MERS Coronavirus in Dromedary Camel Herd, Saudi Arabia

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A prospective study of a dromedary camel herd during the 2013–14 calving season showed Middle East respiratory syndrome coronavirus infection of calves and adults. Virus was isolated from the nose and feces but more frequently from the nose. Preexisting neutralizing antibody did not appear to protect against infection.

Ongoing transmission of Middle East respiratory syndrome coronavirus (MERS-CoV) to humans underscores the need to understand the animal sources of zoonotic infection (*1*,*2*). MERS-CoV RNA has been detected in dromedary camels (*3*,*4*), and dromedary infection precedes human infection (*5*). We conducted a prospective study in dromedary herds in Al-Hasa, Saudi Arabia, through the peak calving season (December 2013–February 2014) to document virologic features of MERS-CoV infection in these animals.

The Study

We studied dromedaries at 2 farms in Al-Hasa, 4–5 km apart. Farm A had 70 animals; 4 were 1 month of age, 8 were ≈1 year of age, and the rest were adults (≥2 years of age). The herd did not go to pasture in the desert ("zero-grazing"; type of grazing may influence types of potential exposures). The animals were sampled on 5 occasions during November 2013–February 2014. Farm B ("semi–zero-grazing") had 17 adults and 3 calves; its herd was sampled in February 2014. Nasal, oral, or rectal swab samples and blood samples were collected (Table 1; online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/20/7/14-0571-Techapp1.pdf). Swab and serum samples were stored frozen at −80°C until testing.

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Hydrolysis probe–based real-time reverse transcription PCR (RT-PCR) targeting MERS-CoV upstream of E (UpE) and open reading frame (ORF) 1a genes and a broad-range RT-PCR reacting across the CoV family to detect other CoVs were used as described (*4*). Specimens initially positive for MERS-CoV were re-extracted and retested to confirm the positive results.

The full genome of MERS-CoV was obtained directly from the clinical specimens with 3–4 times coverage by sequencing PCR amplicons with overlapping sequence reads and sequence assembly (*4*). Dromedary MERS-CoV full genomes obtained in this study (GenBank accession nos. KJ650295–KJ650297) were aligned with human MERS-CoV genomes retrieved from GenBank. We constructed full-genome phylogenies using MEGA5 with neighbor-joining and bootstrap resampling of 500 replicates (*6*). Virus isolation was attempted in Vero E6 cells. We tested serum samples for neutralizing antibody titers using a validated MERS-CoV spike pseudoparticle neutralization test (*7*) (online Technical Appendix).

At farm A, we detected MERS-CoV in 1 of 4 dromedaries sampled on November 30, none of 11 sampled on December 4, nine of 11 sampled on December 30, and none of 9 sampled on February 14 (Table 1). Of the 10 dromedaries that tested positive for MERS-CoV, 9 had parallel nasal and fecal specimens tested, with virus detected in the nasal swab specimens from 8 and the fecal specimen from 1. At the December 30 sampling, 7 of 8 calves and 2 of 3 adults tested positive for MERS-CoV, indicating that when MERS-CoV circulates on a farm, both calves and adults can be infected (online Technical Appendix Table). Because all 12 adults with serum collected before December 30 were seropositive (titers ≥320), it is likely, though not certain, that the MERS-CoV infections in the 2 adults (nos. 21, 19Dam) sampled on December 30 were reinfections, as has been reported for other CoVs (*8*). The seronegative 1-year-old calves, nos. 13 and 14, had the highest nasal viral loads (UpE assay 1.3×10^8 to 1.78×10^8/mL specimen), and a 2-week-old calf, no. 22, with (presumably passively acquired) titers of 1,280 became infected but had a much lower viral load. Overall, these data suggest that prior infection or passively acquired maternal antibody might not provide complete protection from infection (online Technical Appendix Table).

Four MERS-CoV–positive calves had mild respiratory signs (cough, sneezing, respiratory discharge), abnormally elevated body temperature, and loss of appetite at the December 30 sampling, which resolved over a few days. Three calves from which paired serum samples were available (Table 2; nos. 13, 15, 17) demonstrated ≥4-fold rising antibody titers to MERS-CoV. Calf no. 13 (1 year of age) had

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Table 1. RT-PCR of dromedary camel samples for MERS-CoV, Al-Hasa, Saudi Arabia*

Farm, sampling date	Age†/no. sampled	No. specimens positive/no. tested		
		Nasal	Oral	Fecal
Farm A				
2013 Nov 30	Calf, 0	ND	ND	ND
	Adult, 4	1/1	0/2	0/4
2013 Dec 4	Calf, 9	ND	0/9	0/7
	Adult, 2	ND	0/2	0/2
2013 Dec 30	Calf, 8	7/8	0/1	0/6
	Adult, 3	1/3‡	0	1/3‡
2014 Feb 14	Calf, 7	0/7	ND	0/7
	Adult, 2	0/2	ND	0/2
Farm B: 2014 Feb 11	Calf, 3	0/3	ND	0/3
	Adult, 3	0/3	ND	0/3

*Data on individual dromedaries are provided in online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/20/7/14-0571-Techapp1.pdf. RT-PCR, reverse transcription PCR; MERS-CoV, Middle East respiratory syndrome coronavirus; ND, not done.
†Adults are 6–14 y of age; calves are 40 d to 2 y of age.
‡Two different dromedaries were positive in nasal and fecal swabs.

a high viral load and was seronegative at the first MERS-CoV–positive result (indicating that it had been recently infected) but was MERS-CoV RNA negative 6 weeks later, suggesting that virus shedding is not prolonged. We did not detect virus RNA by RT-PCR in the 3 acute-phase serum samples from infected dromedaries (nos. 1, 16, 17), suggesting that acute infection is not associated with prolonged viremia. Dromedaries from farm B were sampled once on February 11; all results were negative.

The full genomes of MERS-CoV sequenced directly from a nasal swab specimen collected on November 30 were identical to those from a nasal swab specimen and a fecal specimen collected on December 30. In addition, the complete spike gene was sequenced from 4 other MERS-CoV–positive nasal swab specimens, and these spike genes were genetically identical.

Virus isolation in Vero E6 cells was attempted from 7 positive nasal swab and fecal specimens that had $>10^6$ copies/mL in the original sample in the UpE RT-PCR. Viruses were isolated from 2 nasal swab (nos. 13, 14) and 1 fecal swab (no. 19Dam) specimens collected on December 30; these were the specimens with high numbers of MERS-CoV copies (9.27×10^7 to 1.78×10^8 copies/mL). The full-genome sequence of 1 virus culture isolate was obtained in parallel with that of the original virus in the original clinical specimen. We observed 3 nucleotide changes in ORF1b, spike, and membrane protein genes in the isolates after 2 passages in Vero E6 cells, of which 2 were nonsynonymous, leading to changes in spike (S1251F) and membrane proteins (T8I). This finding highlights the importance of sequencing the viral genome directly from clinical specimens.

MERS-CoVs circulating in dromedaries on farm A during a 1-month period were genetically identical over the full 30,100-nt genome in 3 viruses and the spike protein of 4 more viruses, giving a mutation rate of 0 nt substitutions per site per day (95% credible interval 0 to 2.7×10^{-6}). The estimated mutation rate for epidemiologically unlinked human MERS-CoV was 3.1×10^{-6} (95% CI 2.4×10^{-6} to 3.8×10^{-6}) (*9*).

Conclusions

The unusual genetic stability of MERS-CoV in dromedaries, taken together with its high seroprevalence (*7*,*10*–*13*), raises the hypothesis that dromedaries might be the natural host for this virus. Further longitudinal studies of MERS-CoVs in dromedaries are needed to confirm this hypothesis.

Genome organization of the dromedary MERS-CoV detected in this study was identical to that of the virus in humans. The virus strains clustered phylogenetically within clade B (*9*) and were most closely related to the strain MERS-CoV_FRA/UAE and to MERS-CoV detected in Buraidah (Saudi Arabia) and Al-Hasa (Figure). The farm is ≈300 km from United Arab Emirates and 600 km from Buraidah. Dromedaries move between Al-Hasa and Buraidah and, more limitedly, between Al-Hasa and United Arab Emirates.

The full-genome sequence of MERS-CoV from dromedaries in this study is 99.9% similar to genomes of human clade B MERS-CoV. The spike gene is the major determinant for virus host specificity. In comparison with other publically available human MERS-CoV sequences, we found 6-nt mutations in the spike gene unique to these dromedary viruses. Of these, 3 (S457G, L773F, and V810I) were nonsynonymous. These amino acid changes are located outside the binding interface between MERS-CoV spike protein and human DPP4 receptor, suggesting these amino acid differences are unlikely to affect receptor binding. Thus, these dromedary viruses may retain capacity to infect humans, as Chu et al. suggested for dromedary MERS-CoV in Egypt (*4*).

MERS-CoV may be isolated from nasal swab specimans and feces, indicating that both could be possible sources of virus transmission to humans and other animals, but virus detection rates were higher in nasal swab specimens.

Table 2.Longitudinal sampling of MERS-CoV–positive dromedary camel calves on farm A, Al-Hasa, Saudi Arabia*

Calf no.	Sample collection date	Sex/age	RT-PCR result	Titer
13	2013 Dec 30	F/1 y	Positive	<20
	2014 Feb 14	F/1 y	Negative	640
15	2013 Dec 30	F/1 y	Positive	20
	2014 Feb 14	F/1 y	Negative	160
17	2013 Dec 30	F/40 d	Positive	80
	2014 Feb 14	F/3 mo	Negative	1,280
19	2013 Dec 30	F/1 y	Positive	NA
	2014 Feb 14	F/1 y	Negative	320

*MERS-CoV, Middle East respiratory syndrome coronavirus; RT-PCR, reverse transcription PCR.

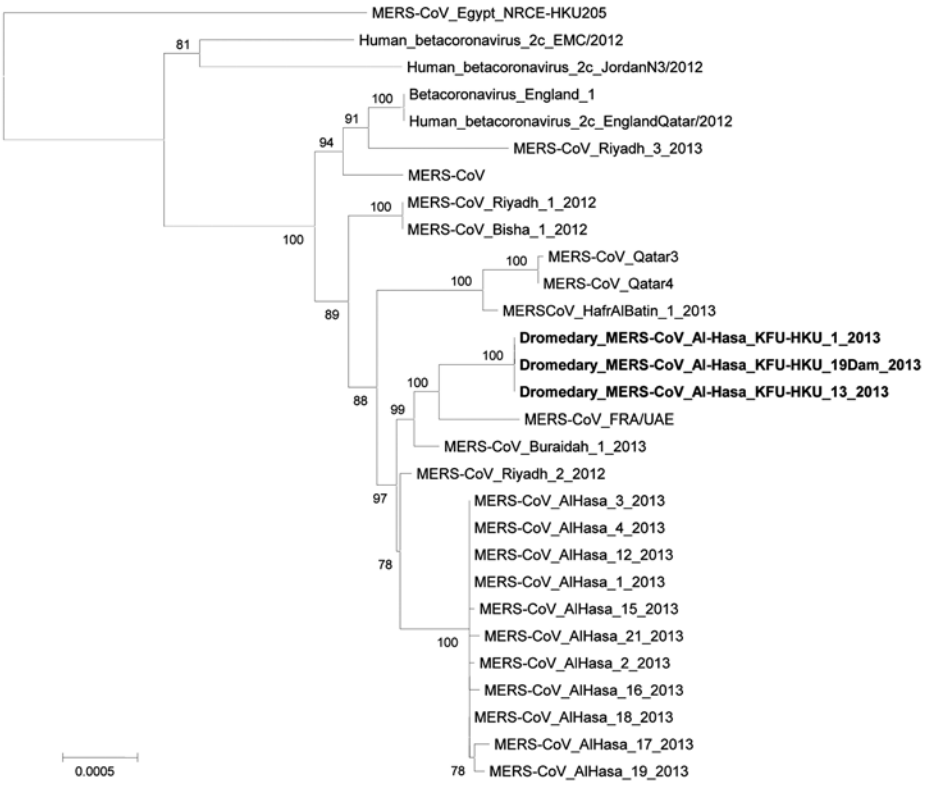

Figure. Phylogenetic tree of Middle East respiratory syndrome coronavirus (MERS-CoV) full genomes (29,901 nt after trimming the ends) or near–full genomes from humans and dromedary camels. The tree was constructed by using neighbor-joining methods with bootstrap resampling of 500 replicates. The most divergent MERS-CoV, Egypt NRCE-HKU205, was used as outgroup. Bold type indicates camel MERS-CoV genomes from this study. GenBank accession numbers of genome sequences included in this study are KJ477102, KF600652, KF600630, KF600651, KF186567, KF600627, KF186564, KF600634, KF600632, KF600644, KF600647, KF600645, KF186565, KF186566, KF745068, KF600620, KF600612, KC667074, KC164505, KF192507, KF600613, KF600628, KF961222, KF961221, KC776174, and JX869059. Scale bar indicates nucleotide substitutions per site.

Our preliminary data suggest that preexisting MERS-CoV antibody might not completely protect against re-infection; however, this question needs more investigation.

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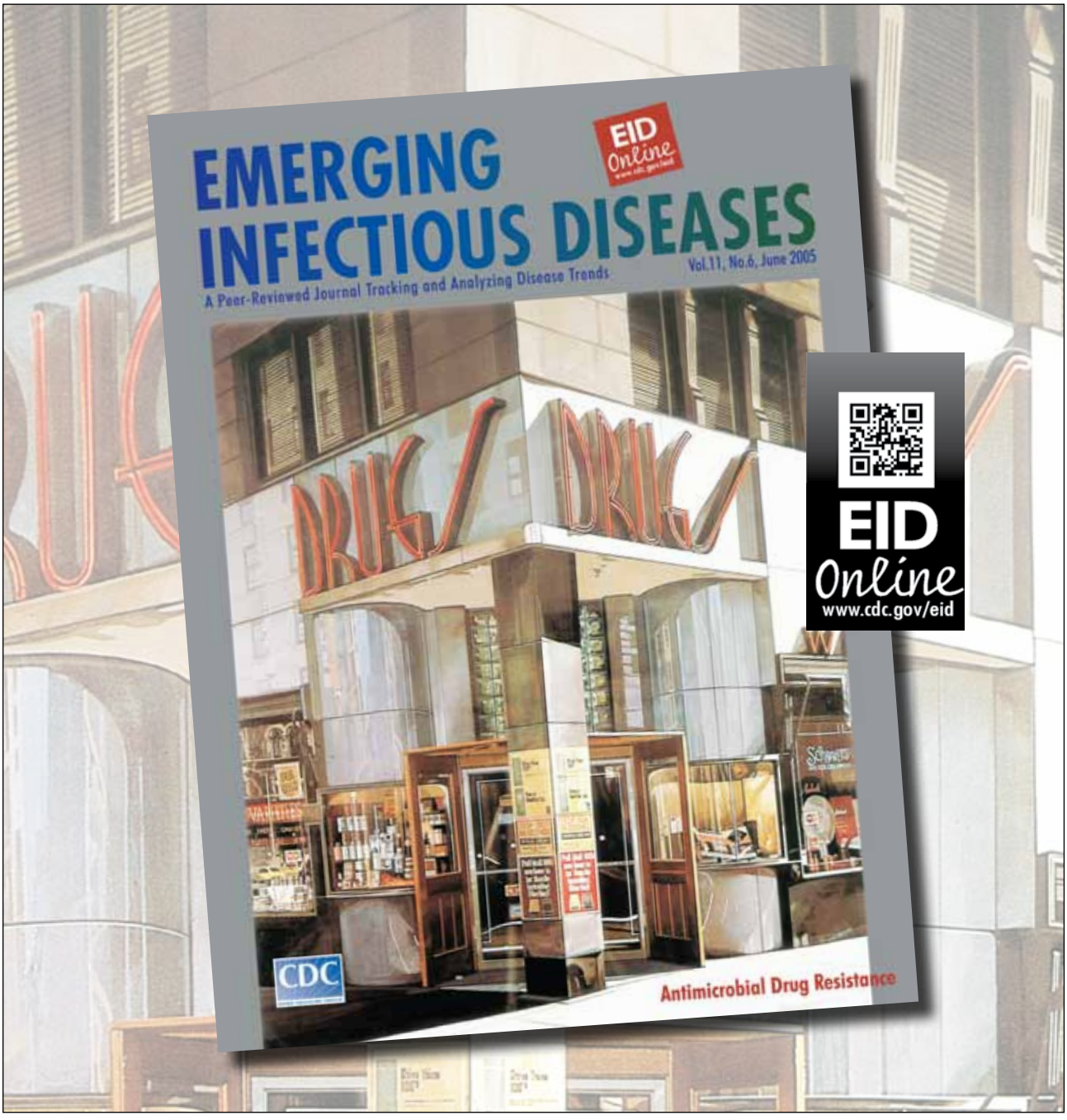

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Technical Appendix

Technical Appendix Table. Testing of dromedary camels by RT-PCR and serologic testing for MERS-CoV, Al-Hasa, Saudi Arabia*

Farm, sampling date	Camel no.	Calf/Adult†	Age	RT-PCR result: Nasal sample	RT-PCR result: Oral sample	RT-PCR result: Fecal sample	Copy/mL‡	Antibody titers§
Farm A								
2013 Nov 30	1	Adult	13 y	Pos¶	Neg	Neg	2.61×10^7	>5,120
	2	Adult	12 y	–	–	Neg		>5,120
	3	Adult	10 y	–	Neg	Neg		>5,120
	4	Adult	14 y	–	–	Neg		>5,120
2013 Dec 4	5	Adult	8 y	–	Neg	Neg		640
	6	Adult	9 y	–	Neg	Neg		2,560
	2	Adult	10 y	–	Neg	–		>5,120
	7Calf	Calf	1–2 y	–	Neg	Neg		1,280
	7Dam	Adult	9.5 y	–	Neg	–		2,560
	8	Adult	7 y	–	Neg	Neg		1,280
	9	Adult	6 y	–	Neg	Neg		1,280
	10	Adult	8 y	–	Neg	Neg		640
	11Calf	Calf	1–2 y	–	Neg	Neg		–
	11Dam	Adult	–	–	Neg	Neg		–
	12	Adult	12 y	–	Neg	Neg		320
2013 Dec 30	13	Calf	1 y	Pos¶#	–	–	1.30×10^8	<20
	14	Calf	1 y	Pos#	–	Neg	1.78×10^8	<20
	15	Calf	1 y	Pos	–	Neg	6.07×10^6	20
	16	Calf	1 y	Pos	–	Neg	3.78×10^7	>5,120
	17	Calf	40 d	Pos	–	Neg	4.86×10^4	80
	18	Calf	40 d	Neg	Neg	–		–
	19Calf	Calf	1 y	Pos	–	Neg	2.41×10^7	–
	19Dam	Adult	–	Neg	–	Pos¶#	9.27×10^7	–
	20	Adult	8 y	Neg	–	Neg		>5,120
	21	Adult	7 y	Pos	–	Neg	3.31×10^3	320
	22	Calf	2 wk	Pos	–	Neg	3.38×10^3	1,280
2014 Feb 14	26	Calf	9 mo	Neg	–	Neg		>5,120
	13	Calf	1 y	Neg	–	Neg		640
	27	Calf	1 0 mo	Neg	–	Neg		40
	15	Calf	1 y	Neg	–	Neg		160
	17	Calf	3 mo	Neg	–	Neg		1,280
	11Dam	Adult	12 y	Neg	–	Neg		1,280
	19Calf	Calf	1 y	Neg	–	Neg		320
	28Calf	Calf	3 mo	Neg	–	Neg		20
	28Dam	Adult	10 y	Neg	–	Neg		1,280
Farm B, 2014 Feb 11	23Calf	Calf	2.5 mo	Neg	–	Neg		–
	23Dam	Adult	7 y	Neg	–	Neg		>5120
	24Calf	Calf	2 mo	Neg	–	Neg		–
	24Dam	Adult	6 y	Neg	–	Neg		1,280
	25Calf	Calf	2 mo	Neg	–	Neg		–
	25Dam	Adult	6 y	Neg	–	Neg		640

*RT-PCR, reverse transcription PCR; MERS-CoV, Middle East respiratory syndrome coronavirus; Pos, positive; Neg, negative; –, specimen not collected or age information not available.
†Calf defined as dromedary camel <2 y of age; adult defined as dromedary camel ≥2 y of age.
‡Data deduced from the upstream of E assay.
§Pseudotype neutralization antibody titers.
¶Full genome sequenced.
#Virus isolated.

Methods

PCR

Hydrolysis probe-based real-time PCRs targeting upstream of E gene (UpE) and open reading frame (ORF) 1a were used as recommended by the World Health Organization (WHO). We also tested each specimen with broad-range reverse transcription PCR (RT-PCR) conserved across the coronavirus family to detect other coronaviruses. The nucleic acid extraction methods, primers, and PCR testing protocols used have been described (*1*).

In brief, nucleic acid was extracted from 140-μL aliquots of swab supernatants using QIAamp Viral RNA Minikit (QIAGEN, Hilden, Germany) following the procedure recommended by the manufacturer. Viral RNA was eluted in 60 μL elution buffer provided in the kit, and 12 μL was used for preparing a 20-μL reverse transcription reaction with Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Complementary DNA (cDNA) produced was subjected to the detection of Middle East respiratory syndrome coronavirus (MERS-CoV) by using probe-based real-time PCRs targeting UpE and ORF1a as recommended by WHO. For the upE qPCR, a 15-μL reaction was set up containing 1 μM of each of the forward (5′-GCAACGCGCGATTCAGTT-3′) and reverse (5′-GCCTCTACACGGGACCCATA-3′) primers, 0.5 μM probe (5′6-FAM/CTCTTCACATAATCGCCCCGAGCTCG/3′6-TAMSp), and 2 μL cDNA in 1× reaction buffer (Takara, Kyoto, Japan). The reaction was carried out in ViiA 7 real-time PCR system (Life Technologies) with 40 cycles of amplification. For the ORF1a quantitative PCR, the reaction was set up as above except with forward primer (5′-CCACTACTCCCATTTCGTCAG-3′), reverse primer (5′-CAGTATGTGTAGTGCGCATATAAGCA-3′), and probe (5′6-FAM/TTGCAAATTGGCTTGCCCCCACT/3′6-TAMSp) targeting the ORF1a gene. Cycle threshold was generated by the ViiA7 system automatically with default settings.

The full genome of MERS-CoV in dromedary camel swab samples was deduced by sequencing PCR amplicons with overlapping sequence reads. Genome sequence of the virus was constructed by concatenating the amplicon sequences obtained. The sequencing was done with 3–4 times coverage. Genes of the dromedary MERS-CoV was identified by ORF prediction and with reference to published human MERS-CoV genomes. Dromedary MERS-CoV genomes were aligned with human MERS-CoV genome sequences retrieved from

GenBank. Phylogenetic trees were constructed by using MEGA5 with neighbor-joining and bootstrap resampling (*2*).

Virus Isolation

A 200-µL aliquot of original sample was filtered through 0.4-µm filters (Millipore, Billerica, MA, USA) and 100 µL aliquots of neat 1:10 diluted and 1:100 diluted samples were inoculated into Vero E6 cell (ATCC CRL-1586) in T25 flasks with ≈80% confluence. The inoculated cells were kept in minimum essential medium without fetal bovine serum and incubated at 37°C for 6 days. The cells were examined daily to detect virus cytopathic effect (CPE). Upon appearance of CPE, or at day 6 if no CPE was observed, the cells were harvested and passaged for a second time in Vero E6 cells. The cells and supernatant of flasks showing CPE was harvested, aliquoted, and stored at –80°°C.

Serology

HIV pseudoparticles bearing the MERS-CoV spike protein was prepared as described (*3*). HIV/MERS pseudoparticles (5 ng of p24) were pre-incubated with serially diluted heat inactivated serum for 30 min at 4°C and then added to Vero E6 cells (ATCC CRL-1586) in triplicate in 96-well microtiter plates. Residual virus replication was assayed at 2 days post infection as described (*3*). The highest serum dilution giving a 90% reduction of luciferase activity was regarded as the ppNT antibody titer.

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