotropica*, *Bordetella brochiseptica*, *Treponema cuniculi*, *Clostridium piliformis*, cilia-associated respiratory bacillus, *Salmonella* spp., *Encephalitozoon cuniculi*, *Eimeria stiedae*, *Eimeria magna*, *Eimeria intestinalis*, *Eimeria irresidua*, *Eimeria flavescens*, *Eimeria piriformis*, *Toxoplasma gondii*, *Passalurus ambiguous*, other helminths, parainfluenza virus 1, parainfluenza virus 2, reovirus, *Psoroptes cuniculi*, *Cheyletiella parasitovorax*, *Leporacarus gibbus*, and *Dermatomycosis* spp.

The rabbits' care and use were approved by the LSUHSC Institutional Animal Care and Use Committee and were consistent with the Guide for the Care and Use of Laboratory Animals (*8*) and the Animal Welfare Act (http://www.aphis.usda.gov/animal_welfare/). The LSUHSC is fully accredited by American Association for the Assessment of Laboratory Animal Care, Int. The animals were singly housed in stainless steel rabbit cages (Suburban Surgical, Wheeling, IL, USA). On the day the rabbits arrived, blood was collected from each rabbit and stored for HEV testing. Rabbits from vendor A were treated for *P. cuniculi* and *L. gibbus* with selamectin (Pfizer New York, NY, USA). After arrival, the rabbits were acclimated to the facility for 7 days. After this period, they were used for research protocols approved by the Institutional Animal Care and Use Committee.

Sample Collection

Approximately 0.5–1 mL of blood was collected through the rabbits' middle auricular artery with a 25-gauge butterfly catheter. The blood was placed into serum separator tubes for centrifugation. Serum was then placed into microcentrifuge tubes and stored at –20°C until analysis. Laboratory personnel changed gloves between blood collection from each rabbit and between contact with each sample when transferring serum.

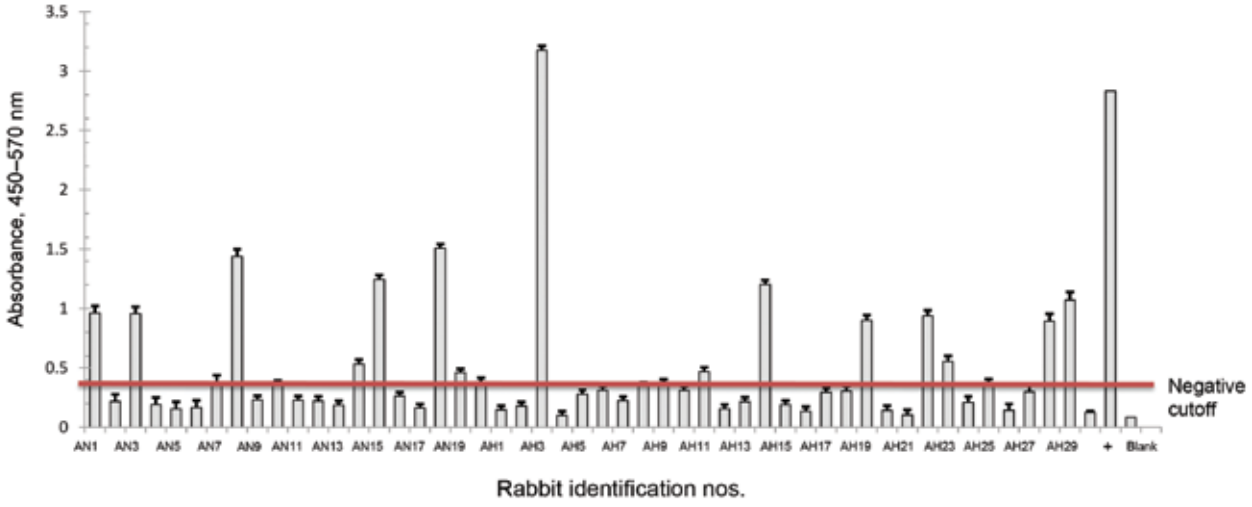

Figure 1. Hepatitis E virus antibody detection for rabbits from supplier A, shipment 1 (June 2012, Louisiana State University Health Sciences Center). Rabbits were purchased from supplier A. Serum was isolated and antibodies in the serum were measured by using ELISA. Rabbit identification numbers are listed on the *x*-axis and the mean + SD optical density of each sample is listed on the *y*-axis. The negative cutoff point is indicated by the horizontal line. +, positive control.

HEV Infection and Outcome of Rabbit Model of Ocular HSV-1

The records of 30 animals from supplier A (AH1–AH30) were obtained retrospectively from an investigator who used the rabbits in an HSV-1 ocular keratitis study. Survival data and HEV antibody status were examined statistically to determine whether HEV status had any effect on the survival of the rabbits in the study. Briefly, 4 different strains of HSV-1 with different virulence levels were tested, including MCK D-gK, ICP0-delete, 17 syn + mutant, and McKrae. Rabbits were infected with these viruses, and keratitis score and survival rate were recorded.

ELISA

ELISA was performed by using the protocol from Cossaboom et al. with a few modifications (*1*). Plates (Fischer Scientific, Waltham, MA, USA) were coated with 6 μg HEV antigen (Clone# rHEV-ORF2; Feldan Bio Inc.,

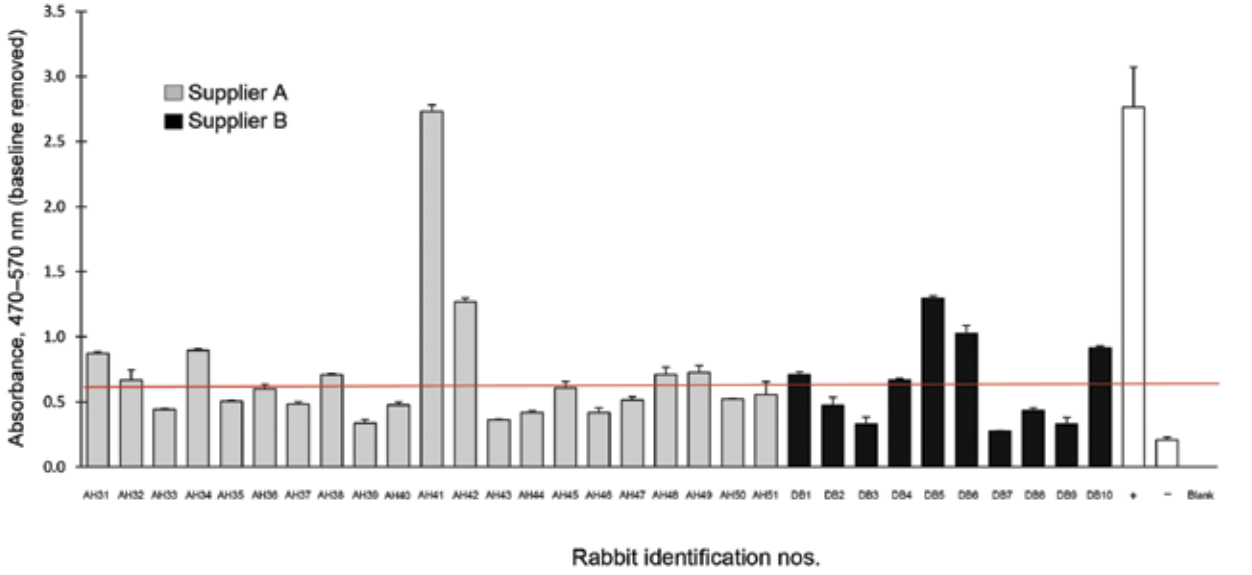

Figure 2. Hepatitis E virus antibody detection for rabbits from suppliers A and B, shipments 2 (October 2012, Louisiana State University Health Sciences Center [LSUHSC]) and 3 (8/2012 LSUHSC). Rabbits were purchased from supplier A (gray bars) and supplier B (black bars). Serum was isolated and antibodies in the serum were measured by using ELISA. Rabbit identification numbers are listed on the *x*-axis and the mean + SD optical density of each sample is listed on the *y*-axis. The negative cutoff point is indicated by the horizontal line. White bars indicate controls. +, positive control; –, negative control.

Quebec, QC, Canada), incubated with 100 µL of rabbit serum diluted 10-fold in blocking buffer, and detected with horseradish peroxidase–conjugated secondary antibody (goat anti–rabbit IgG diluted 1:1000, Southern Biotech, Birmingham, AL, USA). The plates were read at 450 nm and 540 nm for correction on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis

ELISAs for HEV were performed in duplicate on each serum sample, and the results were averaged. A 1-way analysis of variance was performed to find the optical densities that were not statistically different from those of the negative controls. The values of the negative controls and the negative samples were then used to determine the cutoff points for the ELISA (mean of the negative samples + 3 SDs).

To evaluate the possible effect of HEV serology status on the survival of rabbits used in the HSV-1 study, we used the Fisher exact test when HSV-1 survival status was cross-tabulated with the HEV serology status. Odds ratios are also reported (SAS, version 9.3, SAS Institute, Cary, NC, USA); α was set at 0.05.

Prevalence of HEV in US Laboratory Rabbits

We found that the seroprevalence of HEV in the 70 rabbits from supplier A was 40%. The 10 rabbits from supplier B exhibited a 50% seroprevalence for HEV (Figures 1 and 2).

HEV Infection and Disease Outcome of HSV-1–infected Animals

HSV-1 outcome results revealed no significant association between positive serologic HEV status and frequency of HSV-1 signs. Prevalence of positive HEV status was 32% (6/19) , compared with 36% (4/11) (p = 0.298, odds ratio 1.238, 95% CI 0.2592–5.9132) for frequency of HSV-1 signs.

Conclusions

We sought to determine the seroprevalence of HEV in research rabbits within LSUHSC. We were surprised to identify HEV-seropositive rabbits from the SPF research rabbit supplier (supplier B) because of the strict health quality standards employed at this supplier. The difference in HEV seroprevalence between the 2 vendors may be a function of sample numbers. Testing a larger number of samples from supplier B would answer this question. HEV may be widespread in research facilities throughout the United States.

Statistically, we could not demonstrate any effect of HEV serology status on the survival outcome or keratitis scores of HSV-1–infected rabbits. However, the interpretation of these results is limited because only tested for HEV only by serologic testing and not reverse transcription PCR. Controlled studies in which rabbits are experimentally infected with HEV and then later infected with another pathogen are needed to determine the true effects of HEV co-infection on the outcome of systemic infection experiments in rabbits.

Rabbits are commonly used in research and they often housed in a conventional setting. Because HEV is classified as a Biosafety Level-2 pathogen, wearing personnel protective protection equipment, such as masks, laboratory coats, and gloves, when working with rabbits is warranted (*9,10*). Laboratory animal care personnel, researchers, and support staff represent a new population at risk for HEV infection, and research facilities should be diligent in measures to prevention of this possibly zoonotic pathogen.

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Dr Birke is the associate director for the Division of Laboratory Animal Care at LSUHSC. Her research interests include HEV and *L. gibbus* infections in rabbits.

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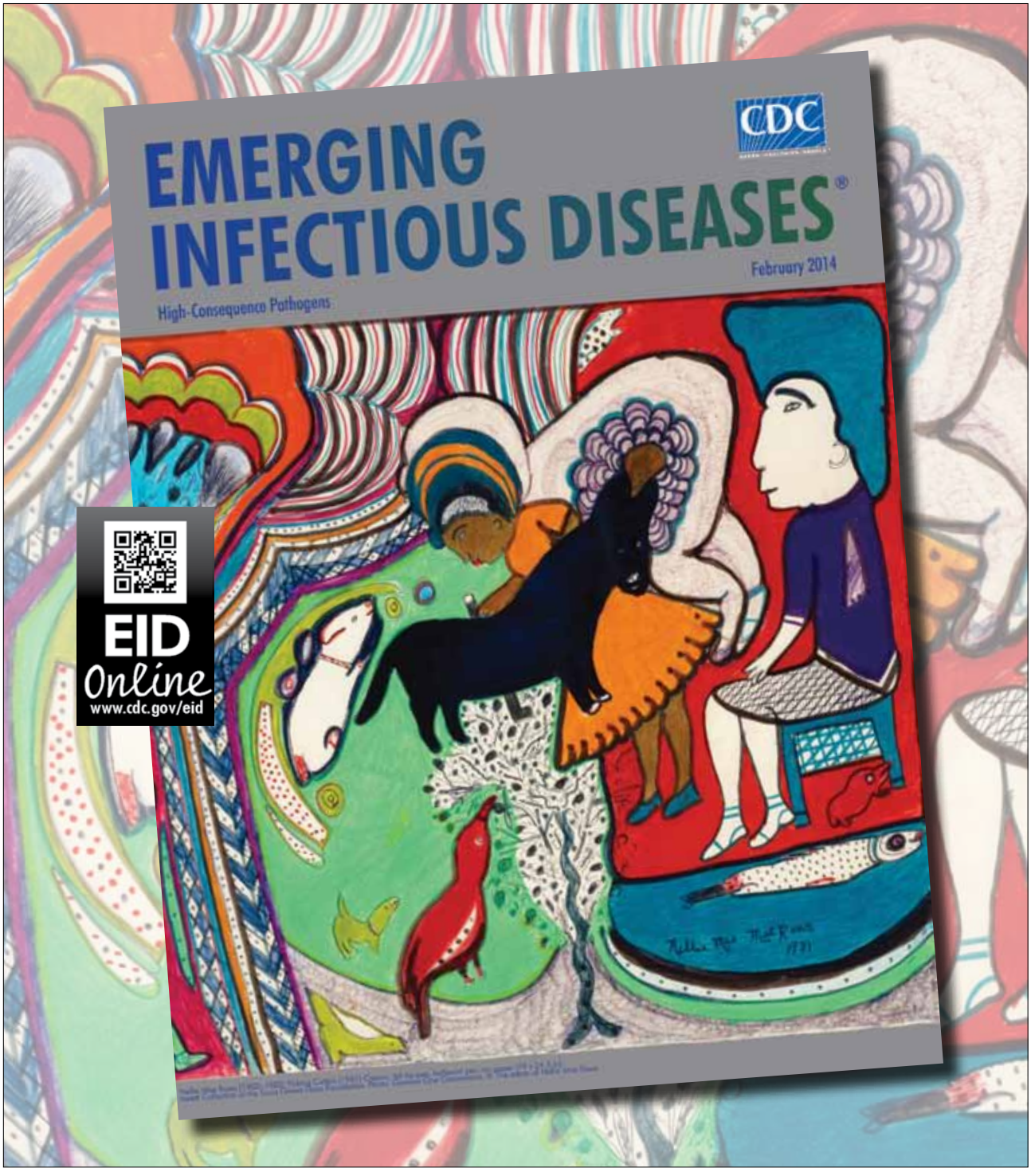
Address for correspondence: Leslie Birke, Louisiana State University Health Sciences Center, Division of Animal Care, 533 Bolivar St, New Orleans, LA 70112, USA; email: lbirke@lsuhsc.edu



New Alphacoronavirus in *Mystacina tuberculata* Bats, New Zealand

Richard J. Hall, Jing Wang, Matthew Peacey, Nicole E. Moore, Kate McInnes, and Daniel M. Tompkins

Because of recent interest in bats as reservoirs of emerging diseases, we investigated the presence of viruses in *Mystacina tuberculata* bats in New Zealand. A novel alphacoronavirus sequence was detected in guano from roosts of *M. tuberculata* bats in pristine indigenous forest on a remote offshore island (Codfish Island).

Human settlement in New Zealand is relatively recent compared with that of many countries and extends back ≈800 years to when early Polynesian explorers first arrived (*1*). A wide variety of exotic flora and fauna have since been introduced, and major changes to the landscape and ecology have occurred, particularly once European settlers arrived 150 years ago. However, large areas of New Zealand that are representative of a prehuman state still remain, in particular offshore, islands such as Whenua hou (Codfish Island), which is situated off the southern coast of New Zealand. Before human settlement, only 3 species of terrestrial mammals were present, all of which were bats. Why nonvolant mammals have been absent is unknown, but this absence was probably caused by major extinction events and subsequent geographic isolation that prevented recolonization (*2,3*).

Only 2 bat species remain in New Zealand and both are considered vulnerable (www.iucnredlist.org/). The long-tailed bat (*Chalinolobus tuberculatus*) belongs to the family Vespertilionidae and is believed to have arrived from Australia ≈1 million years ago (*4*). The lesser short-tailed bat (*Mystacina tuberculata*) is the sole surviving member of the family Mystacinidae in New Zealand (*5*). *M. tuberculata* bats are believed to have diverged from other bat species ≈20 million years ago (*6*) and have lived in geographic isolation from other bat species until the arrival of *C. tuberculatus* bats (*3*). *M. tuberculata* bats also exhibit one of the widest feeding ranges of any bat species and occupy a niche similar to rats and mice because these bats can walk on the ground by using their wings (*7*).

Little is known about the microorganisms present in these bats. One study found no evidence of pathogenic bacteria or lyssaviruses in them, but reported a *Sarcocystis* sp. (*8*). Given the recent intense interest in bats as a reservoir of emerging diseases and the advent of high-throughput sequencing as a virus discovery tool, we investigated the presence of viruses in *M. tuberculata* bats.

The Study

Four bat guano samples were collected from known roost sites on the remote offshore island of Whenua hou (Codfish Island) (46°47′S, 167°38′E), which is situated at the southern coast of New Zealand. This small island is heavily forested and largely unmodified by humans. It has special conservation status for the protection of endangered species, and public access is not permitted (*9*).

Bat guano was held at 4°C during transport (<48 h), resuspended in 2 mL phosphate-buffered saline, and subjected to centrifugation at 6,000 × *g* for 5 min. RNA was extracted from 400 μL of supernatant by using the iPrep PureLink Virus Kit (Life Technologies, Carlsbad, CA, USA) and eluted into 50 μL reverse transcription PCR molecular-grade water (Ambion, Austin, TX, USA). Metagenomic sequencing was then conducted for 1 of the samples by using an Illumina MiSeq Instrument (New Zealand Genomics Ltd., Massey Genome Service, Massey University, Palmerston North, New Zealand) after a series of steps involving DNase I treatment, reverse transcription, multiple displacement amplification (QIAGEN, Valencia, CA, USA), and Illumina TruSeq library preparation (New Zealand Genomics, Ltd.).

A total of 10,749,878 paired-end sequence reads of 250 bp were generated and assembled into contigs by using Velvet 1.2.07 (*10*). Assembled contigs were searched for viral sequence by comparison to the nonredundant nucleotide database in Genbank (downloaded March 2013) by using the nucleotide basic local alignment search tool (National Center for Biotechnology Information, Bethesda, MD, USA). Forty-six contigs showed similarity to known genus *Alphacoronavirus* sequences (Table). (Raw sequence data are available on request from the authors.)

Virus sequence from the genus *Alphacoronavirus* was confirmed in all 4 original guano samples by using a specific reverse transcription PCR based on the metagenomic data specific for 582 bp of the RNA-dependent RNA polymerase (*RdRp*) gene (GenBank accession nos. KF515987–KF515990). The *Rdrp* sequence was identical in all 4 guano samples, and the closest relative was bat coronavirus HKU8 (GenBank accession no. DQ249228;

Author affiliations: Institute of Environmental Science and Research, Upper Hutt, New Zealand (R.J. Hall, J. Wang, M. Peacey, N.E. Moore); Department of Conservation, Wellington, New Zealand. (K. McInnes); and Landcare Research, Dunedin, New Zealand. (D.M. Tompkins)

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Table. Summary of BLASTn output for contigs from bat guano that show identity to alphacoronaviruses, New Zealand*

Alphacoronavirus gene	No. contigs matching alphacoronaviruses	Range of contig lengths, bp	Highest scoring BLASTn hit recorded against alphacoronaviruses	
			e-value	Nucleotide identity (%)†
Open reading frame 1ab	33	182–1054	8×10^{-93}	581/828 (77)
Spike protein	5	580–1629	1×10^{-19}	362/551 (66)
Matrix	4	251–840	2×10^{-99}	532/746 (71)
Nucleocapsid	4	536–890	6×10^{-6}	79/109 (72)

*BLASTn, nucleotide basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).
†No. nucleotides identical between query and database sequence.

79% nt identity, 426/542). Phylogenetic analysis was performed for *Rdrp* (Figure 1) and for the spike protein, as derived from metagenomic data (GenBank accession no. KF575176) (Figure 2). We propose that these data support identification of a new alphacoronavirus, which has been designated as *Mystacina* bat CoV/New Zealand/2013.

Conclusions

The discovery of unknown coronaviruses provides information for a model of coronavirus evolution (*11*) and contributes to understanding the process of disease emergence, as in detection of Middle East respiratory syndrome coronavirus (*12*). The alphacoronavirus

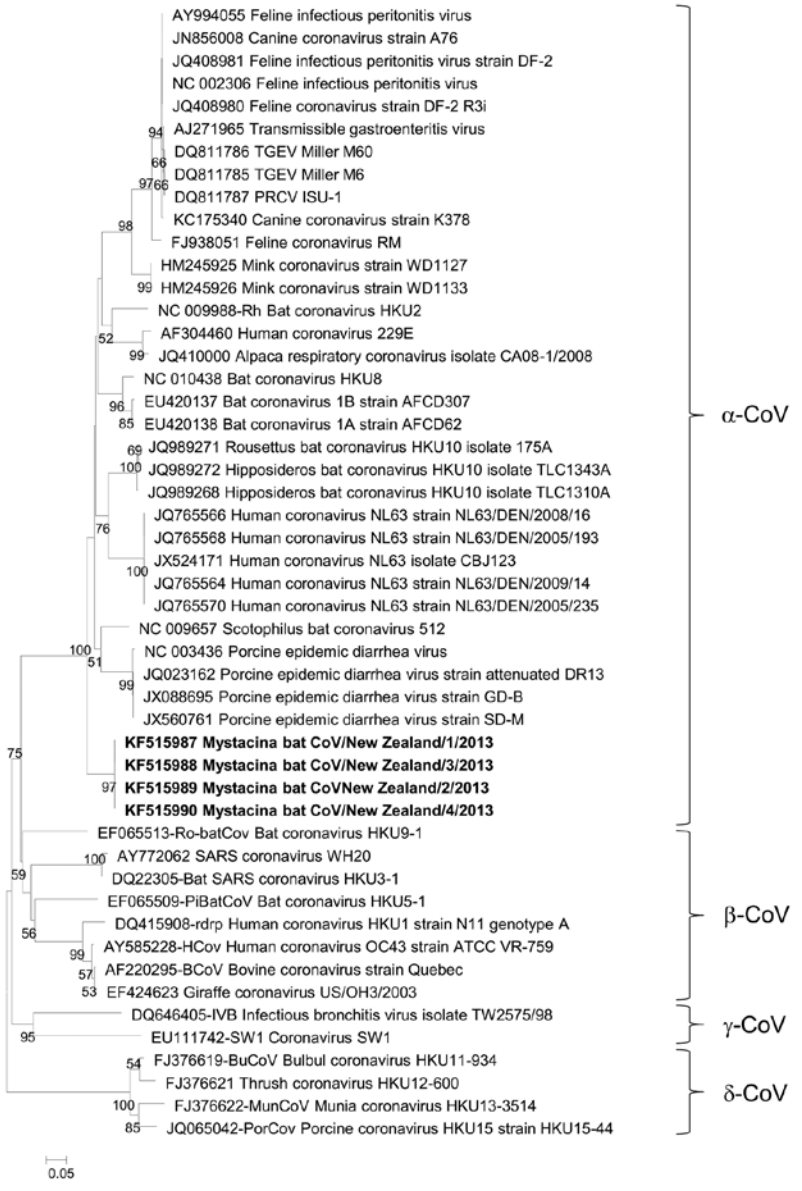

Figure 1. Phylogenetic tree showing genetic relatedness of RNA-dependent RNA polymerase amino acid sequences for *Mystacina* sp. bat coronavirus (CoV)/New Zealand/2013 (shown in boldface) with those of known coronaviruses. Evolutionary history was inferred for 183 informative amino acid sites by using the maximum-likelihood method based on the Whilan and Goldman model with gamma distribution in MEGA 5.05 software (www.megasoftware.net). Bootstrap values are calculated from 1,000 trees (only bootstrap values >50% are shown). Scale bar indicates nucleotide substitutions per site. TGEV, transmissible gastroenteritis CoV; PRCV, porcine respiratory CoV; SARS, severe acute respiratory syndrome.

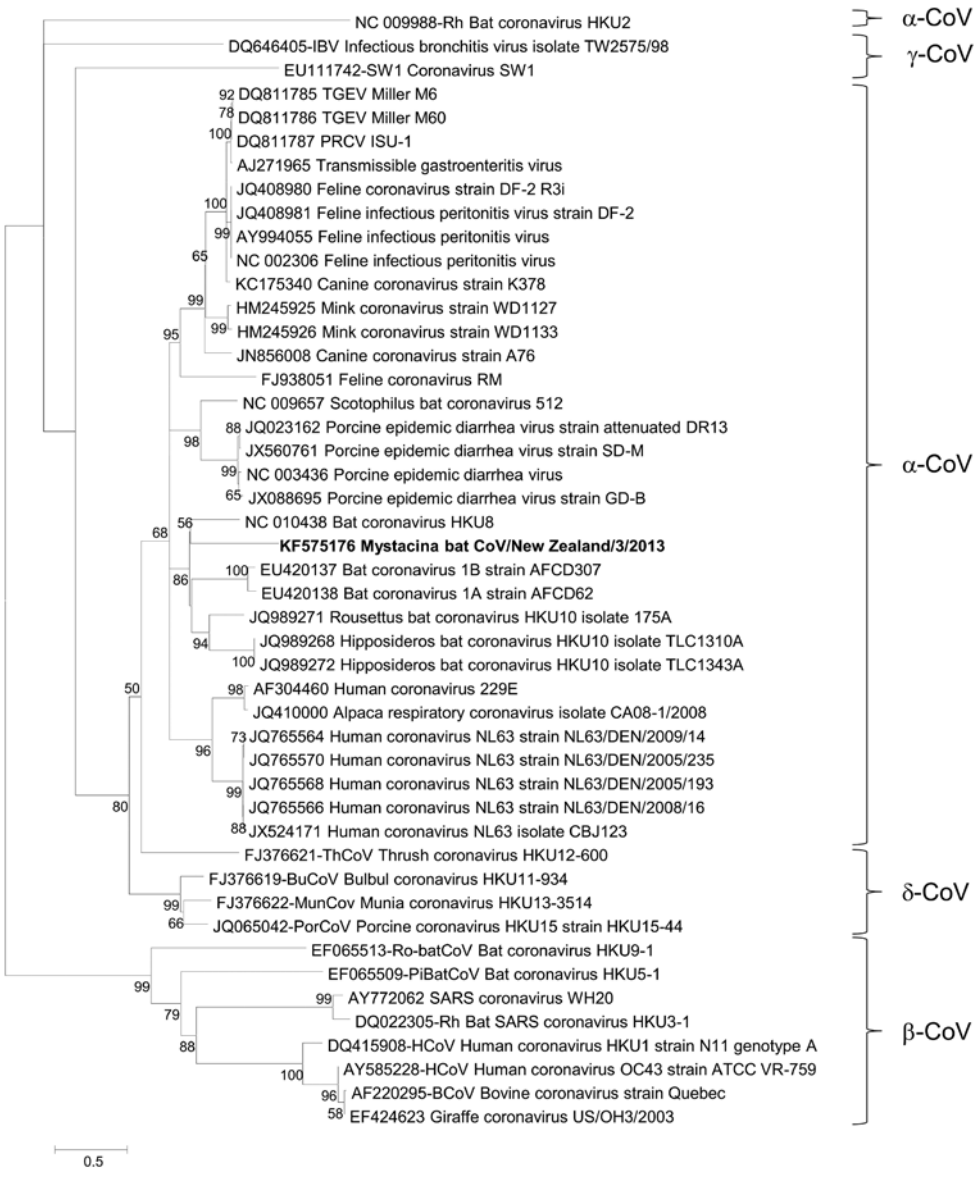

Figure 2. Phylogenetic tree showing genetic relatedness of spike protein amino acid sequence for *Mystacina* sp. bat coronavirus (CoV)/New Zealand/2013 (shown in boldface) with those of known coronaviruses. Evolutionary history was inferred for 492 informative amino acid sites by using the maximum-likelihood method based on the Whilan and Goldman + F model with gamma distribution and invariant sites in MEGA 5.05 software (www.megasoftware.net). Bootstrap values are calculated from 1,000 trees (only bootstrap values >50% are shown). Scale bar indicates nucleotide substitutions per site. TGEV, transmissible gastroenteritis CoV; PRCV, porcine respiratory CoV; SARS, severe acute respiratory syndrome.

identified in this study from *M. tuberculata* bat guano is unique in respect to the extreme geographic and evolutionary isolation of the host bat species, which along with *C. tuberculatus* bats, has been separated from all other mammalian species for ≈1 million years.

The current estimate for a common ancestor for all coronaviruses is 8,100 BCE (*13*). To be consistent with this estimate, *Mystacina* bat coronavirus would need to have been introduced to bats on Whenua hou within the past 800 years since humans first arrived on this island (given that the island had no other mammals before this time) (*11*) or is extant to modern alphacoronavirus phylogenetic radiation (genesis and expansion). Apart from humans, only 2 other terrestrial mammals have ever inhabited Whenua hou: the brushtail possum (*Trichosurus vulpecula*) and the Polynesian rat (*Rattus exulans*), both of which were eliminated from the island in the late 1980s (*11*); neither mammal has been reported as a host of alphacoronaviruses. Members of the genus *Alphacoronavirus* infect only mammals. Thus, an avian origin for this virus is unlikely.

An alternative theory of an ancient origin for all coronaviruses has recently been proposed that involves an alternative evolutionary molecular clock analysis, which places the most recent common ancestor many millions of years ago (*14*). The discovery of *Mystacina* bat CoV/New Zealand/2013 virus could lend support to such a theory; despite potentially millions of years of isolation, it has diverged relatively little from other extant alphacoronaviruses, as shown by the close relationship of the *Rdrp* and spike protein genes to those of other extant

alphacoronaviruses (Figures 1, 2). An expanded survey for *Mystacina* bat coronavirus in mammals in New Zealand and subsequent characterization of viral genomes would provide further insights into the origin of coronaviruses.

No instances of human zoonotic disease from New Zealand bat species have been reported. Only a small number of conservation staff handle these bats, these staff use standard personal protective equipment and work practice. Staff are also offered prophylactic rabies vaccination as a precautionary measure, even though New Zealand is free from rabies. The genus *Alphacoronavirus* includes several human and animal pathogens, but on the basis of phylogenetic data in this study, it is not possible to estimate the risk posed by *Mystacina* bat coronavirus to human or animal health.

New Zealand is not considered a hot spot for emerging infectious diseases. This country is free from many human and animal diseases, such as rabies and foot-and-mouth disease, and infections with human arboviruses because of recent colonization by humans and strict biosecurity border controls (*15*). Thus, indigenous wildlife in New Zealand has generally been viewed as an almost sterile and unique biosphere. Given detection of this coronavirus, more thorough characterization of the ecology of viruses and other microorganisms in native wildlife should be considered to fulfill conservation needs and further safeguard human and domestic animal health against cross-species transmission.

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Dr Hall is a virologist at the Institute of Environmental Science and Research based at the National Centre for Biosecurity and Infectious Disease in Upper Hut, New Zealand. His primary research interest is discovery of new viruses that cause human and animal disease through application of new sequencing technologies.

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Address for correspondence: Richard J. Hall, Institute of Environmental Science and Research, National Centre for Biosecurity and Infectious Disease, PO Box 40158, Upper Hutt 5140, New Zealand; email: richard.hall@esr.cri.nz

Invasive *Salmonella enterica* Serotype Typhimurium Infections, Democratic Republic of the Congo, 2007–2011

Benedikt Ley, Simon Le Hello, Octavie Lunguya, Veerle Lejon, Jean-Jacques Muyembe, François-Xavier Weill, and Jan Jacobs

Infection with *Salmonella enterica* serotype Typhimurium sequence type (ST) 313 is associated with high rates of drug resistance, bloodstream infections, and death. To determine whether ST313 is dominant in the Democratic Republic of the Congo, we studied 180 isolates collected during 2007–2011; 96% belonged to CRISPOL type CT28, which is associated with ST313.

Salmonella enterica serotype Typhimurium multilocus sequence type (ST) 313 has been reported as an emerging cause of invasive salmonellosis in sub-Saharan Africa (*1*). ST313 is almost exclusively from sub-Saharan Africa, is characterized by a degraded genome capacity similar to that of *S. enterica* ser. Typhi, has high rates of antimicrobial drug resistance, and is associated with bloodstream infections and mortality rates >25% (*2*). Whole-genome sequence analysis of 129 ST313 strains, isolated during 1988–2010 from 7 countries of sub-Saharan Africa, identified 2 dominant genetic lineages, I and II. These lineages emerged ≈52 and ≈32 years ago, respectively, possibly coevolving with the spread of HIV (*3*). Although lineage I has not been observed since the mid-2000s, lineage II has been observed with increasing frequency. However, data from Central Africa, particularly the Democratic Republic of the Congo (DRC) are scarce, and information is limited to 10 genomes from strains isolated >20 years ago (*3*). To determine whether ST313 is the dominant ST among invasive *Salmonella* Typhimurium in the DRC, we studied 180 isolates collected during 2007–2011.

Author affiliations: Institute of Tropical Medicine, Antwerp, Belgium (B. Ley, J. Jacobs); Institut Pasteur, Paris, France (S. Le Hello, F.-X. Weill); Institut National de Recherche Biomédicale, Kinshasa, Democratic Republic of the Congo (O. Lunguya, J.-J. Muyembe); and Institut de Recherche pour le Développement, Montpellier, France (V. Lejon)

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The Study

We earlier described a series of invasive non-Typhi *Salmonella* isolates from blood cultures collected in 7 of the 11 provinces in the DRC during 2007–2011 (Figure 1) (*4*). In that study, a health care facility–based survey was administered to persons who met the eligibility criteria of suspected bacteremia at time of presentation and patient age >2 months. Blood culture vials were shipped to Kinshasa and processed according to standard identification procedures (*4*). A total of 233 non-Typhi *Salmonella* isolates were recovered, 184 (79%) of which belonged to serotype Typhimurium (*4*). The serotypes for all *S. enterica* ser. Typhimurium isolates were determined locally and later confirmed at the Institute of Tropical Medicine, Antwerp, Belgium. Most (180/184, 98.7%) *S. enterica* ser. Typhimurium isolates were subsequently shipped to the Pasteur Institute, Paris, France, for further analysis.

The population structure of these 180 *S. enterica* ser. Typhimurium isolates was assessed by CRISPOL typing. CRISPOL is a recently developed high-throughput assay based on clustered regularly interspaced short palindromic repeat (CRISPR) polymorphisms (*5*). This bead-based hybridization assay is designed to detect the presence or absence of 72 short variable DNA sequences (spacers) from both CRISPR loci of *S. enterica* ser. Typhimurium. Initially, 245 different CRISPOL types (CTs) were identified in a 2012 study that included 2,200 isolates (*5*); just before we conducted the study reported here, the CRISPOL *Salmonella* Typhimurium database of the Pasteur Institute contained >7,000 strains comprising >750 different CTs.

A total of 174 (96.7%) *S. enterica* ser. Typhimurium isolates from the DRC belonged to the CT28 group, of which 163 (90.5%) were CT28. A total of 11 (6.1%) isolates belonged to 7 other CTs that were single-spacer variants (loss of a single spacer), single-event variants (loss of ≥2 contiguous spacers), or double-event variants of CT28 (Figure 2). Six (3.3%) isolates belonged to 2 CTs not related to CT28. CT28 had been associated with ST313 in a multidrug-resistant DT56 *S. enterica* ser. Typhimurium isolate from Senegal and in the D23580 ST313 lineage II genome (*5*). In contrast, the analysis of raw pyrosequence data for genome A130 (*3*), representative of ST313 lineage I, corresponded to CT698, distinct from the CT28 group.

To confirm the association of ST313 to the CT28 group, we performed multilocus sequence typing (MLST) (*6*) on 12 isolates. A total of 3 isolates belonged to CT28, and 1 isolate of each single-spacer, single-event, and double-event variant was tested, resulting in 10 isolates from the CT28 group. We also performed MLST on 1 isolate of

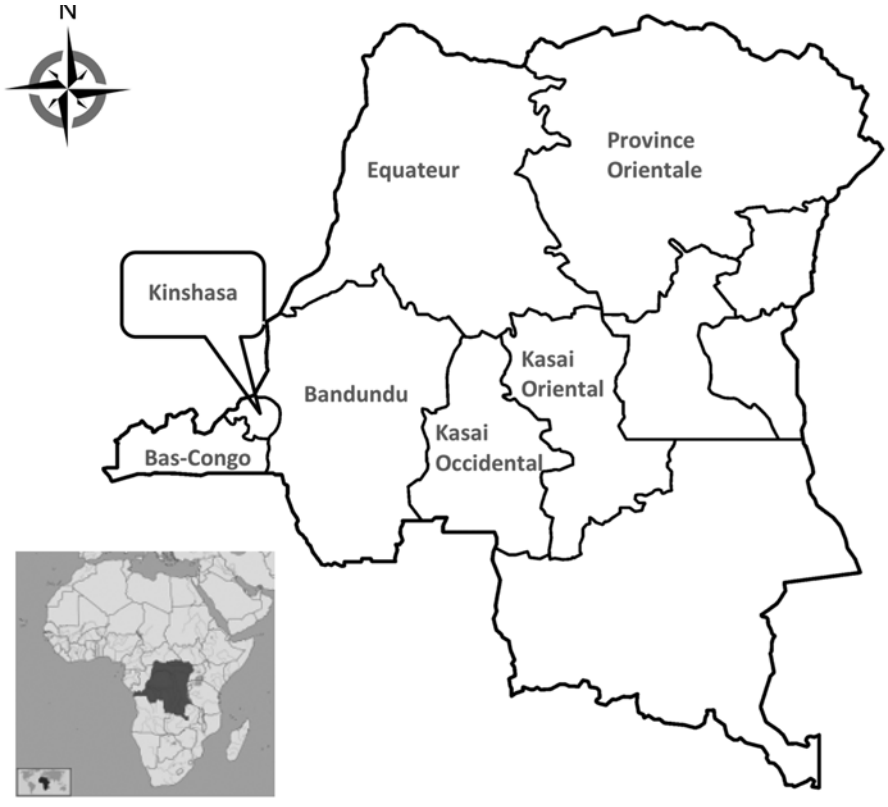

Figure 1. Provinces with sample collection sites (circled) in the Democratic Republic of the Congo, 2007–2011 (*4*).

each of the 2 non–CT28 group isolates. All 10 CT28 group isolates tested were ST313; both non–CT28 group isolates tested were ST19.

Antimicrobial drug susceptibility has been studied with a limited panel of 7 drugs (*4*). We performed additional susceptibility testing by disk diffusion with a panel of 32 antimicrobial agents (Bio-Rad, Marnes-La-Coquette, France) (*7*). Extended-spectrum β-lactamase (ESBL) phenotype was assessed by using the double-disk synergy method (*8*). For all ESBL-producing isolates, MICs of ceftriaxone, ceftazidime, azithromycin, and imipenem were determined by the Etest macromethod (bioMérieux, Marcy L'Etoile, France). Results were interpreted according to break points defined by the Antibiogram Committee of the French Society for Microbiology (www.sfm-microbiologie.org/). Susceptible strains were defined as having a ceftriaxone MIC ≤1 mg/L, ceftazidime MIC ≤4 mg/L, azithromycin MIC ≤16 mg/L, and imipenem MIC ≤2 mg/L. Resistance was defined as having a ceftriaxone MIC >2 mg/L, ceftazidime MIC >4 mg/L, azithromycin MIC >16 mg/L, and imipenem MIC >8 mg/L. The presence of macrolide resistance genes was assessed by PCR and sequencing as described elsewhere (*7*). Of the 174 CT28 group isolates, 167 (96%) were resistant to ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole in combination with other drugs (Figure 2); the remaining isolates were resistant to 1 or 2 of these drugs. Two isolates were resistant to extended-spectrum cephalosporins (ceftriaxone MIC 6–32 mg/L, ceftazidime MIC 4–32 mg/L); both contain the ESBL $bla_{\text{SHV-2a}}$ gene (*4*). We report that both isolates contain the *mph*(A) gene encoding a macrolide 2′-phosphotransferase that inactivates macrolides (azithromycin MIC 96–128 mg/L). All 6 non-CT28 group isolates were susceptible to all drugs tested (Figure 2).

Conclusion

Our data are based on the analysis of *S. enterica* ser. Typhimurium isolates recovered from >9,600 blood cultures collected during a 4-year period from distinct parts of the DRC. We found that >96% of the *S. enterica* ser. Typhimurium isolates belonged to the CT28 group. Because of the strong association between CT28 group and ST313, our findings suggest high rates of ST313 among invasive salmonellosis in the DRC.

Of the 10 genomes from the DRC isolated during 1988–1992 (*3*), genetic lineages I and II were identified at approximately equal rates. Of the more recent isolates (2007–2011) described here, all ST313 isolates belonged to the CT28 group, associated with lineage II. A notable feature of lineage II is chloramphenicol resistance resulting from a *cat* gene within a specific Tn21-like element, carried by the virulence-associated plasmid pSLT (*3*). In the

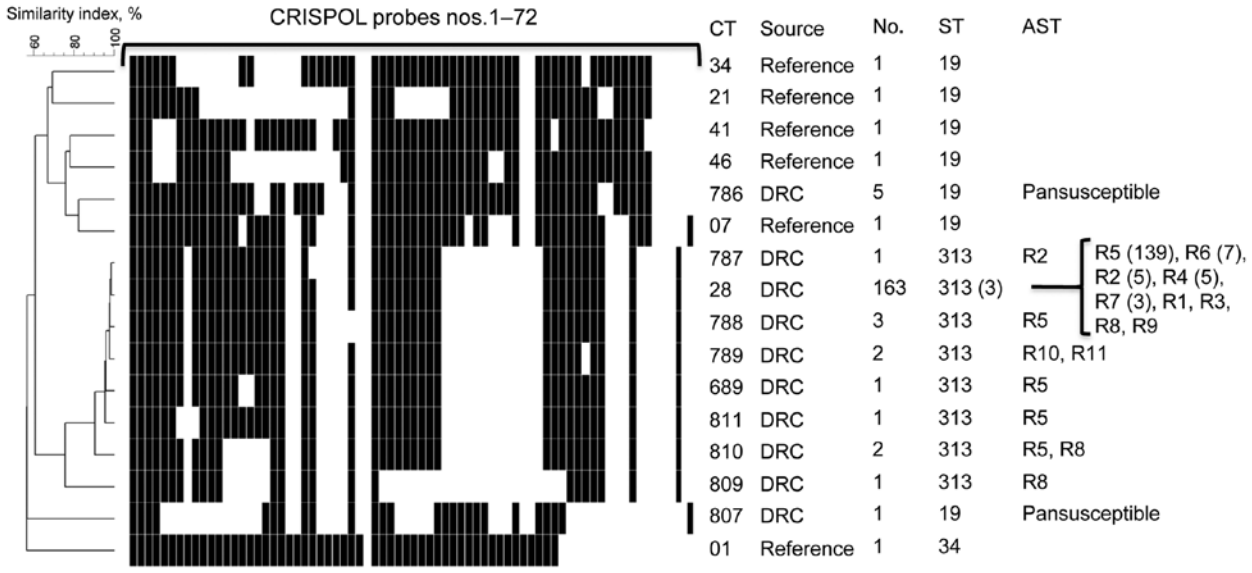

Figure 2. Representative CRISPOL profiles of *Salmonella enterica* serotype Typhimurium isolates studied. CRISPOL is a recently developed high-throughput assay based on clustered regularly interspaced short palindromic repeats (CRISPR) polymorphisms. Black squares indicate presence of the CRISPR spacer, detected by the corresponding probe; white squares indicates absence of the spacer. The dendrogram was generated by using BioNumerics version 6.6 software (Applied Maths, Sint-Martens-Latem, Belgium) as described (*5*). The CRISPOL types (CTs) detected among the 180 isolates from the Democratic Republic of Congo (DRC) are labeled as DRC in the Source column. Six common CTs of the Pasteur Institute CRISPOL database (labeled as reference) are also shown. These CTs are from strains of serotype Typhimurium 02–1800 (CT34, DT120), 02–5270 (CT21, DT104), LT2 (CT41, DT4), 02–2561 (CT46, DT12), 02–1749 (CT7, DT14) or its monophasic variant of antigenic formula 1,4,[5],12:i:-, 07–1777 (CT1, DT193). For each distinct CT, the numbers of corresponding isolates, their sequence types (STs), and their antimicrobial drug susceptibility testing (AST) data are indicated. For the ST and AST columns, the numbers in parentheses refer to the number (≥2) of tested isolates with such result. AST data are shown only for DRC isolates. The resistance types were as follows: R1, ASKTNGSulTmpC; R2, ASKTNGSulTmpCTe; R3, AC; R4, ASSulTmp; R5, ASSulTmpC; R6, ASSulTmpCNal; R7, ASSulTmpCTe; R8, ASulTmpC; R9, SSulTmpC; R10, ACroCazSKTNGSulTmpCTeAzi; and R11, ACroSKTNGSulTmpCTeNaAzi. Abbreviations used in the descriptions of resistance types are as follows: A, amoxicillin; Cro, ceftriaxone; Caz; ceftazidime; S, streptomycin; K, kanamycin; T, tobramycin; N, netilmicin; G, gentamicin; Sul, sulfamethoxazole; Tmp, trimethoprim; C, chloramphenicol; Te, tetracycline; Nal, nalidixic acid; Azi, azithromycin.

set described herein, we observed chloramphenicol resistance in >97% of all isolates belonging to the CT28 group. The almost complete replacement of lineage I isolates by lineage II isolates from Kenya and Malawi has also been reported (*1*,*9*).

Our data are based on invasive *S. enterica* Typhimurium isolates collected in a nonsystematic health care facility–based approach and do not include noninvasive strains of *S. enterica* Typhimurium. Wain et al. (*10*) recently cited unpublished data showing that ST313 *S. enterica* Typhimurium might be a common cause of gastroenteritis among immune-competent patients. A human reservoir for multidrug-resistant *S. enterica* Typhimurium and Enteritidis in Kenya has been suggested because of the presence of similar strains in asymptomatic siblings and parents of index case-patients (carriage prevalence 6.9%) (*11*). Whole-genome sequencing of ST313 strains has shown genome degradation, including pseudogene formation and chromosomal deletions as have been observed for human-restricted *S. enterica* Typhi (*12*,*13*), suggesting that ST313 might be undergoing an evolution toward niche specialization or, more likely, human adaptation (*1*).

Our results indicate very high rates of multidrug-resistant *S. enterica* Typhimurium ST313 among invasive non-Typhi *Salmonella* infections in the DRC. Future field studies involving patients with uncomplicated *Salmonella* spp. infections will help determine whether ST313 *S. enterica* Typhimurium in Central Africa is an opportunist or a primary pathogen. Systematic analyses of potential non-human and human reservoirs of *S. enterica* Typhimurium might provide a better understanding of the transmission dynamics of this emerging pathogen.

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Dr Ley is a medical biologist, epidemiologist, and researcher at the Institute of Tropical Medicine, Antwerp, Belgium. His research interests include infectious diseases in tropical countries.

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Address for correspondence: François-Xavier Weill, Centre National de Référence des *Escherichia coli*, *Shigella* et *Salmonella*, Unité des Bactéries Pathogènes Entériques, Institut Pasteur, 28 Rue du Docteur Roux, 75724 Paris, CEDEX 15, France: email: francois-xavier.weill@pasteur.fr

Salmonella Subtypes with Increased MICs for Azithromycin in Travelers Returned to the Netherlands

Robert-Jan Hassing, Wil H.F. Goessens, Wilfrid van Pelt, Dik J. Mevius, Bruno H. Stricker, Nicky Molhoek, Annelies Verbon, and Perry J.J. van Genderen

Antimicrobial susceptibility was analyzed for 354 typhoidal *Salmonella* isolates collected during 1999–2012 in the Netherlands. In 16.1% of all isolates and in 23.8% of all isolates that showed increased MICs for ciprofloxacin, the MIC for azithromycin was increased. This resistance may complicate empirical treatment of enteric fever.

Enteric fever caused by *Salmonella enterica* serotypes Typhi and Paratyphi A, B, and C is mainly a disease of the developing world, and it is occasionally diagnosed as an imported disease in countries where the disease is not endemic (*1*). Its empirical treatment has been hampered by resistance to ampicillin, chloramphenicol, and trimethoprim and by decreased ciprofloxacin susceptibility (MIC for ciprofloxacin 0.125–1.0 μg/mL), and ciprofloxacin resistance (MIC for ciprofloxacin >1.0 μg/mL) (*2*,*3*). As a consequence, third-generation cephalosporins are used as first-line drugs for intravenous treatment, and azithromycin is frequently used for empirical treatment of uncomplicated enteric fever.

Although no clinical breakpoints are available to define azithromycin susceptibility or resistance, several clinical studies have demonstrated good efficacy of azithromycin for the treatment of uncomplicated enteric fever in clinical and in vitro studies (*4*–*10*). Regarding MIC breakpoints, the European Committee on Antimicrobial Susceptibility Testing states that isolates with an MIC ≤16 μg/mL for azithromycin should be considered as wild-type organisms that are responsive to treatment (*3*). The Clinical and Laboratory Standards Institute (www.clsi.org/) does not provide clinical breakpoints for macrolides for the group of *Enterobacteriaceae*. In previous studies of typhoidal *Salmonella* isolates, MICs for azithromycin ranged from 4 μg/mL to 64 μg/mL (*8*–*13*).

The first clinical case for which treatment of illness caused by typhoidal *Salmonella* spp. with azithromycin (MIC 256 μg/mL) failed was reported during 2010, evidenced by testing a *S. enterica* Paratyphi A isolate from Pakistan (*14*). Further, in a study of isolates from blood samples collected in India during 2005–2008, MICs ≥16 μg/mL for azithromycin were observed in 34.7% (35/101 isolates) of the typhoidal *Salmonella* isolates; clinical nonresponse was reported in 19 of 36 patients treated with azithromycin (*13*). Whether this problem of increasing MICs for azithromycin is limited to India or is emerging globally is not clear. The objective of our study was to investigate azithromycin susceptibility and trends in antibacterial drug resistance over time in isolates collected during 1999–2012 in the Netherlands.

The Study

Enteric fever is a notifiable disease in the Netherlands. During January 1999–December 2012, a total of 354 isolates were submitted by microbiology laboratories to the Salmonella National and Community Reference Laboratory (www.eurlsalmonella.eu/): 177 (50%) *S. enterica* Typhi isolates, 98 (27.7%) *S. enterica* Paratyphi A isolates, 78 (22.0%) *S. enterica* Paratyphi B isolates, and 1 (0.3%) *S. enterica* Paratyphi C isolate. There was no statistically significant difference in sex among patients whose tests showed *S. enterica* Typhi isolates (56.3% male, 43.7% female, p = 0.18) and *S. enterica* Paratyphi isolates (51.6% male, 48.4% female, p = 0.18). Patients ranged in age from 0 to 92 years. The median ages, 28.4 and 29.5 years, respectively (p = 0.60), did not differ between patients in whose samples *S. enterica* Typhi and Paratyphi were isolated. Trends in cumulative 1-year incidence were determined by linear regression analysis; data were weighted on the number of isolates collected each year. The cumulative 1-year incidence of enteric fever was relatively stable during 1999–2012, with an average of 25 isolates (4–39/year) for that period (p = 0.42).

All MICs were determined by using the broth microdilution method with Mueller-Hinton II cation–adjusted broth (Difco, Franklin Lakes, NJ, USA). We applied European Committee for Antimicrobial Susceptibility Testing guidelines for category interpretation for different

Author affiliations: Erasmus Medical Centre, Rotterdam, the Netherlands (R.-J. Hassing, W.H.F. Goessens, B.H. Stricker, A. Verbon); Inspectorate of Health Care, The Hague, the Netherlands (R.-J. Hassing, B.H. Stricker); National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands (W. van Pelt); Utrecht University, Utrecht, the Netherlands (D.J. Mevius); Central Veterinary Institute of Wageningen UR, Lelystad, the Netherlands (D.J. Mevius); Travel Clinic Havenziekenhuis, Rotterdam (N. Molhoek, P.J.J. van Genderen); and Institute for Tropical Diseases, Havenziekenhuis, Rotterdam (P.J.J. van Genderen)

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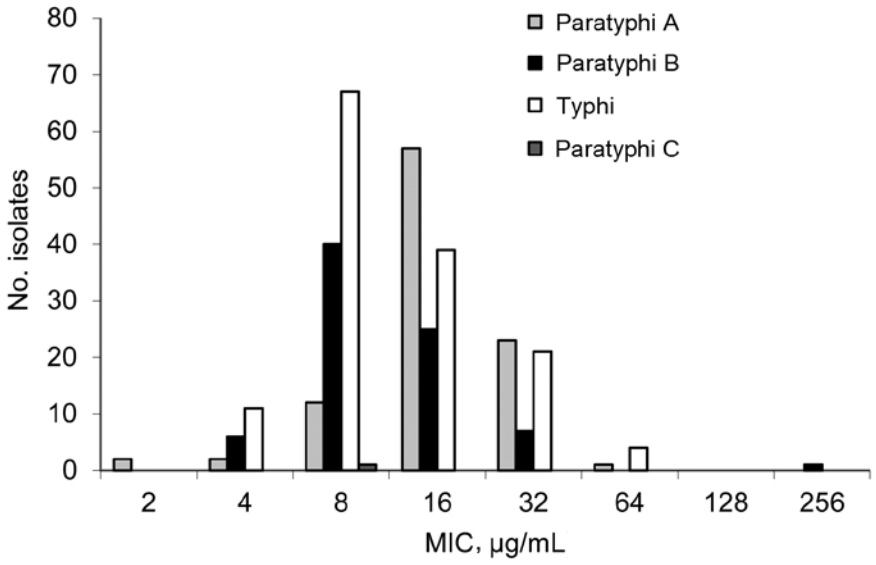

Figure 1. MICs of azithromycin of 354 *Salmonella enterica* serotypes Typhi and Paratyphi A, B, and C isolates from samples collected from ill returned travelers in the Netherlands, 1999–2012. For wild type isolates, MIC≤16 µg/mL.

antibacterial drugs (*3*). Azithromycin MICs were 2–256 µg/mL among the 354 isolates and were increased in 57 (16.1%) of the isolates (Figure 1). The distribution of azithromycin MICs of *S. enterica* Typhi and Paratyphi A and B peaked at 8, 16, and 8 µg/mL, respectively (Figure 1). Trend analysis showed no increased MIC over time for all isolates (p = 0.21) or for *S. enterica* Typhi (p = 0.35) or Paratyphi (p = 0.70). One Paratyphi A isolate, from a sample acquired in Malaysia in 2007, required an MIC of 256 µg/mL. Decreased susceptibility to ciprofloxacin was observed in 116 (32.8%) and ciprofloxacin resistance in 6 (1.7%) of the 354 isolates. Cumulative 1-year incidence of isolates with decreased susceptibility or resistance to ciprofloxacin increased significantly from 0% (0/12 isolates) in 1999 to 64.3% (18/28 isolates) in 2012 (p<0.001) (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/20/4/13-1536-Techapp1.pdf). Among isolates with decreased susceptibility or resistance to ciprofloxacin, 23.8% (29/122 isolates) showed an increased MIC for azithromycin; MIC increased for 12.1% (28/232 isolates) of the ciprofloxacin-susceptible isolates (p = 0.004) (Figure 2). No significant increase in amoxicillin, trimethoprim, or chloramphenicol resistance was observed (amoxicillin, p = 0.97; trimethoprim, p = 0.95; and chloramphenicol, p = 0.99) (online Technical Appendix Figure). For all isolates, the MICs for erythromycin ranged from 64 to ≥512 µg/mL. Resistance to third-generation cephalosporins was not observed in the isolates.

The origins of isolates for which the country of acquisition was known (205 of 354 strains) were distributed among geographic regions by using the United Nations geoscheme (http://unstats.un.org/unsd/methods/m49/m49regin.htm). Besides imported cases from countries in which enteric fever is highly endemic, such as India, Indonesia, and Pakistan, rates of importation were high for travelers from Turkey and Morocco (Table).

Percentages of elevated MICs for azithromycin were highest for isolates acquired in regions that had concurrent high proportions of isolates with decreased susceptibility or resistance to ciprofloxacin (Table). In isolates acquired in countries from Southern Asia, increased MICs for ciprofloxacin and increased MICs for azithromycin were observed in 21.4% (18/84 isolates) of the isolates.

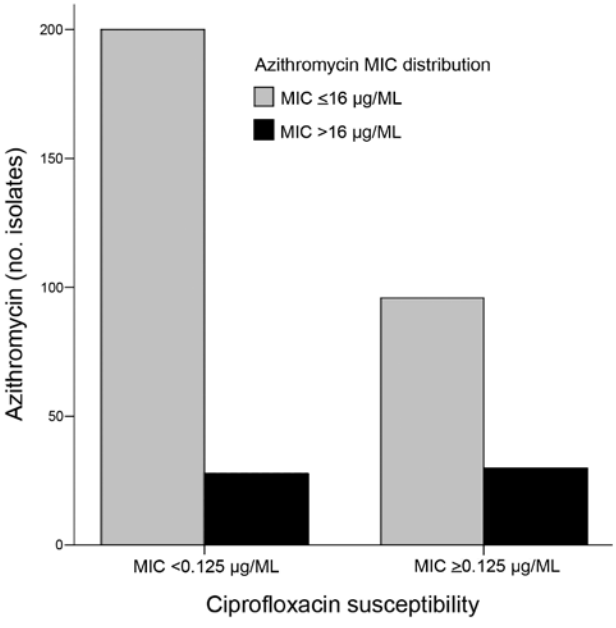

Figure 2. MICs of azithromycin in relation to ciprofloxacin susceptibility of 354 *Salmonella enterica* serotypes Typhi and Paratyphi isolates. Increased MICs for azithromycin (MIC >16 µg/mL) in isolates with decreased ciprofloxacin susceptibility or ciprofloxacin resistance (MIC ≥0.125 µg/mL) versus ciprofloxacin-susceptible isolates (MIC <0.125 µg/mL) (p = 0.004).

Table. Isolates with elevated MICs for ciprofloxacin and isolates with an MIC for azithromycin above that for wild type (>16 mg/L) in *Salmonella enterica* serotype Typhi and Paratyphi isolates collected in travelers returning to the Netherlands, 1999–2012

Region	No. (%) isolates	No. (%) isolates with elevated MICs		
		Azithromycin	Ciprofloxacin	Azithromycin and ciprofloxacin
All	205	36 (17.6)	83 (40.5)	20 (9.8)
Asia	161 (78.5)	31 (19.3)	80 (49.7)	20 (12.4)
Southern Asia*	84 (40.0)	21 (25)	69 (82.1)	18 (21.4)
South-Eastern Asia†	44 (21.5)	5 (12.2)	3 (6.8)	1 (2.3)
Western Asia‡	27 (13.2)	1 (3.7)	6 (22.2)	1 (3.7)
Eastern Asia§	5 (2.4)	1 (20)	2 (40)	0
Unknown	1 (0.5)	0	0	0
Africa¶	35 (17.1)	5 (14.3)	1 (2.9)	0
Europe**	4 (2.0)	0	2 (50)	0
Latin America††	5 (2.4)	0	0	0

*India, Pakistan, Bangladesh, Nepal, Afghanistan, Nepal, Sri Lanka.
†Indonesia, Cambodia, Malaysia, Thailand.
‡Turkey, Iraq, Syria.
§China.
¶Morocco, Ghana, Nigeria, Senegal, Tunisia, Malawi, Tanzania.
**Gibraltar (Great Britain), Greece, Italy, Romania.
††Peru, Mexico, unknown.

Conclusions

We found high percentages of elevated azithromycin MICs in typhoidal *Salmonella* isolates collected in the Netherlands during 1999–2012. MICs >16 μg/mL for azithromycin were found in 16.1% of all isolates and in 23.8% of isolates with elevated MICs for ciprofloxacin. This observation may be explained by increased use of azithromycin in countries from which samples yielded high rates of typhoidal *Salmonella* isolates with decreased susceptibility or resistance to ciprofloxacin. Moreover, our findings are aligned with an alarming report from India on increasing MICs for azithromycin (*13*). Our study shows higher MICs than anticipated based on another Western case series (*11*), implying that these potentially resistant strains are not confined to India.

Besides treatment with third-generation cephalosporins, empirical treatment options may be scarce for patients with potential azithromycin-resistant *Salmonella* serotypes. Reuse of antibacterial drugs, such as ampicillin, chloramphenicol, or trimethoprim may be a valuable treatment option upon proven susceptibility, but widespread use of these antibacterial drugs as first-line treatment will likely result in rapid reemergence of multidrug resistance and associated drug-related adverse effects. Further, increasing the dose of ciprofloxacin or using alternative fluoroquinolones has been suggested as an effective treatment in some cases (*15*). This option will not be feasible for empirical treatment because it applies only in a minority of cases and may be associated with drug toxicity. The danger of losing azithromycin to antimicrobial resistance could be detrimental in countries faced with endemic or epidemic enteric fever and complicated by poverty; therefore, azithromycin should be used with care. The results of this study also implicate the importance of developing more effective vaccines as control measures for enteric fever. Future research is needed to evaluate clinically relevant breakpoints of azithromycin by analyzing the treatment outcome of azithromycin in relation to their MICs.

In conclusion, typhoidal *Salmonella* isolates in ill returned travelers from the Netherlands already show a high percentage of increased MICs for azithromycin. Because the highest proportions of increased MICs for azithromycin are found in isolates with increased MICs for ciprofloxacin and in regions where decreased susceptibility or resistance to ciprofloxacin is already widely prevalent among *S. enterica* Typhi and Paratyphi isolates, this resistance may further limit future treatment options for enteric fever.

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Dr Hassing is a PhD candidate and an infectious diseases specialist at the Erasmus Medical Centre, Rotterdam, the Netherlands, and a pharmacovigilance inspector at the Health Care Inspectorate, The Hague, the Netherlands. His research interests include antimicrobial resistance in *Enterobacteriaceae*.

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Address for correspondence: Robert-Jan Hassing, Erasmus Medical Centre, PO Box 2040, 3000 CA Rotterdam, the Netherlands; email: r.hassing@erasmusmc.nl

Complete Genome of Hepatitis E Virus from Laboratory Ferrets

Tian-Cheng Li, Tingting Yang, Yasushi Ami, Yuriko Suzaki, Masayuki Shirakura, Noriko Kishida, Hideki Asanuma, Naokazu Takeda, and Wakita Takaji

The complete genome of hepatitis E virus (HEV) from laboratory ferrets imported from the United States was identified. This virus shared only 82.4%–82.5% nt sequence identities with strains from the Netherlands, which indicated that the ferret HEV genome is genetically diverse. Some laboratory ferrets were contaminated with HEV.

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus that belongs to the family *Hepeviridae*, genus *Hepevirus*, and is the causative agent of hepatitis E (*1,2*). Because the transmission of HEV from deer, swine, and wild boars to humans is well known, hepatitis E is recognized as a zoonosis. Zoonotic hepatitis E is associated mainly with genotype 3 (G3) and G4 HEV infection (*3,4*). In addition to deer, swine, and wild boars, other animals, including monkeys, rats, ferrets, chickens, and bats, harbor HEV or HEV-like viruses (*5–9*). The genus *Hepevirus* might include 3 additional species (avian HEV, bat HEV, and rat/ferret HEV) (*10*). However, whether HEV from these animals is transmitted to humans is not clear.

HEV has been detected in ferrets (*Mustela putorius*) in the Netherlands (*6*). The ferret HEV genome contains 3 open reading frames (ORFs 1–3). ORF1 encodes a nonstructural protein of 1,596; ORF2 encodes a capsid protein of 654 aa, and ORF3 encodes a functionally unknown phosphoprotein of 108 aa. A putative ORF4 observed in the ferret HEV genome was also found in the rat HEV genome. Nucleotide sequence analyses indicated that the ferret HEV genome shares the highest nucleotide sequence identity (72.3%) with rat HEV. The nucleotide sequence identity between the ferret HEV and G1–4 HEV, rabbit HEV, and avian HEV ranges from 54.5% to 60.5% (*6*). However, the antigenicity, pathogenicity, and epidemiology of ferret HEV remain unclear.

Ferret HEV was also recently detected in the United States in serum (*11*), suggesting that ferret HEV infection is not restricted to the Netherlands and might be distributed in ferrets worldwide. Because ferrets are susceptible to several respiratory viruses, including human and avian influenza viruses, and severe acute respiratory syndrome coronavirus (*12,13*), ferrets have been used as a small-animal model for these viruses. Ferrets are also kept as pets in many countries. Thus, information about ferret HEV epidemiology, distribution, transmission, and pathogenesis is urgently needed.

In this study, we amplified and analyzed the complete genome of the US strains of ferret HEV to confirm whether US strains are new ferret HEV genotypes. Phylogenetic analysis demonstrated that HEVs detected in laboratory ferrets from the United States are genetically different from those detected in the Netherlands, suggesting that the ferret HEV genome is genetically diverse.

The Study

Sixty-three fecal samples were collected from laboratory ferrets (*Mustela putorius furo*) at the National Institute of Infectious Diseases, Tokyo, Japan, on May 24, 2013. These ferrets had been imported from a farm in the United States for influenza research 7 days before sample collection. Fecal specimens were diluted with 10 mmol/L phosphate-buffered saline to prepare a 10% suspension, shaken at 4°C for 1 h, and clarified by centrifugation at 10,000 × *g* for 30 min. The supernatant was passed through a 0.45-μm membrane filter (Millipore, Bedford, MA, USA), and stored at –80°C until use.

RNA was extracted by using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations. Reverse transcription was performed by using the Superscript II RNase H^- reverse transcription procedure (Invitrogen, Carlsbad, CA, USA) and primer TX30SXN as described (*14*). Ferret HEV RNA was detected by using a nested, broad-spectrum reverse transcription PCR (*15*). Forty (63.5%) of 63 fecal specimens were positive for ferret HEV RNA. Sequences were similar to those detected in ferret serum samples in the United States (*11*), which suggested that the laboratory ferrets were infected in the United States and then transported to Japan.

RNA from 2 ferret HEVs was randomly selected, and the full-length genome was amplified by using reverse transcription PCR with primers based on nucleotide sequences derived from strains from the Netherlands and United States (Table 1). Sequence of the 5′-terminal noncoding regions of the genome was determined by using Rapid Amplification of cDNA Ends Kits (Invitrogen) according to the

Author affiliations: National Institute of Infectious Diseases, Tokyo, Japan (T.-C. Li, Y. Ami, Y, Suzaki, M. Shirakura, N. Kishida, H. Asanuma, W. Takaji); Affiliated Hospital of Qingdao University Medical College, Qingdao, China (T. Yang); and Osaka University, Osaka, Japan (N. Takeda)

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Table 1. Oligonucleotides used to amplify ferret hepatitis E viruses

Primer (nucleotide positions)*	Sequence, 5′→3′	Product length, bp†
Forward FF1 (1–21)	GGCAGACCCCTAATGGAGACA	
Reverse FR628 (628–648)	GTTGCGTGCGACATAGGCCTT	626
Forward FF541 (541–561)	AGCAATGTATCGCCATGGCAT	
Reverse FR1535 (1535–1554)	ATCTGCATCAGTCGGGCACA	1,014
Forward FF1518 (1518–1538)	AGGATCTGACAGTAGACCTGT	
Reverse FR2555 (2555–2577)	TGCAATGCCAAATTAGCTGTGT	1,060
Forward FF2401 (2401–2421)	GGCGATGAGTTGTACCTGTTA	
Reverse FR3424 (3432–3445)	GAGCAGCCGGTAACATACTCAA	1,045
Forward FF3336 (3336–3355)	GCACAATTTCTATCTCACCA	
Reverse FR4210 (4210–4230)	ACTCCGAATCAGATGATACA	985
Forward FF4181 (4181–4202)	GGCTGGTGCACCTGAATGGCT	
Reverse FR5800 (5800–5821)	TCAGGCAGACGGCGTATCTTAT	1,641
Forward FF4812 (4812–4831)	ATGGAGCATGTGTACAAGAT	
Reverse TX30SXN	GACTAGTTCTAGATCGCGAGCGGCCGCCC TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	≈2,050
Reverse FR451‡	ACACCGTGTGAATCCCTCCGT	
Abridged amplification	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	
Reverse FR279 (279–300)	ATAGATCTAGGATGCGCACCAA	§
Abridged universal amplification	GGCCACGCGTCGACTAGTAC	
Reverse FR191 (191–211)	CGGATGCGACCAAACAACAGA	≈240

*Values in parentheses indicate positions of the primer corresponding to the entire genome of hepatitis E virus (JN998607) isolated from ferret.
†Blank cell indicates that 1 primer pair produced 1 product.
‡Used only for cDNA synthesis.
§PCR product was not detected.

manufacturer's instructions. All PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and cloned into the TA cloning vector pCR2.1 (Invitrogen). Nucleotide sequencing was conducted by using an ABI 3130 Genetic Analyzer Automated Aequencer (Applied Biosystems, Foster City, CA, USA).

Both ferret HEV genomes consisted of 6,820 nt and a poly (A) tail (GenBank accession nos. AB890001 and AB890374), and nucleotide sequence identity was 99.6%. Genomic structure of strains from the United States was similar to that of 2 strains from the Netherlands. The amino acid alignment of ORF2 indicated that ferret HEV ORF2 has an additional 6 amino acids at the N terminus. However, because the seventh codon is AUG, it is unclear which codon was used for the ORF2 translation initiation.

The ORF1 of strains from the United States encodes 1,589 aa, which is 7 aa shorter than ORF1 of both strains from the Netherlands. In addition, the ferret HEV strains from the United States have 2 aa insertions between amino acid residues 596–597 (Thr) and 631–632 (Ile) and 9 aa deletions in amino acid residues 645–653 (Cys-Leu-Arg-Ser-Ser-Pro-Lys-Pro-Pro), which corresponds to those of strains from the Netherlands. Similar to ferret HEV from the Netherlands, an additional putative 183-aa ORF 4 (nt 30–581) was found in strains from the United States. Analysis of 5 other entire ORF2 sequences (GenBank accession nos. AB890375–AB890379) showed that nucleotide identities among them were 98.9%–99.5%, which indicated that genetically similar ferret HEV strains had circulated at the ferret farm in the United States.

Nucleotide and deduced amino acid sequence identities between ferret HEV from the United States and other HEVs are shown in Table 2. The entire genome of strains from the United States shared relatively high nucleotide

Table 2. Nucleotide and deduced amino acid sequence identities between ferret HEV from the United States and other HEVs*

HEV strain (GenBank accession no.)	Full-length genome, %	Ferret HEV (AB890374)					
		Nucleotides, %			Amino acids, %		
		ORF1	ORF2	ORF3	ORF1	ORF2	ORF3
Genotype 1 (NC-001434)	53.6	51.4	58.9	50.3	51.4	56.9	22.2
Genotype 2 (M74506)	53.8	51.7	59.3	49.4	56.0	57.2	25.2
Genotype 3 (AF060668)	55.2	53.6	59.4	47.9	54.5	58.1	22.9
Genotype 4 (AJ272108)	53.9	51.5	59.5	49.4	53.5	57.5	28.8
Wild boar HEV (AB573435)	54.4	51.9	60.5	49.1	57.2	57.3	24.3
Wild boar HEV (AB602441)	54.0	51.9	59.1	50.9	57.4	57.0	31.5
Rabbit HEV (FJ906895)	54.8	57.0	60.5	51.2	55.2	57.7	23.3
Rat HEV (GU345042)	61.2	57.0	71.3	63.3	70.1	79.4	40.4
Rat HEV (JX120573)	62.6	58.1	72.8	64.9	74.1	80.0	43.5
Ferret HEV (998606)	82.4	81.4	84.9	85.9	91.5	94.2	73.1
Ferret HEV (998607)	82.4	81.3	85.1	85.9	91.3	94.8	73.1
Ferret HEV (AB890001)	99.6	99.7	99.5	100.0	99.7	99.8	100.0
Avian HEV (AY535004)	50.8	50.5	54.2	47.0	43.1	47.9	24.2

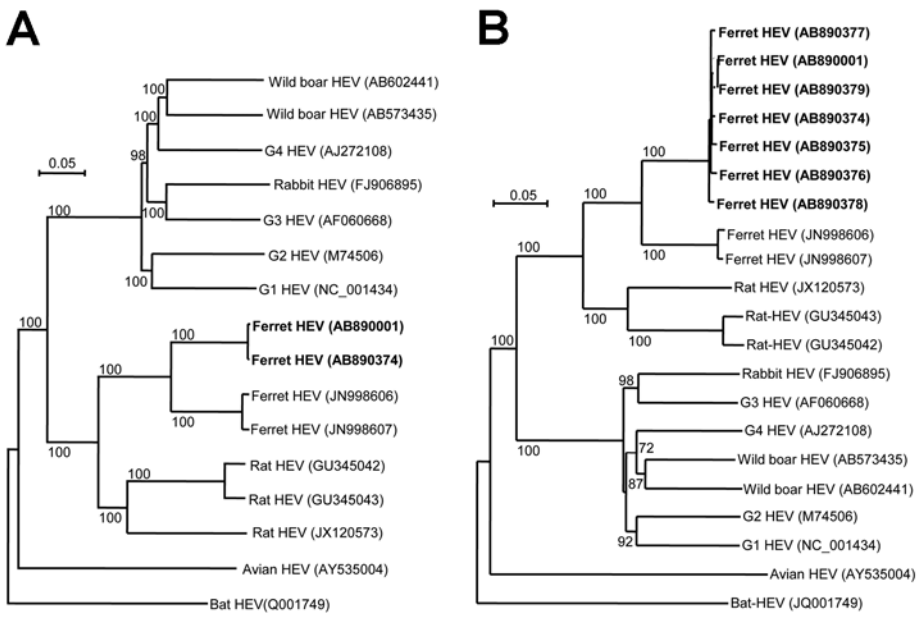

Figure. Phylogenetic relationships among genotypes 1–4 and wild boar, rabbit, rat, avian, bat, and ferret isolates of hepatitis E virus. Nucleic acid sequence alignment was performed by using Clustal X 1.81 (www.clustal.org/clustal2/). Genetic distance was calculated by using Kimura's 2-parameter method. Phylogenetic trees with 1,000 bootstrap replicates were generated by using the neighbor-joining method (Njplot 2.3, http://njplot.sharewarejunction.com/) based on A) the entire genome and B) open reading frame 2. Items in boldface indicate strains isolated in this study. Numbers along branches indicate bootstrap values. Scale bars indicate nucleotide substitutions per site.

sequence identities (82.4%–82.5%) with strains from the Netherlands. We generated phylogenetic trees based on ORF2 or the entire genome. These trees showed that although strains from the United States were closely related to strains from the Netherlands, they formed a new and distinct cluster (Figure). We observed similar phylogenetic clustering when we analyzed nucleotide sequences of ORF1 and ORF3 separately. Although we cannot conclude whether ferret HEV from the United States is a new genotype, these results indicated that there is genetic variety in ferret HEV. Researchers should also bear in mind that some laboratory ferrets are contaminated with ferret HEV.

Conclusions

We amplified the entire genome of 2 ferret HEV strains isolated from laboratory ferrets imported from the United States. Nucleotide sequence comparisons showed that 2 ferret HEV strains from the United States had high (99.6%) identity and shared 98.6%–100% identities with partial sequences of ORF1 that were detected in the United States (*11*), which indicated that genetically similar ferret HEV was circulating in laboratory ferrets.

Although nucleotide sequence identities of the entire genome for strains from the United States and the Netherlands was 82.4%–82.5%, ORF2 showed relatively high amino acid identities (94.2%–94.8%), which suggested that isolated from the United States and the Netherlands share similar antigenicity. Ferret HEV–like particles derived from 1 of the isolates from the Netherlands were cross-reactive with serum from HEV-infected laboratory ferrets in the United States (*11*).

In conclusion, we isolated and identified 2 ferret HEV strains from laboratory ferrets imported from the United States. These strains were genetically distinct from ferret HEV isolates from the Netherlands. Some laboratory ferrets were contaminated with ferret HEV. Further studies are needed to confirm the pathogenicity and zoonotic potential of ferret HEV.

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Dr Li is a senior researcher at the National Institute of Infectious Diseases, Tokyo, Japan. His research interests are epidemiology, expression of viral proteins, and 3-dimensional structure of HEV.

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Address for correspondence: Tian-Cheng Li, Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan; email: litc@nih.go.jp

Incorporating Research and Evaluation into Pandemic Influenza Vaccination Preparedness and Response

Tom T. Shimabukuro and Stephen C. Redd

This issue of Emerging Infectious Diseases contains 2 articles that address critical elements of implementing large-scale local-level vaccination programs in response to a public health emergency. In addition to describing activities undertaken during the 2009 pandemic of influenza A(H1N1) virus (pH1N1) and subsequent program evaluation and lessons learned, the articles highlight the critical role for scientific evaluation in improving our ability to respond to emergencies (*1*,*2*).

Saha et al. report on assessment of the efficiency of public health–managed large-scale vaccination clinics, referred to as points of dispensing (PODs), to administer pH1N1 vaccine in densely populated Los Angeles County, California, USA (*3*). The authors examined rates of visits to PODs according to patients' socioethnic characteristics and assessed factors affecting vaccination throughput (doses administered per hour). Their evaluation provides information about optimal placement of PODs in the community and possible strategies to improve their operational efficiency.

Marcello et al. describe the experience of New York City, New York, USA, in using its Citywide Immunization Registry to capture information about pH1N1 vaccine doses administered during the response (*4*). Immunization information systems (IISs) are commonly used to document administration of recommended childhood vaccinations; however, routine adult participation has historically been low (*5*). During New York City's pH1N1 vaccination program, the health department required all providers to register with the Citywide Immunization Registry and report doses administered. The New York City experience demonstrates the feasibility and potential usefulness of expanding mandatory IIS reporting to all types of providers during a pandemic influenza vaccination program as a means of monitoring progress and managing supply and distribution. The article also reveals limitations of IISs as they existed in 2009 and 2010.

Los Angeles County and New York City were able to conduct meaningful program evaluation because public health officials had the foresight to incorporate evaluation into emergency planning and response and commit valuable time and resources to conduct health services research during the height of pH1N1 vaccination. Los Angeles County health officials coordinated a meticulous data collection effort from 101 POD events held during a 6-week period from October through December 2009. In New York City, a substantial outreach and education program was necessary to incorporate providers of vaccines to adults and others not accustomed to IIS reporting into the program to acquire the most comprehensive and timely information possible about vaccine doses administered.

For health departments, the decision to commit to planning for and conducting research and evaluation during a public health emergency is complicated by the competing priority of providing direct services to persons and populations in need. In addition to balancing the effort needed to plan and conduct the public health response and the research or evaluation effort, other uncertainties impose limitations on research efforts during emergencies. In the case of research conducted during the 2009 influenza A(H1N1) pandemic, the inherent difficulty of projecting demand for vaccination, combined with delays and uncertainty around the timing of availability of pH1N1 vaccine, were serious challenges for the vaccination program and for its evaluation (*6*).

Although researchers have to make assumptions about the event under study, a high degree of flexibility is necessary. Public health emergencies often present unforeseen

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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circumstances and influenza pandemics are among the least predictable of all emergencies. Public health priorities during a pandemic response can change quickly on the basis of disease characteristics, resource constraints, and the potential for social disruption. Planning evaluation efforts for different pandemic scenarios and being nimble enough to rapidly adapt to shifting priorities are essential qualities for any research and evaluation program. Research planners need to be able to identify and address key response questions under conditions of much less certainty than in other research efforts.

Despite these challenges, invaluable knowledge is gained from well-planned and well-conducted (and appropriately resourced) health services research during an event. Tabletop and functional exercises are useful tools for organizations to expand knowledge, assess readiness, and identify deficiencies (*7*,*8*). Yet they rarely approach the intensity, complexity, and duration of a real event. Data obtained during a response to an actual public health emergency provide the best (and perhaps only) source of information for program evaluation under conditions in which the public health system is severely stressed. To address knowledge gaps in preparedness, public health authorities must strike an appropriate balance between conducting research, evaluating program efforts, and providing services during a public health emergency (*2*). Publishing the results of such evaluations is also essential to permit others who are planning vaccination campaigns during emergencies to benefit from the experiences in Los Angeles County and New York City.

Human infection with influenza A(H3N2) variant virus (*9*) and avian influenza A(H7N9) virus (*10*) and continued sporadic cases of infection with highly pathogenic avian influenza A(H5N1) virus (*11*)—all viruses with pandemic potential—remind us that we must remain vigilant in our preparedness. We encourage health officials and leaders of health care organizations at all levels to identify the critical questions that will affect future emergencies and design research efforts into emergency preparedness planning to take advantage of these rare opportunities to learn and improve the nation's response capability. For vaccination programs during influenza pandemics, priorities for evaluation include the following: efforts to improve situational awareness; efforts to identify and vaccinate populations prioritized for vaccination, including vulnerable populations and groups prioritized because of occupation; strategies to balance vaccine allocation to the existing, largely private, vaccination system, with large-scale vaccination venues (i.e., PODs); and strategies to build systems for pandemic influenza response that also improve seasonal influenza vaccination programs.

Although we recognize that smaller and less well-resourced organizations may be challenged in their ability to conduct large-scale sophisticated evaluation, we believe that learning by doing is possible for any organization, provided leaders and planners are willing to make the commitment. Even modest evaluation efforts will increase knowledge and advance preparedness.

Dr Shimabukuro is acting deputy director of the Immunization Safety Office, Centers for Disease Control and Prevention (CDC), Atlanta. During the 2009 influenza A(H1N1) pandemic, he served as deputy director of CDC's H1N1 Vaccine Task Force.

Dr Redd is the director of the Influenza Coordination Unit, CDC, Atlanta. He served as incident commander of CDC's response to the 2009 influenza A(H1N1) pandemic.

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Address for correspondence: Tom T. Shimabukuro, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D26, Atlanta, GA 30333, USA; email: tshimabukuro@cdc.gov

Decline of *Salmonella enterica* Serotype Choleraesuis Infections, Taiwan

To the Editor: Human salmonellosis is a major public health problem of global concern. *Salmonella enterica* serotype Choleraesuis is a nontyphoid serotype; it has a narrow host range and is associated with a high proportion of invasive infections (50%–90% of isolates are from sterile sites [(*1*)]). In 2000, emergence of fluoroquinolone resistance in *S. enterica* ser. Choleraesuis was reported from Chang Gung Memorial Hospital at Linkou, Taiwan (*2*). Since then, we have continuously monitored the trend of these infections at this hospital in northern Taiwan and at Chang Gung Memorial Hospital at Kaohsiung in southern Taiwan. We report the decline of *S. enterica* ser. Choleraesuis infections coinciding with the implementation of several national control programs in Taiwan.

In humans, *S. enterica* ser. Choleraesuis usually causes invasive infections (*1*). Transmission of the organism is probably from pigs to humans (*3*). According to an inspection program conducted by the Council of Agriculture in 2004, illegal slaughter of pigs dying of unidentified diseases and sale of the pork at a reduced price were common in Taiwan and probably were associated with transmission of the organism (*4*). In Taiwan during 1996–1997, a large epidemic of swine foot-and-mouth disease occurred (*5*), and consumption of pork decreased substantially. To prevent further spread of that epidemic, the government implemented massive slaughter of infected pigs and a swine vaccination policy (*5*). Coincidently, in 1996–1998, *S. enterica* ser. Choleraesuis infections among humans decreased significantly ($p<0.001$) (*2*). However, during the following years, infections among humans and antimicrobial drug resistance gradually increased (*2*). In Taiwan, widespread use of antimicrobial drugs in animals, which might contribute to development of antimicrobial drug resistance in human pathogens, was subsequently reported (*6*).

The Taiwan government took further actions to improve the quality of pig husbandry. In 2002, a live attenuated *S. enterica* ser. Choleraesuis vaccine (Suisaloral; Impfstoffwerk Dessau-Tornau GmbH, Rosslau, Germany) for pigs was licensed for importation (veterinary drug license no. 06387) (*7*). In 2004, the following approaches were implemented to consolidate the management of animal husbandry and ensure the quality and safety of meat: improving pollution control and sanitary conditions in pig farms by periodic inspection and education programs; implementing regulations to promote and improve an existing quality food certification system, Certified Agricultural Standards, by tracking and auditing illicit labeling; reinforcing monitoring and tracking systems for the sale and consumption of antimicrobial drugs for animal use (by continuing education and inspections with or without fixed schedules); preventing illegal slaughtering and sale of pigs dying of unidentified diseases (by inspections and monetary penalties for violations); and establishing a Taiwan Agriculture and Food Traceability system (http://taft.coa.gov.tw) to fully record all processes from farm to table (*4*,*8*). A particularly notable control measure launched at that time was a death insurance program for pigs. Although farmers were fully compensated for their losses, pigs dying of unidentified diseases were collected and chemically marked to prevent the possibility of subsequent illegal use (*4*). In 2005, 2 representative counties in central-southern Taiwan tried the program. During the next year, the program was extended to 8 neighboring counties, and in 2007, it was implemented in all counties (*9*). Furthermore, since the establishment of the Taiwan Agriculture and Food Traceability system, various Taiwan Good Agricultural Practice manuals have been gradually developed, and in 2008, a manual for pig farming established official standard operating procedures (*10*).

The control measures seem to be successful, as evidenced by the results of a long-term monitoring program at the 2 large tertiary care hospitals in northern and southern Taiwan (Figure). *S. enterica* ser. Choleraesuis infections have declined significantly since 2005 in southern Taiwan (Figure, panel A) and since 2006 in northern Taiwan (Figure, panel B) (χ^2 test for trend, $p<0.01$). At the southern hospital, the number of nonrepetitive clinical isolates was reduced by almost half in 2005 (Figure, panel A), the year the death insurance program was initiated in central-southern Taiwan. The reduction was even larger at the other hospital. The annual number of clinical isolates obtained decreased from >80 in 2004–2005 to 24 in 2006 (Figure, panel B), when the death insurance program was expanded to more areas. At both hospitals, the annual number of *S. enterica* ser. Choleraesuis infections had decreased consistently in the subsequent years; since 2008, this number has remained <10 (Figure).

After interruption of the identified infection chain, the substantial decline of infection among humans became evident. Despite the absence of national surveillance data for nontyphoid human salmonellosis in Taiwan, the decrease in *S. enterica* ser. Choleraesuis infections reported herein has also been noted at other hospitals in Taiwan (L.-H. Su, pers. comm.). This finding demonstrates that application of effective control measures on farms and in agricultural practices can lead to successful control of *S. enterica* ser. Choleraesuis infection among humans.

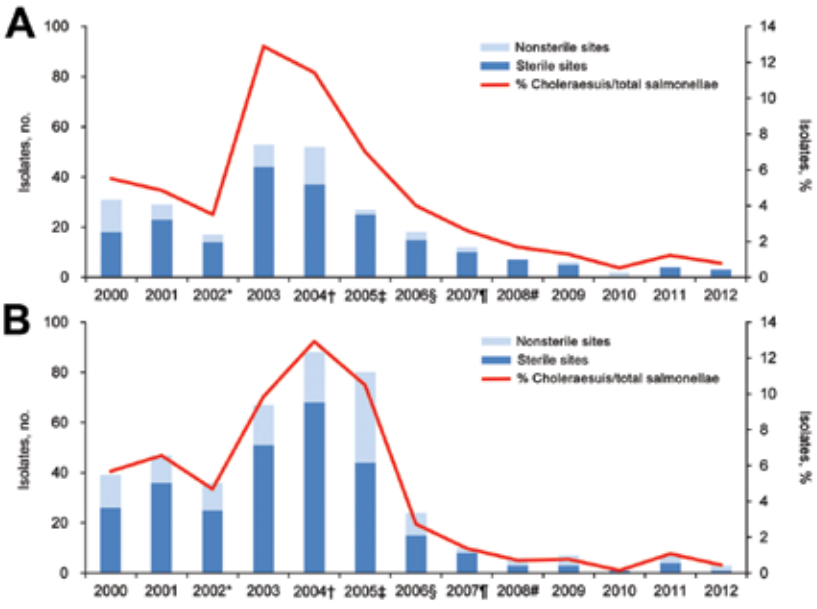

Figure. Trends of annual numbers and percentages of *Salmonella enterica* serotype Choleraesuis isolates from 2 tertiary care hospitals in Taiwan. A) Data from Chang Gung Memorial Hospital at Kaohsiung, southern Taiwan. B) Data from Chang Gung Memorial Hospital at Linkou, northern Taiwan. *Approval and importation of vaccine for swine. †Promotion of the Certified Agricultural Standards quality food certification system (*4*), monitoring of sale of antimicrobial drugs for animal use (*4*), inspection of chemical residues in swine farms and pork market, launch of educational programs about safe use of drugs in animals (*4*); inspection of illegal slaughtering and sale of farmed animals dying of unidentified disease (*4*); and establishment of Taiwan Agriculture and Food Traceability system (*4,8*). ‡Initiation of death insurance program for pigs in 2 representative central-southern counties (*9*). §Extension of death insurance program to another 8 neighboring counties (*9*). ¶Full implementation of death insurance program throughout all Taiwan counties (*9*). #Establishment of Taiwan Good Agricultural Practice for pig husbandry (*10*).

Lin-Hui Su, Tsu-Lan Wu, and Cheng-Hsun Chiu

Author affiliations: Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Taoyuan, Taiwan (L.-H. Su, T.-L. Wu); and Chang Gung Children's Hospital, Chang Gung University College of Medicine, Taoyuan (C.-H. Chiu)

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Address for correspondence: Cheng-Hsun Chiu, Division of Pediatric Infectious Diseases, Department of Pediatrics, Chang Gung Children's Hospital, 5 Fu-Hsin St, Kweishan, Taoyuan 333, Taiwan; email: chchiu@adm.cgmh.org.tw

Detection of *Rickettsia sibirica mongolitimonae* by Using Cutaneous Swab Samples and Quantitative PCR

To the Editor: Tick-borne rickettsioses are caused by the obligate intracellular bacteria spotted fever group (SFG) *Rickettsia* spp. These zoonoses are now recognized as emerging or reemerging human infections worldwide, with ≈15 new tick-borne rickettsial species or subspecies recognized as human pathogens during the 30 past years (*1*). New approaches have emerged in recent years to definitively identify the causative agents, including emerging pathogens. Using cutaneous swab specimens from patients for quantitative PCR (qPCR) testing rather than cutaneous biopsy specimens is

a major innovation in the diagnosis of SFG rickettsioses (*2*–*4*). Using this approach, we report 1 of the few documented infections caused by *Rickettsia sibirica mongolitimonae*.

A 16-year-old boy with no medical history was admitted to the Department of Infectious diseases at University Hospital in Marseille on May 25, 2012, with a fever (40°C) and skin lesions on his lower right eyelid. He had been fishing 7 days earlier at a pond situated in southern France near Marseille (43°26′N, 5°6′E). He had been given amoxicillin/clavulanic acid by his family doctor and showed no improvement after 2 days. The only sign on physical examination was the presence of 2 eschars on his lower right eyelid, associated with right periorbital edema (Figure) and painful right-sided cervical lymphadenopathies. Results of standard laboratory tests were normal except for the C-reactive protein level (21 mg/L; reference value <10 mg/L). He reported that the black spots on his lower eyelid were most likely related to bites from ticks that he got while fishing. He removed the ticks the next day. Because a tick-borne rickettsiosis was suspected, oral empirical treatment with doxycycline (200 mg/daily) was started. The patient improved in 48 hours and remained well (Figure).

The first serologic test result for *Rickettsia* spp. was negative. Because of the location of the eschars, it was not possible to obtain biopsy specimens from them. Nevertheless, real-time qPCR that was performed on 2 eschar swab specimens showed positive results for *Rickettsia* spp in 24 hours. The specific qPCR test results were positive for *Rickettsia sibirica mongolitimonae* in both samples (*1*).

Amplification and sequencing of a fragment of *ompA* gene on these samples showed 100% (533/533) identity with *R. sibirica mongolitimonae* HA-91 (RHU43796). Four days later, after doxycycline treatment, 1 additional swab specimen was positive by specific qPCR for *R. sibirica mongolitimonae.* The convalescent-phase serum specimen (obtained 14 days after admission) was positive by indirect immunofluorescence assay for rickettsial antigens against SFG, suggesting seroconversion.

Figure. Palpebral eschars caused by *Rickettsia sibirica mongolitimonae* infection in a 16-year-old febrile boy with fever, southern France, spring, 2012 (left). He recovered after doxycycline treatment (right).

R. sibirica mongolitimonae is an intracellular bacterium that was recognized as a human pathogen in 1996 (*1*). The inoculation eschar at the tick bite site is a hallmark of many tick-borne SPG rickettsioses. However, because lymphangitis was also observed in a few of the patients reported subsequently, *R. sibirica mongolitimonae* infection was named lymphangitis-associated rickettsiosis (*5*). To date, 24 cases have been reported in Europe (France, Spain, Portugal, Greece) and 3 in Africa (Egypt, Algeria, South Africa) (*6*,*7*). Vectors include ticks in the genus *Hyalomma* and also *Rhipicephalus pusillus*, a species of tick found on the European wild rabbit (also can be found on wild carnivorous animals, dogs, and domestic cats), which may bite humans (*7*). The life-threatening Mediterranean spotted fever caused by *R. conorii* peaks in the warmer months of July and August because of a heat-mediated increase in the aggressiveness and, therefore propensity to bite humans, of the brown dog tick vector, *R. sanguineus* (*8*). In contrast, *R. sibirica mongolitimonae* infection is more frequently reported in the spring (*7*).

The diagnosis of rickettsioses is most commonly based on serologic testing (*1*). However, serologic evidence of infection generally appears in the second and third weeks of illness, as in the case-patient described here. The use of molecular tools or cell culture on a skin biopsy specimen from an eschar is the best method of identifying *Rickettsia* spp. However, this invasive and painful procedure needs to be performed in sterile conditions with local anesthesia. Swabbing an eschar is easy and painless; the physician only needs a dry sterile swab that must be directed, while being rotated vigorously, to the base of the eschar, after the crust is removed (*4*). The sensitivity of this technique is comparable with that of rickettsial detection on skin biopsy samples by molecular tools. If the eschar lesion is dry, a wet compress, previously humidified with sterile water, should be placed on the inoculation eschar for 1 minute before swabbing, to increase the quantity of material swabbed. In addition, the crust eschar also can be used for rickettsial diagnosis. Because sufficient material can be obtained during swabbing, this test can be used by any practitioner at the patient's bedside. As soon as the samples are sent to a laboratory with qPCR capability, results can be obtained quickly. In any case, when a physician is confronted with a patient with a fever and an eschar, doxycycline treatment

should be initiated immediately because β-lactam antimicrobial drugs are inefficient for the treatment of rickettsioses (*9*).

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D.R. is a cofounder of the biotechnology company Inodiag (www.inodiag.com) and is the inventor of a serologic diagnostic method for which the patent is held by Aix-Marseille University; this method was not used in this study. All other authors have no conflicts of interest.

Julie Solary, Cristina Socolovschi, Camille Aubry, Philippe Brouqui, Didier Raoult, and Philippe Parola

Author affiliations: Aix-Marseille Université, Marseille, France

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Address for correspondence: Philippe Parola, Unité de Recherche en Maladies Infectieuses et Tropicales Emergentes (URMITE), WHO Collaborative Center for Rickettsioses and Other Arthropod-borne Bacterial Diseases, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille Cedex 05, France; email: philippe.parola@univ-amu.fr

Pandemic *Vibrio parahaemolyticus*, Maryland, USA, 2012

To the Editor: Since 1996, an increasing number of infections caused by *Vibrio parahaemolyticus* strains belonging to a pandemic clonal complex (CC), CC3, typically O3:K6, have been observed worldwide (*1–3*); most of these strains are sequence type (ST) 3. In the summer of 1998, outbreaks linked to O3:K6 occurred in Galveston Bay, Texas, and Oyster Bay, New York, USA; the illnesses were associated with oyster consumption (*4*). Strains belonging to CC36 are the leading cause of *V. parahaemolyticus* infections in the United States. These strains are endemic to the West Coast (*2*) and have been historically linked to outbreak-associated *V. parahaemolyticus* infections caused by consumption of raw oysters harvested from the region (*5*).

In August 2012, a *V. parahaemolyticus* outbreak involving 6 persons occurred in Maryland, USA. The patients (members of 2 dining parties) had eaten in the same restaurant on the same day; raw and cooked seafood was served at the restaurant. Party A comprised 4 diners, of whom 2 had laboratory-confirmed illness and 2 were probable case-patients. Party B comprised 2 diners, of whom 1 had laboratory-confirmed illness and 1 was a probable case-patient. Probable case-patients were epidemiologically linked to confirmed case-patients, but *V. parahaemolyticus* was not detected in their stool samples. The epidemiologic investigation did not conclusively identify the specific food responsible for the outbreak. The affected diners had not eaten oysters, lobster, or mussels, but they had eaten cooked clams, fish, crab, and shrimp. Because the patients had not eaten oysters, a traceback investigation was not conducted. The outbreak possibly was caused by cross-contamination during food preparation. No other cases were reported from this restaurant or the surrounding area.

V. parahaemolyticus was isolated from stool samples of 3 of the patients. The isolates were characterized by real-time PCR for virulence-related genes (*tdh* and *trh*). All 3 isolates were *tdh* positive and lacked the *trh* gene. Pulsed-field gel electrophoresis (PFGE) was run, using *SfiI* and *NotI*; the resulting K16S12.0138 (*SfiI*) and K16N11.0143 (*NotI*) patterns were indistinguishable. The PFGE pattern combination was queried against combination entries made in PulseNet (www.cdc.gov/pulsenet/) during February 4, 2010–April 16, 2013, and found to be indistinguishable from other clinical entries (online Technical

Appendix 1 Table, wwwnc.cdc.gov/EID/article/20/4/13-0818-Techapp1.pdf). This PFGE pattern combination has been seen 25 times; all patterns were for strains from humans (N. Facundo, pers. comm.). In 2012, this PFGE pattern combination was observed in 3 US states—California (6 cases), Arizona (6 cases), and Texas (5 cases)—but those isolates were not further tested (S.G. Stroika, pers. comm.), suggesting that other cases of pandemic *V. parahaemolyticus* infections have occurred in the United States but were not identified as being caused by pandemic clones.

The whole genomes of the 3 Maryland strains were sequenced by using the Ion Torrent personal genome machine (Life Technologies, Grand Island, NY, USA); in silico multilocus sequence typing (MLST) (*2*) showed that the isolates were all ST3, the most common ST belonging to CC3. Bioinformatic analysis of the whole genomes was conducted with the Bacterial Isolate Genome Sequence Database (*6*) genome comparator tool available within the *V. parahaemolyticus* MLST database (http://pubmlst.org/vparahaemolyticus) (*7*,*8*). Results confirmed that these outbreak isolates were linked to the O3:K6 pandemic clone of *V. parahaemolyticus* (Figure). We identified 2,613 variable loci in this analysis by using as reference genome the prototype pandemic *V. parahaemolyticus* clonal strain RIMD221633 (available from GenBank, www.ncbi.nlm.nih.gov/genome/?term=vibrio parahaemolyticus) (*10*). Differences in variable loci and the absence of certain genes indicated that, although indistinguishable by MLST and PFGE, these strains are easily differentiated from RIMD2210633 (online Technical Appendix 1). The draft genome sequences for the 3 strains are available at the *V. parahaemolyticus* MLST database (identification nos. 1187 [Vp16MD], 1188 [Vp17MD], and 1189 [Vp18MD]).

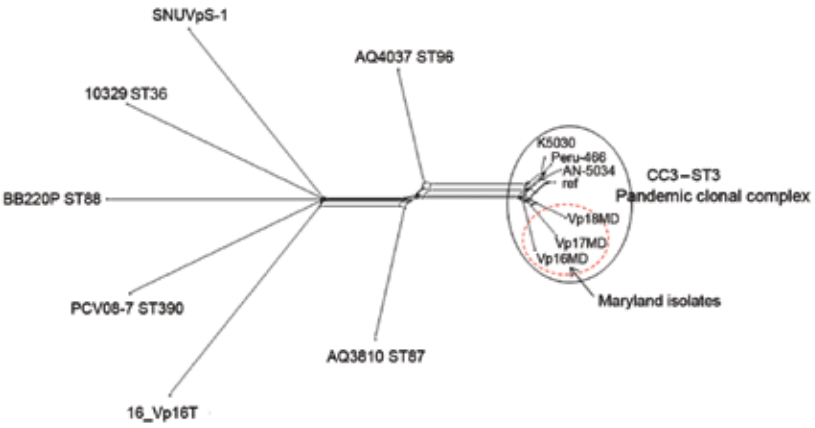

Figure. Neighbor-Net graph generated with the Bacterial Isolate Genome Sequence Database genome (BIGSdb) comparator tool implemented within the *Vibrio parahaemolyticus* MLST database (http://pubmlst.org/vparahaemolyticus) (*7*,*8*) using 2,613 variable loci. These loci were identified by using as a reference (ref) the *V. parahaemolyticus* strain RIMD2210633 chromosome I (3,080 genes) and conducting a whole-genome MLST (wgMLST) for *V. parahaemolyticus* genomes available through GenBank (AN-5034 O4:K68 ST3, Peru-466 ST3, K5030 ST3, 16_VP16T, AQ3810 ST87, AQ4037 ST96, PCV08–7 ST390, BB220P ST88, and SNUVpS-1) and 3 Maryland outbreak strains (Vp16MD, Vp17MD, and Vp18MD). This typing showed that these 3 strains belonged to the pandemic CC3. A similar graph was obtained by using chromosome II of the same strain as reference (data not shown). In brief, the BIGSdb genome comparator tool performs wgMLST, which produces a color-coded wgMLST output (online Technical Appendix 2, wwwnc.cdc.gov/EID/article/20/4/13-0818-Techapp2.xlsx) that facilitates comparison among isolates. This loci output is further categorized into loci that are 1) variable among all isolates, 2) identical among all isolates, 3) missing in all isolates, and 4) incomplete because of being located at the ends of contigs. The variable loci among all isolates are the loci used for assessing relationships and producing a distance matrix based on the number of variable alleles; the strains are resolved into a network by using the NeighborNet algorithm (*9*). MLST, multilocus sequence typing; CC, clonal complex; ST, sequence type.

V. parahaemolyticus strains belonging to the pandemic CC have caused thousands of infections and a *V. parahaemolyticus* pandemic (*3*). Foodborne illnesses caused by pandemic *V. parahaemolyticus* are uncommonly reported in the United States. In Maryland, 12 and 21 cases of *V. parahaemolyticus*–associated gastroenteritis were reported in 2012 and 2013, respectively. We report that the pandemic CC was still causing US outbreaks as recently as August 2012. It is possible that complete availability of PFGE patterns during the outbreaks (online Technical Appendix 1) could have provided additional insight into the scope of the outbreak and implicated food sources. The application of rapid, whole-genome sequencing technology aided our discovery that the Maryland outbreak strains were part of the pandemic CC and likely related to *V. parahaemolyticus* strains that shared common PFGE patterns and that were reported as the cause of illnesses in several states around the same time as the Maryland outbreak.

The presence of this virulent *V. parahaemolyticus* strain in Maryland is an ongoing public health concern, requiring continued microbiological surveillance. This pandemic strain also indicates the need for establishing a *V. parahaemolyticus* genome database that is accessible worldwide. Such a database would enable improved tracking and faster responses to emergent and dangerous pandemic clonal strains.

Julie Haendiges, Marvin Rock, Robert A. Myers, Eric W. Brown, Peter Evans, and Narjol Gonzalez-Escalona

Author affiliations: Department of Health and Mental Hygiene, Baltimore, Maryland, USA (J. Haendiges, M. Rock, R.A. Myers); and Food and Drug Administration, College Park, Maryland, USA (E.W. Brown, P. Evans, N. Gonzalez-Escalona)

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Address for correspondence: Narjol Gonzalez-Escalona, Food and Drug Administration, Center for Food and Applied Nutrition, 5100 Paint Branch Pkwy, College Park, MD 20740, USA; email: narjol.gonzalez-escalona@fda.hhs.gov

Serologic Evidence of Leptospirosis in Humans, Union of the Comoros, 2011

To the Editor: Leptospirosis is a worldwide bacterial zoonosis caused by infection with pathogenic *Leptospira* spp. (Spirochaetales, Leptospiraceae). Most mammals can be infected, but rats are considered the main reservoir, maintaining *Leptospira* spirochetes in the lumen of renal tubules and contaminating the environment with bacteria-infected urine. Transmission to humans is accidental, occurring through contact with animal secretions or with contaminated environmental materials.

In temperate countries, human leptospirosis is a sporadic disease; incidence is much higher in the tropics because climate and environmental conditions are conducive to the survival of bacteria, resulting in increased exposure of humans to leptospirosis-causing pathogens (*1*). Among islands in the southwestern Indian Ocean, human leptospirosis is endemic to Mayotte, France, and La Réunion (*2–4*) and to the Seychelles, where the incidence of leptospirosis is one of the highest worldwide (*5*). Leptospirosis is poorly documented in other islands in the region, including Mauritius, Madagascar, and the Union of the Comoros (*2*,*6–8*). Whether the scant documentation indicates underdiagnosis or reflects local epidemiologic specificities is unknown. To improve knowledge of *Leptospira* infection in the region, we conducted a study in the Union of the Comoros to serologically assess the presence or absence of leptospirosis in humans. The Union of the Comoros consists of 3 islands: Grande-Comore, Mohéli, and Anjouan. Together with a fourth, southern island, Mayotte, these islands form the Comoros Archipelago.

For feasibility reasons, we used excess serum samples. Seventy-six samples were from healthy volunteers who gave informed consent; 318 clinical blood samples from patients had been obtained by private laboratories and by the surveillance laboratory of the National Malaria Control Programme (PNLP) during August 1–October 8, 2011. The Ministère de la Santé, de la Solidarité et de la Promotion du Genre of the Union of the Comoros, authorized the serologic investigation (authorization no. 1175/MSSPG/DNS).

We used the microscopic agglutination test (MAT) to test serum samples; the MAT was based on a panel of 15 *Leptospira* strains, enabling the screening of all recently reported serogroups for human and animal cases on neighboring Mayotte (*2*,*4*,*9*). A list of the tested strains follows, shown as *Genus species* Serogroup/Serovar (type strain): *L. borgpetersenii* Ballum/Castellonis (Castellon 3), *L. borgpetersenii* Sejroe/Hardjobovis (Sponselee), *L. borgpetersenii* Sejroe/Sejroe (M 84),

L. borgpetersenii Tarassovi/Tarassovi (Perepelicin), *L. interrogans* Australis/Australis (Ballico), *L. interrogans* Autumnalis/Autumnalis (Akiyami A), *L. interrogans* Bataviae/Bataviae (Van Tienen), *L. interrogans* Canicola/Canicola (Hond Utrecht IV), *L. interrogans* Hebdomadis/Hebdomadis (Hebdomadis), *L. interrogans* Icterohaemorrhagiae/Copenhageni (Wijnberg), *L. interrogans* Pyrogenes/Pyrogenes (Salinem), *L. kirschneri* Cynopteri/Cynopteri (3522C), *L. kirschneri* Grippotyphosa/Grippotyphosa (Moskva V), *L. kirschneri* Mini/Undetermined serovar (200803703) (*9*), *L. noguchii* Panama/Panama (CZ214K). Each serum sample was tested at dilutions ranging from 1:50 to 1:3,200 and considered positive when the MAT titer was ≥100.

Our serologic findings showed evidence of *Leptospira* infection in humans on the 3 islands of the Union of the Comoros (MAT titers 100–1,600, geometric mean titer [GMT] 194). The positivity rate was 10.3% (95% CI 4.8–15.9) for samples from Mohéli, 4.2% (95% CI 1.4–7.0) for samples from Grande-Comore, and 3.4% (95% CI 0.1–6.7) for samples from Anjouan; no significant difference was found between islands or by the age or sex of residents (p>0.05, Fisher exact test). *Leptospira* infection was more prevalent and MAT titers were higher among serum samples from the patient group than the healthy donor group (20 positive samples/318 total vs. 3 positive samples/76 total; GMT 207 vs. GMT 126), but the difference was not significant (p>0.05, Fisher exact test). In 78% of seropositive serum samples, antibodies reacted with serogroups Australis, Bataviae, Grippotyphosa, Panama, Pomona, Pyrogenes, Mini, and/or Sejroe. MAT titers >100, which are suggestive of more specific antibodies to *Leptospira*, were observed for all serogroups except Australis and Sejroe. Pyrogenes serogroup was identified in one third of positive samples from Mohéli and was associated with the highest agglutination titers (Figure).

Our data indicate that *Leptospira* infections do occur in humans in the Union of the Comoros; this finding is consistent with those in studies reporting leptospirosis in persons returning from travel in the Union of the Comoros (*2*,*8*) and with the detection of pathogenic *Leptospira* spp. in bats sampled on these islands (*10*). The human leptospirosis–related serologic findings in Union of Comoros are most comparable to those from neighboring Mayotte, where leptospirosis is mainly caused by serogroups Mini/Sejroe/Hebdomadis complex, Pyrogenes,

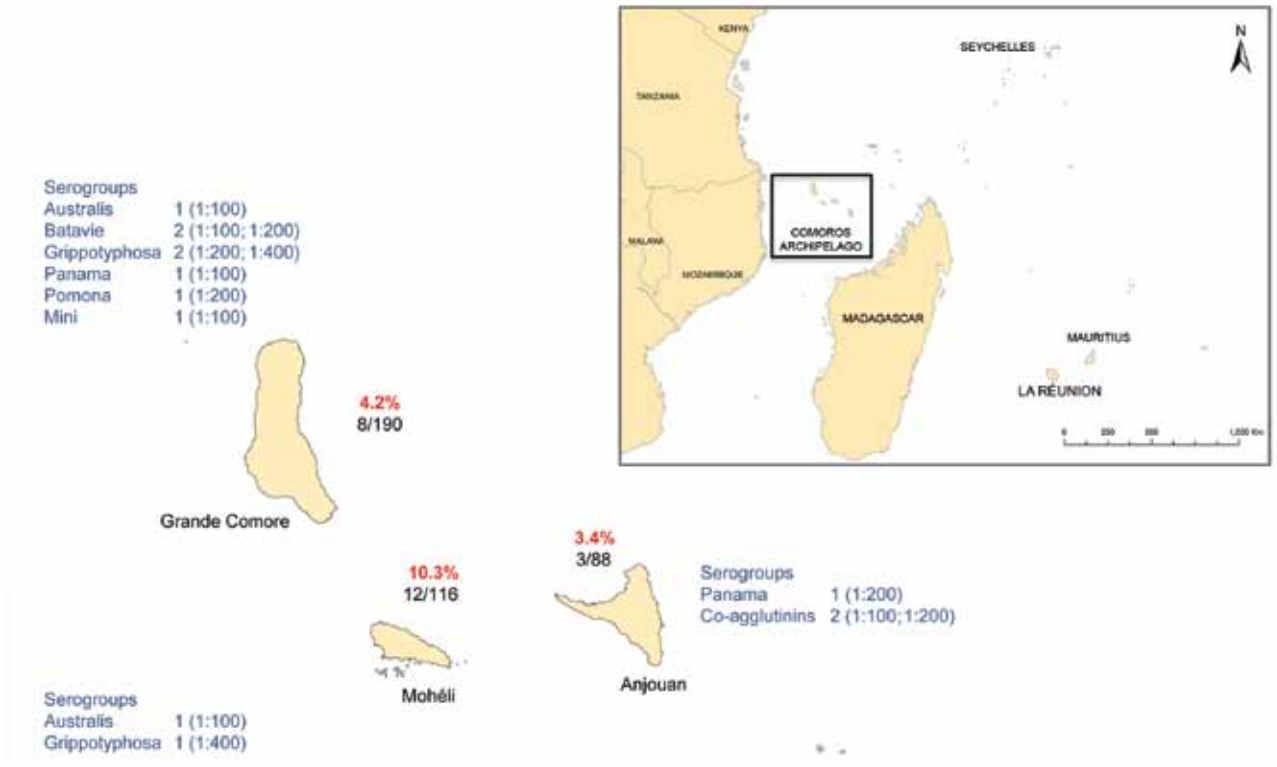

Figure. Microagglutination test results, showing serologic evidence of leptospirosis in humans, Union of the Comoros, 2011. The percentage of positive cases is shown for each island; the number below the percentage indicates the number of positive serum samples/total number tested. The serogroups identified on each island are shown; numbers represent the number of positive serum samples and, in parentheses, the number of corresponding titers. When agglutination was observed with >1 serogroup, the serogroup with a titer difference ≥2 relative to other serogroups was considered to be the infecting serogroup; when no serogroup had a titer difference ≥2 relative to other serogroups, coagglutinins were considered to be present in the serum sample. Data for Mayotte Island are from previous studies (*2*,*4*).

Grippotyphosa, and Pomona and where serogroup Icterohaemorraghiae is not detectable (*2*). These findings contrast with human leptospirosis findings from La Réunion and the Seychelles, where the Icterohaemorraghiae serogroup is most common (*3*).

Our MAT-derived data cannot discriminate between recent and past *Leptospira* infections, nor can these data be used to determine the severity of the disease in the Union of the Comoros. Nonetheless, the data strongly support the presence of human leptospirosis on the 3 islands of the Union of the Comoros and emphasize the need for a proper diagnosis to ascertain the number of leptospirosis cases among the acute febrile illnesses in this country.

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Yann Gomard, Rahamatou Silai, Géraldine Hoarau, Ketty Bon, Florelle Gonneau, Amina Yssouf, Alain Michault, Koussay Dellagi, and Pablo Tortosa

Author affiliations: Centre de Recherche et de Veille sur les Maladies Emergentes dans l'Océan Indien (CRVOI), Ste. Clotilde, La Réunion, France (Y. Gomard, K. Dellagi, P. Tortosa); Programme National de Lutte contre le Paludisme (PNLP), Moroni, Comoros (R. Silai, A. Yssouf); Centre Hospitalier Universitaire, St. Pierre, La Réunion (G. Hoarau, K. Bon, F. Gonneau, A. Michault); Université de La Réunion, Ste. Clotilde (Y. Gomard, P. Tortosa); Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, Marseille, France (A. Yssouf); and Institut de Recherche pour le Développement, Ste Clotilde (K. Dellagi)

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Address for correspondence: Pablo Tortosa, CRVOI, Plateforme de recherche CYROI, 2 rue Maxime Rivière, 97490 Ste Clotilde, France; email: pablo.tortosa@univ-reunion.fr

Nosocomial Drug-Resistant Bacteremia in 2 Cohorts with Cryptococcal Meningitis, Africa

To the Editor: Cryptococcal meningitis is the second leading cause of AIDS-related deaths in Africa. The prolonged hospitalization necessary for optimal management may predispose severely immunocompromised persons to hospital-acquired infections. Limited data are available for sub-Saharan Africa regarding multidrug-resistant infections (*1,2*). We hypothesized that bacteremia was a major cause of death.

We reviewed bacteremia episodes in cryptococcal meningitis cohorts in Kampala, Uganda (n = 115 episodes) and Cape Town, South Africa (n = 72) during November 2010–April 2013. Data were obtained from the prospective cryptococcal optimal antiretroviral therapy timing trial (www.clinicaltrials.gov:NCT01075152), a randomized strategy trial assessing optimal antiretroviral therapy timing (n = 142) and another prospective observational cohort in Cape Town (n = 45).

We enrolled HIV-infected adults who had a first episode of cryptococcal meningitis diagnosed by cerebrospinal fluid culture or cryptococcal antigen testing. Standardized treatment was in accordance with World Health Organization (WHO) guidelines: amphotericin deoxycholate, 0.7–1.0 mg/kg/d for 14 days, and fluconazole, 800 mg/d, requiring a minimum 14-day hospitalization (*3*). Each person provided written informed consent. Institutional review board approval was obtained.

Blood cultures were obtained in accordance with physician discretion, typically with new onset fever (>38°C) unrelated to amphotericin. Two aerobic blood cultures were obtained from 1 peripheral site and not

from central catheters. BACTEC (Becton Dickinson, Franklin Lakes, NJ, USA) or BacT/ALERT (BioMérieux, Durham, NC, USA) bottles were incubated at 37°C in automated instruments for 5 days. Drugs were given empirically at physician discretion and adjusted after culture/susceptibility results were obtained.

Each bacteremic episode was classified as a true pathogen, contaminant, or indeterminate on the basis of clinical scenario and bacterial isolates. Data extraction from case report forms elicited demographics, microbiology results, antimicrobial drug therapy, and clinical outcomes. We determined risk factors between cases and controls who had cryptococcal meningitis without bacteremia/sepsis.

Descriptive statistical analysis reported median and interquartile range (IQR). Risk was expressed as odds ratio with 95% CIs calculated by logistic regression. Significance was defined as $p<0.05$ by Fischer exact test (SPSS 21; IBM, Armonk, NY, USA). Variables with $p<0.10$ by univariate analysis were entered in a multivariable model.

We evaluated 187 persons with cryptococcal meningitis who had a median CD4 count of 27 cells/μL (IQR 9–76). Forty-three blood cultures were prepared for 40 patients with febrile episode(s), of which 37 were positive. Median time from admission to suspected bacteremia was 14 days (IQR 9–17 days). All episodes were detected >72 h after admission and classified as nosocomial bacteremia. Seven isolates were considered contaminants because clinical improvement occurred without appropriate therapy. Thus, 30 cultures for 28 persons (cohort incidence 15%) were classified as true bacteremia with compatible clinical syndrome. Twenty-three bacteremic episodes occurred in Kampala (incidence 18%). Seven episodes occurred in 5 patients in Cape Town (incidence 7%).

The most frequent microbiologic etiologies were *Klebsiella pneumoniae* (9 episodes), *Staphylococcus aureus* (8), and *Pseudomonas* spp. (3) (Table). Methicillin-resistant *S. aureus* constituted 6 of 8 *S. aureus* isolates.

Ceftriaxone was the most common empiric drug used, for which 23 (77%) of 30 isolates were resistant. Eleven (46%) of 24 isolates were resistant to ciprofloxacin. Among bacteremic patients, 12 (43%) of 28 died within 30 days after hospitalization. The 30-day mortality rate

Table. Characteristics of 28 patients with cryptococcal meningitis, Uganda and South Africa, November 2010–April 2013*

Patient	Blood culture isolate	Hospital	30-d outcome	Ceftriaxone susceptibility†	Fluoroquinolone susceptibility†
1	*Staphylococcus aureus* (MRSA)	K	Died	R	NT
2	*S. aureus* (MRSA)	K	Survived	R	NT
3	*S.. aureus* (MRSA)	C	Survived	R	S
4	*S. aureus* (MRSA)	C	Survived	R	R
5	*S. aureus* (MRSA)	C	Died	R	S
6	*S. aureus* (MSSA)	K	Survived	S	NT
7	*S. aureus* (MSSA)	K	Survived	S	NT
8‡	*S. aureus* (MRSA)	C	Survived	R	R
8	*Enterobacter cloacae*	C	Survived	R	S
9	*Klebsiella pneumoniae*	K	Survived	R	R
10	*K. pneumoniae*	K	Died	R	R
11	*K. pneumoniae*	K	Survived	S	S
12	*K. pneumoniae*	K	Died	R	S
13	*K. pneumoniae*	K	Died	R	R
14	*K. pneumoniae*	K	Died	R	R
15	*K. pneumoniae*	K	Survived	R	R
16	*K. pneumoniae*	C	Survived	R	NT
17	*K. pneumoniae*	C	Died	R	S
18	*Pseudomonas putida*	K	Died	S	S
19	*Pseudomonas aeruginosa*	K	Survived	R	R
20	*Pseudomonas* spp.	K	Died	R	NT
21	*Salmonella* spp.	K	Survived	S	S
22	*Salmonella* spp.	K	Died	S	S
23	*Burkholderia cepacia*	K	Survived	R	NT
24	*B. cepacia*	K	Survived	S	S
25	*Citrobacter freundii*	K	Died	R	S
26	*Acinetobacter baumanii*	K	Survived	R	S
27	*Enterobacter* spp.	K	Died	R	R
28‡	*Enterobacter cloacae*	C	Survived	R	S
28	*Stenotrophomonas maltophilia*	C	Survived	R	R

*Sixteen (57%) of 28 patients survived; 7 (23%) of 30 isolates were susceptible to ceftriaxone, and 13 (54%) of 24 isolates were susceptible to fluoroquinolone. MSRA, methicillin-resistant *S aureus*; K, Kampala, Uganda; R, resistant; NT, not tested; C, Cape Town, South Africa; S, sensitive; MSSA, methicillin-sensitive *S. aureus*.

†Drug sensitivity testing performed by using the Kirby-Bauer method in Cape Town, South Africa, and a Phoenix Automated Microbiology System (Becton, Dickinson, Franklin Lakes, NJ, USA) in Kampala, Uganda.

‡Two patients each had 2 episodes of bacteremia.

for persons with cryptococcal meningitis but without bacteremia was 30% (47/158); 1 patient was lost to follow-up. Thus, the estimated attributable mortality rate for bacteremia was 13% (odds ratio 1.8, 95% CI 0.78–4.0, p = 0.17) compared with patients without bacteremia during their initial hospitalization.

Case–control comparisons identified no risk factors for bacteremia (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/4/13-1277-Techapp1.pdf). Although 21 (70%) of 30 bacteremia episodes were preceded by phlebitis at a peripheral intravenous site, phlebitis caused by amphotericin was also common in patients without bacteremia (49%), but these percentages did not differ statistically.

Accurate data regarding incidence of nosocomial infections in Africa are lacking. A systematic review by WHO in 2011 that assessed published data for 1995–2009 identified only 2 high-quality studies. WHO estimated a prevalence of 2.5%–14.8% for nosocomial infections and a cumulative incidence of up to 45.8% in some areas (*4*) and recommended surveillance to estimate the rates of nosocomial infection. WHO acknowledges that health care–associated infections are causes of prolonged hospitalizations, increased antimicrobial drug resistance, financial burdens on health care systems, and causes of excess illness and death (*5*).

Limitations of our study include the retrospective design and inability to identify predictive risk factors for bacteremia. Given the differences in bacteremia incidence between our 2 sites, findings are probably not generalizable to all clinical settings in Africa. However, these findings identify a clinical problem.

The incidence of nosocomial bacteremia was 15% in our hospitalized cryptococcal meningitis cohort at a median time of 14 days after hospitalization. The most frequent etiologies were *S. aureus* and *K. pneumonia*. Less than 25% of isolates were sensitive to ceftriaxone, a standard empiric drug used throughout Africa. Further prospective studies are needed to determine the prevalence and risk factors for nosocomial infections and prevalence of multidrug resistance among hospitalized persons in resource-limited areas.

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Radha Rajasingham, Darlisha Williams, David B. Meya, Graeme Meintjes, David R. Boulware, and James Scriven

Author affiliations: University of Minnesota, Minneapolis, Minnesota, USA (R. Rajasingham, D. Williams, D.B. Meya, D.R. Boulware); Makerere University, Kampala, Uganda (D. Williams, D.B. Meya); University of Cape Town, Cape Town, South Africa (G. Meintjes, J. Scriven); Imperial College London, London, UK (G. Meintjes); and Liverpool School of Tropical Medicine, Liverpool, UK (J. Scriven)

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Address for correspondence: Radha Rajasingham, Beth Israel Deaconess Medical Center, Harvard Medical School, 110 Francis St, Ste GB, Boston, MA 02215, USA; email: radha.rajasingham@gmail.com

Severe Babesiosis in Immunocompetent Man, Spain, 2011

To the Editor: Babesiosis, a malaria-like illness, is transmitted through *Ixodes* ticks by the zoonotic parasites, *Babesia* spp. In humans, these parasites are transferred from mammalian animal reservoirs, and the rate of infection in humans is increasing. Babesiosis also potentially threatens the blood supply because asymptomatic infections in humans are common; such infections can be life-threatening in some recipients (*1*). Most human infection is caused by *B. microti*, but babesiosis caused by *B. divergens*, *B. duncani*, and *B. venatorum* has been reported.

Human babesiosis can be clinically silent or progress to a fulminant malaria-like disease. The infection resolves spontaneously or after treatment with azithromycin/atovaquone or clindamycin/quinine. However, immunocompromised patients may respond suboptimally to these drug regimens (*2*). Given the death rate

associated with babesiosis, no treatment is fully satisfactory (*3*). Infection with *B. divergens* is particularly problematic and is associated with a high death rate in splenectomized or immunocompromised patients (*3*). In Europe, sporadic cases of babesiosis have also been reported in immunocompetent persons (*4*).

In October 2011, a 46-year-old man whose spleen was intact was hospitalized after 3 days of fever, severe abdominal pain, jaundice, and black and red deposits in his urine. The man lived in a rural area in Asturias, Spain, where he was employed as a forest ranger. He reported that he removed ticks from his dogs.

Laboratory findings included hemoglobin 12.3 g/dL (reference range 13.8–17.2 g/dL); creatinine 1.52 mg/dL (reference range 0.7–1.3 mg/dL); total and direct/conjugated bilirubin 18.4 and 12.8 mg/dL (reference ranges total 0.3–1.9 mg/dL; direct/conjugated 0–0.3 mg/dL), lactate dehydrogenase 822 IU/L (reference range 105–333 IU/L; and showed thrombopenia, low haptoglobina, and hematuria. A value of 35% CD4+ T cells (reference range 30%–60%) indicated normal immune status. Results of serologic tests for hepatitis; HIV; and *Bartonella*, *Brucella*, *Leishmania*, *Leptospira*, and *Borrelia* spp. and of blood cultures were negative. Abdominal ultrasound scan revealed mild hepatomegaly and cortical echogenicity compatible with acute kidney failure. Howell-Jolly bodies were identified in blood, and functional splenic studies were conducted. Scintigraphic parameters showed a normal deposit of radioactive hepato-splenic material, compatible with a normal-sized spleen of 13.4 cm.

Giemsa-stained blood smears showed intra-erythrocytic parasites, mainly observed in the 2-celled dividing pyriform stage, leading to the diagnosis of babesiosis with a parasitemia level of 10% (Figure). The complete *B. divergens* 18S rRNA gene was amplified from the patient's blood (*5*), and the nucleotide sequencing (GenBank accession no. KF533077) showed 100% homology with *B. divergens* human strains (GenBank accession nos. FJ944822 and FJ944823) (*5*) and with 2 babesiosis cases reported previously (*4*). Indirect immunofluorescent assays of *B. divergens* cultures showed specific antibodies against *B. divergens* in the patient's serum (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/20/4/13-1409-Techapp1.pdf). The patient was treated with 650 mg oral quinine every 8 hours and 600 mg intravenous clindamycin every 6 hours. The parasitemia diminished gradually and resolved 10 days later, but the hemolytic anemia remained severe, as evidenced by hemoglobin of 7.2 g/dL.

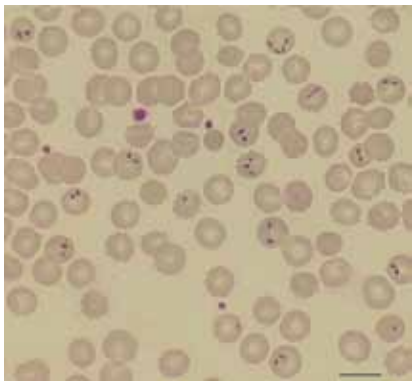

Figure. A Giemsa-stained thin film of blood from a 46-year-old man showing *Babesia divergens*. Double pear shaped intraerythrocytic parasites are indicated by arrows. Slides were examined with a Nikon microscope (Nikon Instruments, Inc., Melville, NY, USA) at 60× magnification. Scale bar indicates 500 nm. A color version of this figure is available online (wwwnc.cdc.gov/eid/article/20/4/13-1409-F1 htm).

The man's illness unexpectedly relapsed on day 18 after treatment. His reticulocyte count was elevated, and parasites were once again detected in blood samples. Thus, treatment was changed to a combination of atovaquone/proguanil 250/100 mg administered every 8 hours plus azithromycin 500 mg every 24 hours. Two weeks later, the patient's hemoglobin was 8.7 g/dL, and no parasites were detectable by microscopy. The treatment was extended for an additional 5 weeks, and the patient was free of parasites on subsequent visits.

We have described what appears to be the third case of human babesiosis in nonsplenectomized patients in Europe. Human presence in tick, cattle, and domestic animal habitats could be responsible for this case. Martinot et al. (*4*) earlier pointed out that in Europe, babesiosis can also occur in persons with intact spleens. A combination of clindamycin and quinine is the recommended treatment of severe babesiosis (*2*,*3*). However, in this case, the recommended therapy failed, and therapy was switched to atovaquone/proguanil plus azithromycin. Other case reports have also related failure, ineffectiveness, adverse reaction, or persistent and relapsing babesiosis to clindamycin and quinine treatment in splenectomized patients infected by *B. divergens* or *B. microti* (*3*,*6*–*9*) or suspected *B. microti* drug resistance in immunocompromised patients (*2*). The recently sequenced *B. microti* genome reveals absence of proteases necessary to digest host hemoglobin and hemozoin formation by the parasite; this absence may explain the ineffectiveness of chloroquine, and perhaps other compounds of the aminoquinoline family used in babesiosis therapy (*10*).

This clinical case report, together with the failure of clindamycin and quinine to successfully eliminate the parasite *Babesia*, again opens the debate about the limitations of conventional treatment for severe human babesisosis in immunocompetent and immunocompromised patients. The capability of *Babesia* spp. to invade erythrocytes is the key step of the disease process. Focusing on *Babesia* spp. molecules involved in the invasion steps may offer new targets for the development of new prophylaxis and treatment for human babesiosis.

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Luis M. Gonzalez, Susana Rojo, Fernando Gonzalez-Camacho, Daniel Luque, Cheryl A. Lobo, and Estrella Montero

Author affiliations: Centro Nacional de Microbiologia, Majadahonda, Spain (L.-M. Gonzalez, F. Gonzalez-Camacho, D. Luque, E. Montero), Hospital Universitario Central de Asturias, Asturias, Spain (S. Rojo); and New York Blood Center, New York, New York, USA (C.A. Lobo)

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Address for correspondence: Estrella Montero, Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Carretera Majadahonda-Pozuelo, Km 2,2, Majadahonda, Madrid, 28220, Spain; email: estrella.montero@isciii.es

Q Fever Endocarditis and New *Coxiella burnetii* Genotype, Saudi Arabia

To the Editor. Q fever is a worldwide zoonosis caused by an obligate intracellular bacterium, *Coxiella burnetii* (*1*). Q fever endocarditis is associated with surgery for 15%–73% of patients, causes death for 5%–65% of patients, and induces a large number of relapses when the endocarditis is inadequately treated (*1*). The most serious risk factor for endocarditis is a substantial underlying valvulopathy, but progression to endocarditis is also found in patients with clinically silent, previously undiagnosed, valvulopathies (*1*). Since the 1960s, Q fever has been recognized as a public health problem in Saudi Arabia, and studies have shown that coxiellosis occurs in livestock (*2*,*3*). Only a few cases of Q fever endocarditis in Saudi Arabia have been reported (*4*–*6*). We report 2 new cases of Q fever endocarditis and detection of a new *C. burnetii* genotype in this country.

The first case was detected in 2007 in a 45-year-old man in Saudi Arabia who had fever, pneumonia, and asthenia. A transesophageal echocardiogram showed endocarditis. Results of an immunofluorescence assay were positive for *C. burnetii*; phase I titers for IgG, IgM, and IgA were 51,200, 100, and 25, respectively, and phase II titers were 102,400, 200, and 50, respectively. Serum and blood samples were negative for *C. burnetii* by real-time PCR for the IS1111 and the IS30A spacers (*7*). For each sample, the quality of DNA extraction was verified by real-time PCR for a housekeeping gene encoding β-actin (*7*). The aortic valve was surgically replaced, and *C. burnetii*–specific PCR results for the valve were positive. According to multispacer sequence typing (*8*), this *C. burnetii* isolate was a new genotype, MST51 (Figure). A *C. burnetii* isolate was cultured from the valve of this patient by the shell-vial method that used human embryonic lung cells (*7*). IgG anticardiolipin testing results were negative (*9*). The patient was given 200 mg oral doxycycline daily and 200 mg oral hydroxychloroquine 3 times daily for 18 months.

The second case was detected in 2012 in a 13-year-old boy in Saudi Arabia who had tetralogy of Fallot, a prosthetic pulmonary valve, 2 intracardiac stents, and long-term fever. Serologic testing results were positive for *C. burnetii*; phase I titers for IgG, IgM, and IgA were 51,200, 400, and 200, respectively, and phase II titers were 102,400, 800, and 400, respectively. Whereas serum and blood

samples were negative for *C. burnetii* by real-time PCR for the IS1111 and the IS30A spacers, the β-actin control was positive (cycle threshold <30). For this patient, we did not receive any material for culture. The patient was given 200 mg oral doxycycline daily and 200 mg oral hydroxychloroquine 3 times daily for 18 months.

To the best of our knowledge, before the 2 cases presented here, only 3 cases of Q fever endocarditis in Saudi Arabia have been described; all patients were from rural regions of Saudi Arabia and had an underlying valvulopathy (*4*–*6*). Moreover, Q fever was not immediately suspected, and as a result, 1 patient died (*6*). However, for 2 other patients, valve replacement was necessary (*4*,*5*). Q fever is prevalent in Saudi Arabia, and the very high prevalence of Q fever among camels was proposed as the reason Q fever is endemic among humans in Saudi Arabia (*2*,*3*). Camels were also suspected as the probable source of acute Q fever in US soldiers returning from Saudi Arabia (*10*). We identified a new *C. burnetii* genotype in the aortic valve of the first patient reported here. More epidemiologic studies are needed to determine whether this novel genotype circulating in Saudi Arabia is endemic to Saudi Arabia and whether it plays a major role in the origin of Q fever and in public health in this country.

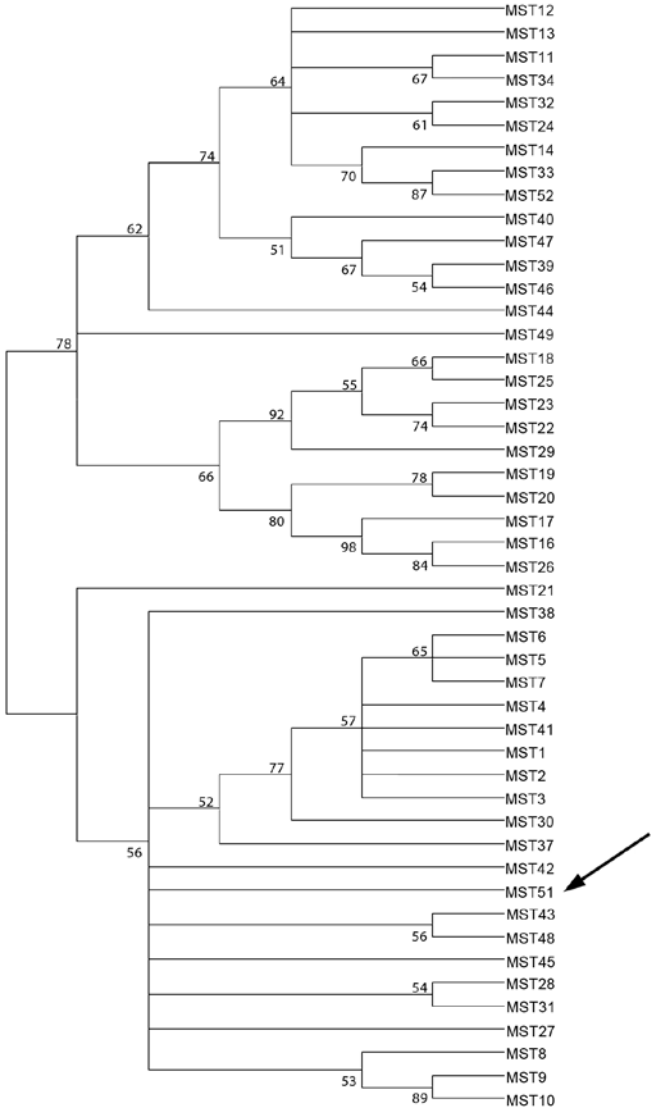

Figure. Neighbor-joining tree of *Coxiella burnetii* genotypes determined by multispacer sequence typing. Arrow indicates new genotype in Saudi Arabia.

Our studies of Q fever cases in southern France have shown that >16% of patients with acute Q fever have endocarditis and that ≈16% 37% of patients with Q fever endocarditis could have had previous symptomatic acute Q fever infection (*1*). Thus, many cases of endocarditis might be avoided if patients with acute Q fever receive antimicrobial drugs as prophylaxis (*1*). For patients >40 years of age, transthoracic echocardiography should be performed because of the increased prevalence of valvulopathy and Q fever endocarditis in this population (*9*). As a result, more studies are needed to determine whether our data can affect local clinical practice.

Emmanouil Angelakis, Sameer Johani, Azeem Ahsan, Ziad Memish, and Didier Raoult

Author affiliations: Unité de Recherche. sur les Maladies Infectieuses et Tropicales Émergentes, Marseille, France (E. Angelakis, D. Raoult); King Saud Bin Abdul Aziz University for Health Science, Jeddah, Saudi Arabia (S. Johani, D. Raoult); King Fahd Medical City, Riyadh, Saudi Arabia (A. Ahsan); and Ministry of Health, Riyadh (Z. Memish)

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Address for correspondence: Didier Raoult, URMITE UMR 6236, CNRS-IRD, Faculté de Médecine, 27 Blvd Jean Moulin, 13385 Marseille Cedex 05, France; email: didier.raoult@gmail.com

Lack of MERS Coronavirus but Prevalence of Influenza Virus in French Pilgrims after 2013 Hajj

To the Editor: Saudi Arabia has reported the highest number of Middle East respiratory syndrome coronavirus (MERS-CoV) cases since the virus first emerged in 2012, with >127 confirmed cases and a case-fatality rate of 42%, as of November 2013 (*1*). Global attention has focused on the potential for spread of MERS-CoV after the Hajj pilgrimage during which Muslims from 180 countries converge in Mecca, Saudi Arabia. Such pilgrims have a high risk for respiratory tract infections because of severe overcrowding. The International Health Regulations Emergency Committee advised all countries (particularly those with returning pilgrims) to strengthen their surveillance capacities and ensure robust reporting of any identified cases (*2*).

We report the results of a prospective cohort study conducted in Saudi Arabia in October 2013. Participants in the survey were adult Hajj pilgrims who traveled together in a group (through 1 travel agency in Marseille, France) from October 3 through October 24, 2013. Pilgrims were included in the study on a voluntary basis and were asked to sign a written consent form. All pilgrims received advice about individual prevention measures against respiratory tract infection before departing, and follow-up was conducted during the journey by a medical doctor who systematically documented travel-associated diseases. Nasal swab specimens were obtained just before the pilgrims left Saudi Arabia, frozen <48 hours after sampling, and processed (*3*,*4*). Each sample was tested for MERS-CoV (upE and ORF1a genes) (*5*,*6*) and influenza A, B (*7*), and A/2009/H1N1 viruses (*8*) by real-time reverse transcription PCR. The protocol was approved by our Institutional Review Board (July 23, 2013; reference no. 2013-A00961–44) and by the Saudi Ministry of Health ethics committee.

On departure from France, the study comprised 129 pilgrims. Their mean age was 61.7 years (range 34–85 years), and the male/female ratio was 0.7:1. Sixty-eight (52.7%) pilgrims reported having a chronic disease, including hypertension (43 [33.3%]), diabetes (34 [26.4%]), chronic cardiac disease (11 [8.5%]), and chronic respiratory disease (5 [3.9%]). Forty-six (35.7%) pilgrims reported receiving influenza vaccination in 2012; none had been vaccinated in 2013 before the Hajj because the vaccine was not yet available in France.

Clinical data were available for 129 persons: 117 (90.7%) had respiratory symptoms while in Saudi Arabia, including cough (112 [86.8%]) and sore throat (107 [82.9%]); 64 (49.6%) reported fever, and 61 (47.3%) had conditions that met the criteria for influenza-like illness (ILI; i.e., the association of cough, sore throat, and subjective fever) (Figure) (*4*). One patient was hospitalized during travel (undocumented pneumonia). Nasal swab specimens were obtained from 129 pilgrims on October 23, 2013 (week 43), 1 day before pilgrims left Saudi Arabia for France; 90 (69.8%) pilgrims were still symptomatic. All PCRs were negative for MERS-CoV.

Eight pilgrims tested positive for influenza A(H3N2), 1 for influenza A(H1N1), and 1 for influenza B virus. No dual infections were reported. 70 (54.3%) pilgrims were seen 3–5 weeks after they returned to France, and the remaining were lost to follow-up. Fifty-five (78.6%) had experienced respiratory symptoms since their return, including cough (50 [71.4%]) and sore throat (14 [20.0%]); 12 (17.1%) reported fever, and illness in 5 (7.1%) pilgrims met the criteria for ILI. The 10 pilgrims who had positive test

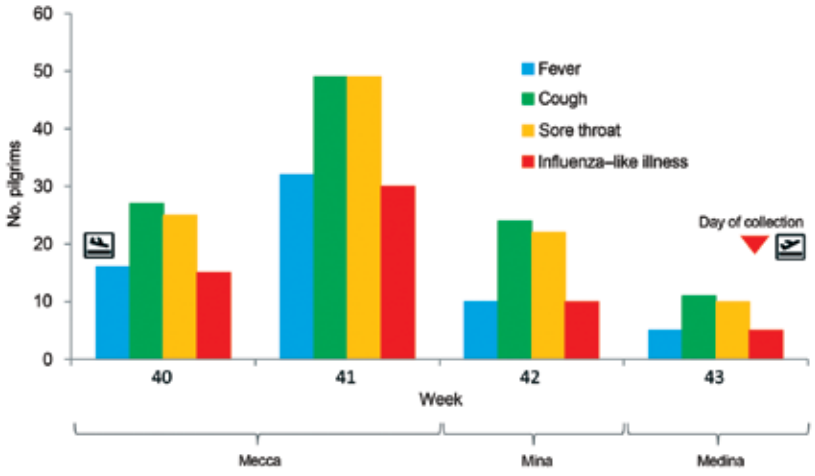

Figure. Onset of respiratory symptoms by week, reported by 129 Hajj pilgrims from France during their stay in Saudi Arabia, October 2013.

results for influenza virus on return had cleared their infection; only 1 additional sample was positive (for influenza A[H1N1]).

Our results support data obtained from a similar cohort in 2012 that showed a lack of nasal carriage of MERS-CoV among Hajj pilgrims from France (*3*). However, a higher prevalence of influenza virus (7.8%) was observed in nasal swab specimens in 2013 than in 2012 when 2 (3.2%) cases of influenza B virus infection were detected and no case of influenza A virus infection was detected among 162 pilgrims returning from the Hajj (*4*).

The estimated incidence of ILI in France during week 43 was 27 per 100,000 inhabitants, far below the epidemic threshold (126/100,000) with few sporadic cases of influenza A virus infection reported in some regions in France (www.grog.org/bullhebdo_pdf/bull_grog_43-2013.pdf). No case was reported in the Marseille area (http://websenti.u707.jussieu.fr/sentiweb). The high prevalence of respiratory symptoms in our cohort probably reflects the close surveillance performed and is consistent with 2012 results (*3*,*4*).

In Marseille, all patients with suspected MERS-CoV infection are referred to the Institut Hospitalo-Universitaire Méditerranée Infection. As of November 8, 2013, of the 14 first returning patients hospitalized for respiratory symptoms and screened for MERS-CoV and other pathogens, including influenza, 4 were infected with influenza A(H3N2), 4 with influenza A(H1N1), and 1 with influenza B virus. All samples tested negative for MERS-CoV.

Our preliminary results indicate that pilgrims from France returning from the 2013 Hajj were free of MERS-CoV but that a proportion were infected with influenza viruses and may represent a potential for early introduction of influenza in southern France. This proportion may have been underestimated because screening was performed at the end of the study period when some infections had cleared. Influenza vaccination should be a priority for pilgrims attending the Hajj (*9*,*10*).

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Author affiliations: Aix Marseille Université, Marseille, France (P. Gautret, R. Charrel, S. Benkouiten, A. Nougairde, N. Salez, J.-C. Lagier, M. Million, D. Raoult, P. Brouqui, P. Parola); Institut Hospitalo-Universitaire Méditerranée Infection, Marseille (P. Gautret, R. Charrel, S. Benkouiten, K. Belhouchat, A. Nougairede, T. Drali, N. Salez, J.-C. Lagier, M. Million, D. Raoult, P. Brouqui, P. Parola); Public Health Directorate, Saudi Ministry of Health, Riyadh, Kingdom of Saudi Arabia (Z.A. Memish, M. al Masri); and College of Medicine, Alfaisal University, Riyadh (A. Memish)

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Address for correspondence: Philippe Gautret, Aix Marseille Université, URMITE, 27 Bd Jean Moulin, 13005 Marseille, France; email: philippe.gautret@club-internet.fr

St. Louis Encephalitis Virus Infection in Woman, Peru

To the Editor: St. Louis encephalitis virus (SLEV) is a flavivirus that can asymptomatically infect humans or cause clinically apparent disease that manifests with fever, headache, nausea, and vomiting (*1*). More severe disease with meningoencephalitic involvement may result in stiff neck, alteration in consciousness, gait disturbance, and other focal neurologic deficits. Heightened levels of human disease are often associated with increased abundance of *Culex* spp. mosquitoes and the summer season.

SLEV was first reported in South America in 1960, when it was isolated from pools of *Sabethes bellisarioi* mosquitoes and *Gigantolaelaps* mites in Pará, Brazil (*2*). SLEV was later recovered from humans in Argentina (1963) (*3*) and Brazil (1978) (*4*). Sporadic infections and large outbreaks occurred over ensuing decades, although no isolates in humans have been reported in other South American countries.

Serologic indication of SLEV circulation in Peru was first obtained from hemagglutination-inhibition and neutralization tests of samples collected in 1965 from residents of eastern Peru (*5*). Later, SLEV was isolated from mosquitoes (*6,7*), and SLEV antibody was detected in serum specimens from humans by plaque reduction neutralization tests (*6*). We report the isolation of SLEV from a person in Peru and describe a unique collection method, using oropharyngeal swab specimens, for detecting this virus.

In March 2006, a 50-year-old woman with a 1-day history of fever, sore throat, cough, malaise, myalgia, and headache sought treatment at her local health center in Quistococha, Peru, in the Amazon Basin (3°49′40″ N; 73°19′6″ E). The woman's recent travel was limited to a 70-km radius from this town. Because influenza was suspected, an oropharyngeal swab specimen was collected as part of an influenza-like illness surveillance project, which had been approved by the US Naval Medical Research Center Institutional Review Board and endorsed by the Peruvian Ministry of Health. No blood specimen was obtained because blood was not collected in this respiratory infection–focused protocol. The swab specimen was inoculated onto Madin-Darby canine kidney cells; no cytopathic effect was observed, and the culture was negative for influenza virus and for other respiratory viruses amenable to culture (e.g., adenovirus and parainfluenza virus).

Nearly 6 years later, as part of a retrospective study of previously negative respiratory specimens, the sample was reevaluated for arboviral infection. Universal transport medium (Copan Diagnostics Inc., Murrieta, CA, USA), containing the swab specimen, was inoculated onto Vero 76 cells; cytopathic effects were revealed on day 7. The cells were harvested, and an indirect immunofluorescence assay was performed by using a panel of mouse polyclonal hyperimmune ascitic fluid specific to a variety of flaviviruses. The initial screening tests indicated reactivity to yellow fever virus, dengue virus, and SLEV. Subsequent immunofluorescence assay analyses using monoclonal antibodies against yellow fever virus and all 4 dengue virus serotypes were negative.

Viral RNA was recovered from the Vero culture supernatant and amplified by conventional reverse transcription PCR/nested PCR with generic flavivirus primers against the nonstructural 5 coding region, which confirmed that the isolate was a flavivirus. Real-time reverse transcription PCR with specific SLEV primers confirmed SLEV.

A total of 10,850 bp, almost the full genome sequence of the virus, were sequenced (GenBank accession no. KF589299), and 10,236 nt from these sequences were compared with other SLEV sequences in GenBank. The strain showed 98.4% similarity with a SLEV strain isolated from a bird in Brazil in 1973, 98.4% similarity with an SLEV strain isolated from mosquitoes in Peru in 1975, and 97.9% similarity with an SLEV strain isolated from mosquitoes in the United States in 2003. The US strain may have been carried by migratory birds from Latin America (*8*). Phylogenetic analysis by the neighbor-joining method with 1,000 bootstraps replicates identified the isolate as genotype V, subgenotype A, which grouped with the strains obtained in Brazil, Peru, and the United States (Figure) and

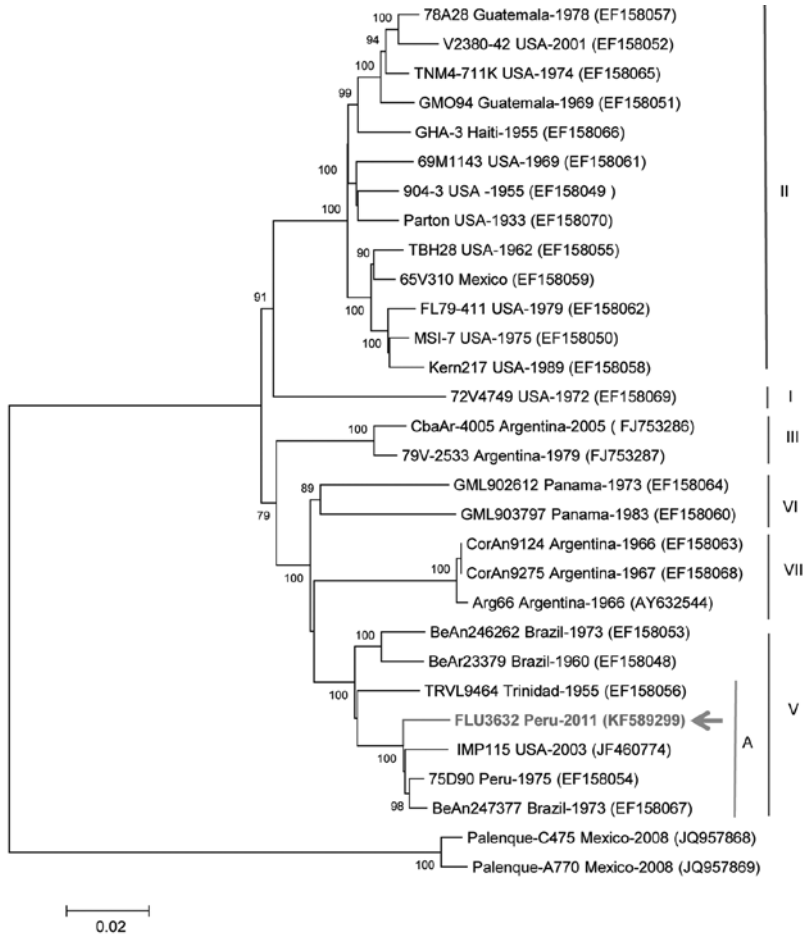

Figure. Phylogenetic analysis from the initially sequenced 10,850-nt region of the St. Louis encephalitis virus (SLEV) genome, isolated from a woman in Peru, 2006. The sequence possessed only 92.8% homology with the NS5 gene region of the sole preexisting SLEV in the laboratory, a genotype II strain similar to TBH28 USA. The Peruvian SLEV sequence described in this case (FLU3632, arrow) groups with Brazil (1975), Peru (1973), and USA (2003) strains, inside the genotype V, subgenotype A. The evolutionary history was inferred by using the neighbor-joining method based on the Kimura 2-parameter model. The entire open reading frame sequence of the Peruvian SLEV isolate was determined in this study by using the Illumina HiSeq 1000 system (Illumina, Inc., San Diego, CA, USA) and assigned GenBank accession no. KF589299. Sequences were analyzed and assembled using the SeqMan Lasergene V.5 and then compared with sequences deposited in GenBank. Multiple sequence alignments were performed by using ClustalX Version 2.0.10 (Conway Institute, University College, Dublin, Ireland; www.clustal.org/) and BioEdit Version 7.0.9.0. (Ibis Biosciences, Carlsbad, CA, USA) Genetic divergence was determined by using MEGA Version 5.02 (www.megasoftware.net/). Scale bar indicates nucleotide substitutions per site.

with an SLEV genotype V strain obtained in Trinidad.

Our findings bolster previous serologic investigations, adding Peru to the South American countries reporting this virus in humans. This discovery is not surprising because *Culex* spp. mosquitoes, the main vectors of SLEV in Brazil and Argentina, have been shown to carry SLEV in Peru (*1*,*7*).

Although SLEV disease in humans is usually confirmed by testing blood or cerebrospinal fluid, the isolate described here was recovered from an oropharyngeal swab specimen. The presence of this virus in human respiratory samples was previously suggested by a study in which nasal wash samples from SLEV-infected persons were injected intranasally into mice, which induced immunity to a subsequent intracerebral challenge with SLEV (*9*). Although this circumstance is uncommon, other arboviruses have also been obtained from the upper respiratory tract, including dengue virus from nasal and throat swab specimens (*10*).

Our findings have many implications. First, SLEV may cause human disease in a wider area of South America than was previously known. In addition, SLEV can cause influenza-like illness and may elude identification unless specific assays are used. Finally, the upper respiratory tract may offer a less invasive way of recovering SLEV isolates than by lumbar puncture or even by drawing blood, although further investigations are needed.

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Vidal Felices, Julia S. Ampuero, Carolina Guevara, Edna R. Caceda, Jorge Gomez, Felix W. Santiago-Maldonado, Patricia V. Aguilar, and Eric S. Halsey

Author affiliations: US Naval Medical Research Unit No. 6, Lima, Peru (V. Felices, J. Ampuero, C. Guevara, E. Caceda,

E. Halsey); Dirección General de Epidemiología-Ministerio de Salud, Lima (J. Gomez); and University of Texas Medical Branch, Galveston, Texas, USA (F. Santiago-Maldonado, P. Aguilar)

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Address for correspondence: Vidal Felices, US Naval Medical Research Unit No. 6, 3230 Lima Place, Washington, DC 20521-3230; email: vidal.felices@med.navy.mil

Whole-Genome Sequencing for Risk Assessment of Long-term Shiga Toxin–producing *Escherichia coli*

To the Editor: Long-term carriage of Shiga toxin–producing *Escherichia coli* (STEC) can greatly affect the social and work lives of infected patients. We describe the use of whole-genome sequencing to assess the risk from long-term STEC carriage in a patient who had been denied surgery because of the infection.

On August 18, 2013, a 64-year-old woman reporting to be a carrier of STEC since March 2013 contacted the University Medical Center Lübeck, Lubeck, Germany, seeking decolonization therapy that had been provided to long-term STEC carriers during the 2011 STEC O104:H4 outbreak (*1*). STEC had initially been identified in the patient during an episode of watery diarrhea. She currently had gonarthrosis grade III, indicating the need for a total knee endoprosthesis; however, the responsible orthopedic department had denied surgery because of the potential risk for development of STEC-associated hemolytic uremic syndrome (HUS) caused by the perioperative use of antimicrobial drug prophylaxis. The patient was also rejected for surgery at another orthopedic clinic. Because of this STEC-associated restriction, the patient requested decolonization therapy.

Before responding to the request, we asked the patient to provide a fecal sample for STEC strain typing. A sample provided on August 22, 2013, was confirmed positive for STEC by culturing an STEC strain on MacConkey agar (bioMérieux, Marcy l'Etoile, France) that did not grow on selective agar (CHROMagar STEC, Mast Diagnostika, Reinfeld, Germany) optimized for the detection of classical enterohemorrhagic *E. coli* strains. Total DNA was extracted from the isolate, and a sequencing library was generated by using the Nextera XT Sample Preparation Kit (Illumina, San Diego, CA, USA). Sequencing was performed (MiSeq Benchtop Sequencer, Illumina) in 2 batches of paired 250-bp sequencing runs. Sequencing reads were further analyzed by using the CLC Genomics Workbench software package (CLC bio, Aarhus, Denmark). De novo assembly resulted in 120 contigs with an average length of 44,331 bp (N50 = 126,317 bp). A predefined dataset of 2,456 sequences was aligned with the generated contigs in a single step by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the alleles and subtypes of genes usually used for *E. coli* and STEC strain typing and for seropathotype detection.

Presence of a Shiga toxin subtype 1a with >99.9% and 100% identity to the *stx*1aA and *stx*1aB subunit genes, respectively (GenBank accession no M19473.1), was confirmed. The STEC strain carried genes with high homology to the O91 antigen–encoding operon (GenBank accession no. AY035396.1) and the H14-flagellin gene (GenBank accession no. AY249998.1). This observation was confirmed by a 100% sequence identity of a 643-bp fragment of the *gnd* gene of the sequenced strain with that of a *gnd* reference sequence of STEC O91:H14 (www.corefacility.ca/ecoli_typer). These 2 sequences are different from the *gnd* sequence of reference strain STEC O91:H21. In vitro multilocus sequence typing (*2*) identified

sequence type (ST) 33. These data were used for risk assessment.

Only strains displaying serotype O91:H21 and a single O91:H10 isolate have been associated with HUS in humans (*3*,*4*). ST33, identified in the patient in this study, has not been associated with HUS in humans despite being the most frequently identified ST of O91 STEC strains in humans (*3*). In addition, the identified strain carried only Shiga toxin 1a, whereas the HUS-associated strain HUSEC034 of serotype O91:H21 carried Shiga toxins 1a, 2a, and 2d (*5*). This data indicated the patient strain was a seropathotype D strain (*6*) with a relative low risk for HUS development in the patient.

The assumption that the patient strain had low pathogenicity was further corroborated by the analysis of additional marker genes (*6*–*9*) indicating the lack of pathogenicity islands associated with high virulence of STEC in humans. None of the 25 marker genes suggested for the LEE locus or pathogenicity islands OI-36, OI-43, OI-44, OI-48, OI-50, OI-57, OI-71 or OI-122 were identified in the patient strain, whereas most of these markers could be detected in highly pathogenic STEC/enterohemorrhagic *E. coli* strains used to establish the method for identifying markers (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/4/13-1782-Techapp1.pdf).

After completing the STEC risk assessment, we advised the patient's general practitioner that antimicrobial drug prophylaxis could be administered for surgery with a low calculated risk for HUS development, as observed for other non-O157 strains (*1*,*10*). In addition, we described our experience with 4 long-term carriers of STEC O91:H14 strains; the patients had been decolonized of STEC by the use of azithromycin decolonization therapy (data not shown).

The patient was added to a waiting list for surgery, and she elected to receive azithromycin as experimental decolonization therapy while awaiting surgery. Azithromycin was administered orally for 3 days (500 mg/day); fecal specimens on post-treatment days 7, 14, and 21 were negative by Shiga toxin ELISA. In addition, an *stx*-specific PCR using enrichment broth confirmed the sustainable eradication of the STEC infection. Our findings show that whole-genome sequencing can be used in the diagnostic process for long-term STEC carriers and might extend or replace other methods used for risk assessment (*6*–*8*,*10*) and treatment decision guidance.

Johannes K.-M. Knobloch, Stefan Niemann, Thomas A. Kohl, Ulrich Lindner, Martin Nitschke, Friedhelm Sayk, and Werner Solbach

Author affiliations: University Hospital Schleswig-Holstein, Lübeck, Germany (J.K.-M. Knobloch, U. Lindner, M. Nitschke, F. Sayk, W. Solbach); Research Center Borstel, Borstel, Germany (S. Niemann, T. A. Kohl); and German Centre for Infection Research (DZIF), Partner Site Hamburg–Lübeck–Borstel, Germany (J.K.-M. Knobloch, S. Niemann, U. Lindner, W. Solbach)

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Address for correspondence: Johannes K.-M. Knobloch, University Hospital Schleswig-Holstein, Campus Lübeck, Department of Medical Microbiology and Hygiene, Ratzeburger Allee 160, 23538 Lübeck, Germany; email: johannes.knobloch@uksh.de

John Frederick Lewis (1804–1876) On the Banks of the Nile, Upper Egypt (detail). Oil on panel, 1876. Yale Center for British Art, Paul Mellon Collection. [B1981.25.418]

Truth in the Details

Sharon Bloom and Emily M. Weeks

"Let him examine, for instance, with a good lens, the eyes of the camels, and he will find there is as much painting beneath their drooping fringes as would, with most painters, be thought enough for the whole head . . . "

—John Ruskin, art critic (1856), regarding the work of John Frederick Lewis.

John Frederick Lewis (1804–1876) was a British painter who specialized in exquisitely detailed paintings of Oriental (Middle Eastern) subject matter, inspired by his ten-year residency in Egypt. Having become an exhibiting member of London's Old Watercolor Society by 1827, Lewis would later turn almost exclusively to oil painting. His early works favored animals and hunting scenes, but his compositions became increasingly diverse after he had traveled to Spain and Europe. In 1841 his travels took him to Cairo, where he remained until 1851, making numerous drawings and gathering the materials that would confirm his growing reputation in London as an "artist-ethnographer."

On the Banks of the Nile, Upper Egypt, painted shortly before Lewis's death in 1876, depicts a springtime scene in rural Egypt, in which a group of traveling Bedouin and their camels pause to meet the local *fellaheen* (peasant farmers). One Bedouin man and his saluki hunting dog rest at the edge of the river, beneath the towering form of a camel, silhouetted against the sky. True to Lewis's realistic style, the details of the man's clothing are rendered with nearly photographic accuracy; he wears the traditional *kufiyeh* (head scarf) and a heavy outer garment of wool and cotton, its voluminous folds showing off a distinctive pattern of broad brown and cream panels. Also typical of the artist's work is the easy parlance between man and beast: the camels wait contentedly for their riders to return, while the dog and waterfowl adopt similarly relaxed demeanors. In the distance are female peasants, who traverse a field of orange and white wildflowers on their way to fetch water from the Nile. They are as much a part of the landscape as are the flora, and they provide a gentle reminder of Lewis's fascination with beauty of all kinds. The viewpoint that

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (S. Bloom); and Independent Art Historian, New Haven, Connecticut, USA (E.M. Weeks)

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Lewis offers–that of an unacknowledged observer upon an idling boat–seems to suggest that we too belong in his composition, as unobtrusive additions to this delicately balanced natural world.

Several Orientalist motifs of Lewis's art are mirrored in this month's issue of Emerging Infectious Diseases. Lewis's juxtaposition of travelers, wild birds, and domesticated animals illustrates ideal opportunities for disease transmission. For example, since 2012, Middle East respiratory syndrome coronavirus (MERS-CoV) has caused an ongoing outbreak of severe acute respiratory tract infection in humans; new findings add to the growing evidence that MERS-CoV, or a closely related virus, infected dromedary camels in the United Arab Emirates long before the first human case of MERS-CoV. While the specific role of camels in MERS-CoV transmission remains unclear, the camel mystery deepens with a report of a novel coronavirus of camels, related to but distinct from MERS-CoV. Wild waterfowl similar to those vividly portrayed by Lewis play a key role in the transmission of avian influenza viruses; active surveillance of avian influenza viruses among domestic poultry in Egypt found that subtype H5N1 still circulates in a widespread manner and that subtype H9N2 is emerging.

The words of art critic John Ruskin, quoted above, allude to Lewis's artistic mission and provide an unexpected link between the disciplines of art and science: In Lewis's meticulous attention to detail and in the careful research of this month's scientists, we witness the tireless pursuit of truth.

Dr Bloom is an associate editor for the journal.

Dr Weeks is an independent art historian and consultant for private collectors, museums, art dealers, and auction houses in America, Britain, Europe, and the Middle East. Her areas of expertise include Orientalism (images of the Middle East created by European artists) and 19th-century British visual culture.

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Address for correspondence: Sharon Bloom, Center for Global Health, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E41, Atlanta, GA 30333, USA; email: sbloom@cdc.gov

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Outbreaks of *Kingella kingae* Infections in Daycare Facilities

Molecular Investigation of Tularemia Outbreaks, Spain, 1997–2008

Bovine Leukemia Virus DNA in Human Breast Tissue

Trends in Mortality Rates of Infectious Diseases, Spain,1980–2011

Carriage Rate and Effects of Vaccination after Outbreaks of Serogroup C Meningococcal Disease, Brazil, 2010

Human Papillomavirus Prevalence in Oropharyngeal Cancers before Vaccine Introduction in the United States

Treatments, Outcomes, and Costs of Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis, United States, 2005–2007

Molecular Characterization of Rabies Virus from Ferret Badgers, Taiwan

Streptococcus mitis Strains Causing Severe Clinical Disease in Cancer Patients

PCR for Detection of Oseltamivir Resistance Mutation in Influenza A(H7N9) Virus

Francisella tularensis subsp. *tularensis* Group A, United States

Novel Avian Influenza A(H7N9) Virus in Tree Sparrow, Shanghai, China, 2013

Full-Genome Analysis of Avian Influenza A(H5N1) Virus from a Human, North America, 2013

Influenza A(H5N2) Virus Antibodies in Humans after Contact with Infected Poultry, Taiwan, 2012

Responses to Threat of Influenza A(H7N9) and Support for Live Poultry Markets, Hong Kong, 2013

Role of Transportation in an Outbreak of Porcine Epidemic Diarrhea Virus Infection, United States

Human Infections with *Rickettsia raoultii*, China

Possible Unnecessary Use of Antimicrobial Drugs by Medicaid Programs

Chronic Wasting Disease Agents in Nonhuman Primates

Shigella spp. with Reduced Susceptibility to Azithromycin, Quebec, Canada, 2012–2013

Acute Lower Respiratory Tract Infections in Soldiers, South Korea, April 2011–March 2012

Complete list of articles in the May issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

May 10–13, 2014
ECCMID
European Congress of Clinical Microbiology and Infectious Diseases
Barcelona, Spain
http://www.eccmid.org/

May 17–20, 2014
114th General Meeting
American Society for Microbiology
Boston, MA
http://www.asm.org/asm2014/

June 24–27, 2014
EMBO conference on
Microbiology after the genomics revolution–Genomes 2014
Institut Pasteur, Paris
http://www.genomes-2014.org

July 27–August 1, 2014
IUMS 2014
International Union of Microbiological Societies
Montréal, Canada
http://www.montrealiums2014.org/side_registration/secure_online_registration_e.shtml

September 5–9, 2014
ICAAC 2014
Interscience Conference on Antimicrobial Agents and Chemotherapy
Washington, DC
http://www.icaac.org

October 8–12, 2014
ID Week
Philadelphia, PA
http://www.idweek.org/

October 31–November 3, 2014
IMED 2014
Vienna, Austria
http://imed.isid.org

November 15–19, 2014
APHA 142nd Annual Meeting & Expo
New Orleans, LA
http://www.apha.org/meetings

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title
Travel-associated Antimicrobial Drug–Resistant Nontyphoidal Salmonellae, 2004–2009

CME Questions

1. Your patient is a 30-year-old, otherwise healthy white woman thought to have nontyphoidal *Salmonella* (NTS). According to the study by Dr. Barlow and colleagues, which of the following statements about the epidemiology and clinical characteristics of NTS infections is correct?

A. Annual incidence of NTS exceeds 1 million in the United States and is estimated to be 98 million worldwide
B. Most NTS infections are transmitted by droplet spray
C. Most NTS infections require antibiotic treatment
D. Ampicillin and chloramphenicol are commonly prescribed in the United States for patients with NTS infection

2. According to the study by Dr. Barlow and colleagues, which of the following statements about antibiotic resistance associated with NTS cases in Oregon between 2004 and 2009 is correct?

A. Clinically important resistance (CIR) occurred in more than one quarter of isolates
B. The odds of acquiring CIR did not change from year to year
C. CIR was defined as decreased susceptibility to ampicillin, ceftriaxone, ciprofloxacin, gentamicin, or trimethoprim-sulfamethoxazole
D. Among cases with travel to East or Southeast Asia, increased resistance was attributable to a single serotype

3. According to the study by Dr. Barlow and colleagues, which of the following variables would most likely be positively associated with antibiotic resistance among NTS cases in Oregon between 2004 and 2009?

A. Outbreaks
B. Travel to Eastern Europe
C. Outpatient treatment
D. Travel to Southeast Asia

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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EMERGING INFECTIOUS DISEASES®

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

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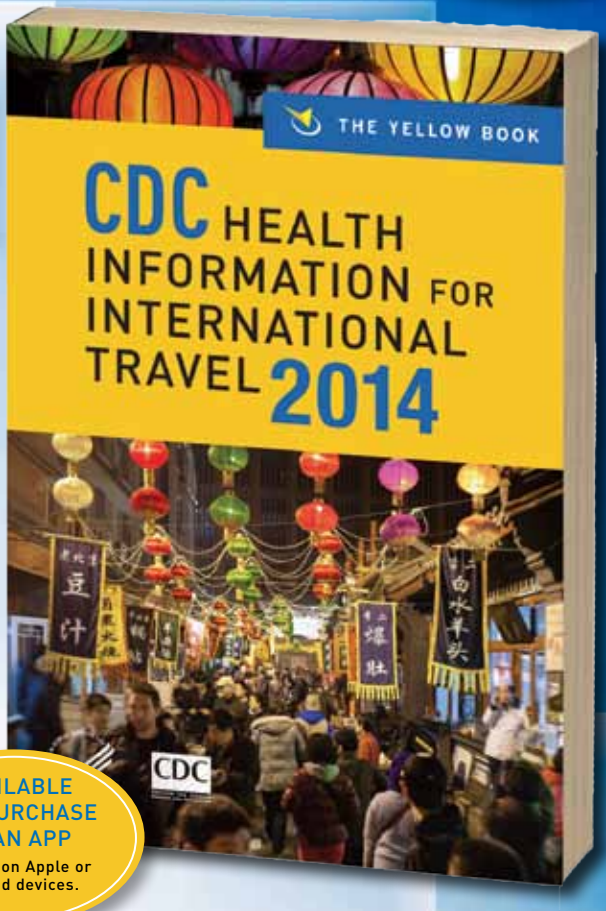

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Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.