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Serologic Surveillance for SARS-CoV-2 Infection among Wild Rodents, Europe

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We report results from serologic surveillance for exposure to SARS-CoV-2 among 1,237 wild rodents and small mammals across Europe. All samples were negative, with the possible exception of 1. Despite suspected potential for human-to-rodent spillover, no evidence of widespread SARS-CoV-2 circulation in rodent populations has been reported to date.

Esitämme tulokset serologisesta tutkimuksesta, jossaseulottiin SARS-CoV-2 tartuntojen varalta 1,237 luonnonvaraista jyrsijää ja piennisäkästä eri puolilta Eurooppaa. Kaikki näytteet olivat negatiivisia, yhtä näytettä lukuun ottamatta. SARS-CoV-2:n läikkymisen ihmisistä jyrsijöihin on arveltu olevan mahdollista, mutta todisteet viruksen laajamittaisesta leviämisestä jyrsijäpopulaatioissa puuttuvat.

Reverse transmission of diverse zoonotic pathogens (bacteria, viruses, eukaryotic parasites, fungi) from humans to animals has been recognized and documented as a global concern for years (*1*). On July 6, 2022, the World Organisation for Animal Health (OIE) stated, "While occasional occurrences of COVID-19 in domestic or zoo animals show little long-term consequence, infections at wildlife population levels indicate the possibility of further evolution of the virus in animals, and a future reintroduction of the virus into humans at a later date" (*2*). From a One Health perspective, "There is an urgent need to develop frameworks to assess the risk of SARS-CoV-2 becoming established in wild mammal populations" (*3*). In particular, wild rodents are suspected of being among the species more susceptible to SARS-CoV-2 infection, and susceptibility to experimental infection has been confirmed among various rodent species (*4*–*6*). Specific courses of infection may differ among rodent host species, but infection usually results in little or no detectable disease, although infectious virus may shed for 4–7 days after infection and disease may be transmitted to naive rodents (*4*–*6*). These characteristics suggest the potential for reverse transmission, broad circulation, and possible long-term establishment of SARS-CoV-2 in rodent populations. Such an event would be of concern: hamsters, for example, have transmitted SARS-CoV-2 to humans,

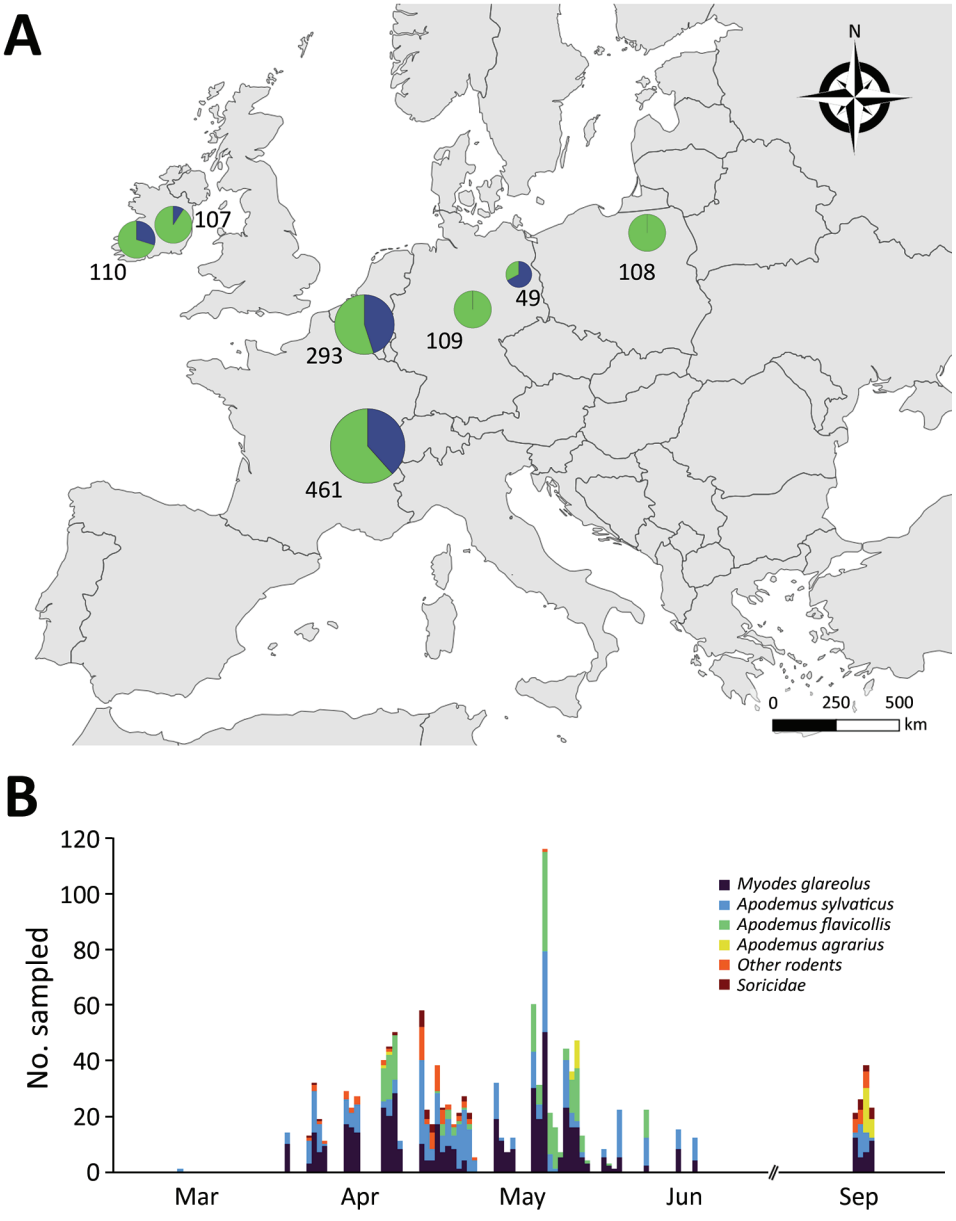

Figure. Sampling of various areas in Europe to detect SARS-CoV-2 antibody response in wild rodents. A) Location of sampling areas. Colors indicate the proportion of samples taken in the 2 habitat types (green: forests; blue: urban parks) and symbol size and numbers indicate sample size. Samples were taken from up to 8 different sites in each country (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/28/12/22-1235-App1.pdf). B) Number of individuals sampled, by date and taxonomy. Details of sampling periods, habitats, and rodent species are provided in Appendix 2 (https://wwwnc.cdc.gov/EID/article/28/12/22-1235-App2.xlsx)

followed by subsequent person-to-person transmission (*7*). Consequently, on December 6, 2021, the joint United Nations Food and Agriculture Organization and OIE (FAO-OIE) Advisory Group on SARS-CoV-2 Evolution in Animals indicated that a large surveillance study of rodent populations exposed to human contact was needed to close a major gap in SARS-CoV-2 research.

Animal experiments have shown that antibodies can be detected consistently for several weeks or longer after rodent infection with SARS-CoV-2, although detectable virus shedding lasts only a few days (*4*–*6*). When field prevalence is low or unknown among the target population, serologic testing is the preferred method to maximize chances of detecting circulation of viruses such as SARS-CoV-2 that cause brief infection but maintain longer-lasting serologic response. A recent survey in Hong Kong found a Norway rat (*Rattus norvegicus*) to be potentially seropositive for SARS-CoV-2 (*8*). Considering the high biodiversity and ubiquity of rodents, this finding called for broader surveillance studies in other continents, habitats, and noncommensal rodent species. To investigate its possible reverse zoonotic transmission and establishment in wild rodents in different settings, we conducted a large-scale serologic survey of SARS-CoV-2 in multiple rodent species across Europe.

We sampled animals in urban parks and zoos, which offer ample opportunity for transmission between humans and rodents, and forests, because other wild forest mammals such as deer have become naturally infected with SARS-CoV-2 (*9*). During 2021, we sampled 1,202 rodents and 35 Soricidae shrews (genera *Sorex* and *Crocidura*) from 23 forests sites and 8 urban parks in 5 countries in Europe (Ireland, Belgium, France, Germany, and Poland) (Figure 1; Appendix 1 Figure 1, https://wwwnc.cdc.gov/EID/article/28/12/22-1235-App1.pdf; Appendix 2, https://wwwnc.cdc.gov/EID/article/28/12/22-1235-App2.xlsx). We then assessed each rodent's SARS-CoV-2 serologic status using an infected cell-based immunofluorescent assay (IFA; Appendix 1) (*10*). We chose the IFA instead of a neutralization assay as the initial screening test because it is scalable to a large number of samples and can be effective in detecting both neutralizing and nonneutralizing antibodies.

All but one of the rodents sampled were IFA negative for SARS-CoV-2. The one IFA-positive rodent (assayed twice on different days to rule out any handling error) was a wood mouse (*Apodemus sylvaticus*) sampled in an urban park near the city of Antwerp, Belgium, on April 6, 2021. We then tested this IFA-positive sample using a seroneutralization assay (Appendix 1), and results were negative, suggesting that the sample had no detectable neutralizing antibodies against the virus strain used in the seroneutralization assay. The sample was also negative by microsphere immunoassay (Appendix 1). The overall serologic status of this wood mouse was therefore unconfirmed. To further investigate possible virus circulation in the area, we used the Luna SARS-CoV-2 RT-qPCR Multiplex Assay Kit (New England BioLabs, https://www.neb.com) to test samples from all 59 rodents captured in the same location as the wood mouse (Appendix 1). PCRs were all negative (including for the IFA-positive wood mouse), which could be expected given the short virus-shedding period described in rodents (*4*–*6*).

Our main conclusion on the basis of this survey is that there is no evidence of a major SARS-CoV-2 spread among wild rodents in northern Europe as of April–September 2021. A similar conclusion had been reached in the study from Hong Kong (*8*), an area with a denser human population and large populations of pest rodents. In that study, serum from 1 urban brown rat was positive in some but not all serologic tests used, and all SARS-CoV-2 PCR tests were negative (*8*). Taken together, these results indicate no evidence of widespread SARS-CoV-2 circulation in rodent populations to date.

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Trapping data from this study will be available in Germany in the Biodiversity Exploratories Information System (https://doi.org/10.17616/R32P9Q).

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Delayed Diagnosis of Acute Q Fever, China

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We report a patient in China with fever of unknown origin who visited 3 hospitals in 3 weeks and was finally given a diagnosis of acute Q fever, determined by metagenomics next-generation sequencing. Our results indicate that physicians are unfamiliar with Q fever and the disease is neglected in China.

Q fever is an important worldwide zoonosis with nonspecific symptoms, making diagnosis challenging (*1*–*3*). Humans become infected mainly by inhalation of *Coxiella burnetii*–contaminated aerosols from animal waste or contaminated soil (*4*). *C. burnetii* is listed as a biologic weapon in the United States, and Q fever is a nationally notifiable disease in the United States, Australia, Netherlands, and Japan, but it is not a notifiable disease in China (*2*,*5*–*7*). Serologic epidemiology indicates that *C. burnetii* is widely distributed in China, but Q fever is rarely reported and might be neglected (*2*,*7*). We report a case of Q fever in a man in Shandong Province, China. The need for ethics approval and informed consent was waived,

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Appendix

This appendix contains methodological details on sample collection (field samples and experimental controls) and the laboratory diagnostic assays (serologic and molecular). The final section consists of the work's legal and ethical statements.

Sampling

Field Samples

Small mammals were trapped using snap-traps (Germany) or live traps (all countries) whose size was adapted to the target species (e.g., large wire mesh traps for rats, Sherman or Longworth traps for *Myodes*, *Microtus*, or *Apodemus* spp.). Traps were set either following predetermined transect lines, or at specific sites where rodents had been seen by site managers (details available upon request). Traps were left on each sampling site for one to eleven nights. Live traps were checked every morning, and re-filled with hydrophobic cotton or straw and food (seeds, carrots, sardine, peanut butter) daily to provide resources and a suitable environment for trapped animals.

Trapped animals were identified using morphological criteria in the field. When field identification was problematic, molecular identification was performed in the lab: *Microtus* species were identified using Sanger sequencing of CO1 fragment (*1*) and *Apodemus sylvaticus* and *A. flavicollis* were distinguished using the AP-PCR as in (*2*).

Live-trapped animals were euthanized with an overdose of isoflurane. Rodents found dead by site managers were also included in the study. All animals were dissected and several organs were collected (including the heart, placed in PBS for serologic assaying, and colon, placed in RNAlater), and stored at –20°C until assayed. Individual characteristics were also recorded (mass, body length, gender, sexual characteristics).

In total we sampled 853 animals from forests and 384 from urban parks. A breakdown of the samples collected by host species, localities and dates is provided in Appendix 2

(https://wwwnc.cdc.gov/EID/article/28/12/22-1235-App1.xlsx). All legal and ethical information regarding this study is collated in a dedicated section at the end of this Appendix.

Experimental Control Samples

Vaccinated Animals

Ten-week old Syrian golden hamsters (*Mesocricetus auratus*) were acclimatized at the University of Helsinki biosafety level 3 (BSL-3) facility for 7 days in individually ventilated biocontainment cages (ISOcage; Scanbur) with one hamster per cage. Animals were then immunized twice 7 days apart with an experimental receptor binding domain-based nasal vaccine (patent pending). Immunized hamsters were euthanized by cervical dislocation 14 days after the second immunization and heart was collected into PBS and stored at –20°C.

Challenged Animals

Virus. The challenge SARS-CoV-2 strain used in these experiments was prepared as described previously (*3*) and used at passage 2.

Animals. Seven week-old female Syrian golden hamsters (strain RjHan:AURA) were purchased from Janviers's breeding Centre (Le Genest, St Isle, France), housed in an animal-biosafety level 3 facility at ANSES, Malzéville, France and left to acclimatize for a minimum of 7 days before challenge. For collection of both sample types (below), the animals were anesthetized with a mix of ketamine + xylazine (150 mg/kg + 10 mg/kg) administered by the intraperitoneal route, killed by exsanguination, and necropsied.

Plasma Samples. Six hamsters were anesthetized using isoflurane and intranasally inoculated with 40 µL containing 10^4 $TCID_{50}$ of SARS-CoV-2 virus (20 µL in each nostril). At fourteen days post-infection, the blood was collected by heart puncture in 4 mL EDTA 3K Vacutest tubes. The plasmas were obtained after centrifugation (15 min, 1000 g) and stored at –16°C until analysis. The presence of SARS-CoV-2 neutralizing antibodies was confirmed in these samples by seroneutralization (see methods details below) before IFA testing.

Heart Samples. Six hamsters were anesthetized with isoflurane and intranasally inoculated with 40 µL containing 10^5 $TCID_{50}$ of virus (20 µL in each nostril). At fifteen days post-infection, after exsanguination, the hearts were collected in vials containing 500 µL of sterile PBS. The samples were then stored at –16°C until analysis. The presence of SARS-CoV-2 antibodies in these samples was confirmed by microsphere immunoassay (see methods details below) before IFA testing. Figure S2 shows representative IFA slides, including three different positive controls and three field samples (two negative and the one positive).

Laboratory Diagnostic Procedures

Immunofluorescent Assay (IFA)

All field samples were screened for SARS-CoV-2 antibodies using an immunofluorescent assay (IFA) based on the SARS-CoV-2/Finland/1/2020 virus as described in (*4*), with the following modifications:

- Samples consisted of whole rodent hearts in PBS, whose supernatant was assayed undiluted, and
- The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, diluted 1/100 in PBS. Anti-mouse conjugates have been used in IFA to detect antibodies against other viruses in both *Myodes* and *Apodemus* samples (*5*,*6*).

The capacity of this test for robust detection of rodent SARS-CoV-2 antibody response was assessed using a range of animal experiments comparing:

- different immunization methods (vaccination versus experimental infection),
- different sample types (plasma versus heart in PBS),
- different secondary conjugates (anti-mouse versus anti-hamster),

Results were consistently positive for all tested combinations of the above factors (Appendix Table). The corresponding animal procedures are detailed in section A.2. above.

Confirmatory Serologic Assays

The IFA-positive field sample was subjected to two further SARS-CoV-2 serologic assays: a microsphere immunoassay and seroneutralization. These assays were also used to confirm the experimental positive controls described above before IFA testing.

Microsphere Immunoassay

This assay was carried out as described in (*7*) with the following modifications: the three recombinant SARS-CoV-2 antigens used to capture SARS-CoV-2 specific antibodies were the Nucleoprotein, the Spike Glycoprotein (S1) RBD and the Spike Glycoprotein (trimer) (obtained from The Native Antigen Company). Bovine Serum Albumine (Sigma) was used as a control antigen.

Seroneutralisation

Vero E6 cells were plated in 96-well microplates in Dulbecco's Modified Eagle medium (DMEM) with 10% FCS (fetal calf serum) and 1% antibiotics (Penicillin/Streptomycin) (20,000 cells in 200 µL per well).

On the following day, serum samples as well as positive and negative internal controls were serially diluted (1 in 3 dilution steps) in culture medium. Fifty microliters of culture medium containing approx. 100 $TCID_{50}$ (back-titrated during the seroneutralization assays) of SARS-CoV-2 virus strain UCN19 (*8*) were then added to the diluted sera. The plates were incubated at 37°C with 5% CO_2 for 1 h to allow neutralisation complexes to form between the neutralizing antibodies and the virus. Afterwards, the cell culture supernatants were removed and replaced with 100 µL of the virus + serially diluted sample (or control) mixes. The microplates were then incubated at 37°C in a humid chamber containing 5% CO_2 for at least 3 days. Plates were then read using an "all or nothing" (binary) scoring method for the presence of viral cytopathic effect (CPE). The neutralisation titers were based on the highest dilution that prevented discernible cytopathic effect. The IFA positive sample was assessed both after a 500 g x 5 min centrifugation starting at a 1:10 dilution, and without centrifugation starting from the neat sample. It was negative in both experiments.

PCR screening of Fort 6 samples (Belgium)

The 59 animals from the Fort 6 location near Antwerp, Belgium, where the seropositive rodent had been detected, were screened for SARS-CoV-2 infection using a specific PCR. Total RNA was extracted from rodent colon samples using the QIAamp 96 Virus QIAcube HT kit (Qiagen). Colon samples were first removed from RNAlater in a BSL2 laboratory and approx. Ten mg were placed in 180 µL ATL buffer + 20 µL proteinase K (supplied with the kit) in secure 2 mL tubes containing two glass bead and autoclaved sand. The tubes were then incubated at 56°C for 30 min for enzymatic lysis, after which they were shaken at 30 Hz for 2 × 2 min using a TissueLyser (Qiagen). Lysates were then spun at 500 g for 5 minutes, and 200 µL clear supernatant were used as starting material for automated QIAcube extraction as per manufacturer's instruction, with the following modification: the final target elution volume was 120 µL. Eluted RNA were then stored at –80°C until assayed by PCR. Tissues from SARS-CoV-2 challenged rodents were used as positive extractions controls with every extraction batch and returned consistent positive PCR.

Extracted RNA were then assayed using the Luna SARS-CoV-2 RT-qPCR Multiplex Assay Kit (New England BioLabs Inc, MA USA) as per manufacturer's instructions with no modification (using the provided kit positive control).

Legal and ethical statements

Field Sampling

Belgium

The procedures were approved by the University of Antwerp Ethical Committee for Animal Experiments (permit number 2020–21). Small mammal trapping was approved by the Flemish regional nature authority (Agentschap voor Natuur en Bos, ANB). The handling of small mammals was carried out in accordance with the recommendations in Directive 2010/63/EU.

France

The procedures complied with the French regulations on care and protection of laboratory animals (French Law 2001–486 issued on June 6th, 2001 and Directive 2010/63/EU issued on September 22nd, 2010). They were also authorised by the regional ethical committee for animal experiments (Languedoc Roussillon, n°36, 2020–2025). The CBGP laboratory, which carried out the sampling in France, has approval (no. D-34–169–003) from the Departmental Direction of Population Protection (DDPP, Hérault, France) for the sampling of rodents and the storage and use of their tissues.

Germany

The study was performed in accordance with the applicable international and institutional guidelines for the use of animals in research. In Brandenburg, collection of rodents was performed under the permission of "Landesamt für Arbeitsschutz, Verbraucherschutz und Gesundheit Brandenburg (LAVG)" (no. 2347-A-16–1-2020, for procedure) and "Landesamt für Umwelt Brandenburg (LfU)" (no. LFU-N1–4744/97+17#194297/2020, for sites and species exemptions). In Thüringen, all procedures were permitted by the "Thüringer Landesamt für Verbraucherschutz (TLV)" (no. 22–2684–04–15–105/16).

Ireland

Ethical approval for the work carried out in Ireland was obtained from the Institute of Technology (Tralee, Ireland) Research Ethics Committee, and following that the Health Products Regulatory Authority (HPRA) granted authorisation (Authorisation Number: AE22171/I004) for euthanasia of the rodents to be sampled.

Poland

This study was carried out according to the recommendations in the Guidelines for the Care and Use of Laboratory Animals of the Polish National Ethics Committee for Animal Experimentation.

Animal Experiments

The hamster vaccination experiment (carried out in Helsinki, Finland) was approved by the Animal Experiment Board of Finland (license number: ESAVI/28687/2020).

The SARS-COV-2 challenge experiments (carried out in Malzéville, France) complied with the 2010/63/CE regulation of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes. The experiments were approved by the Anses/ENVA/UPEC ethics committee and the French Ministry of Research (license numbers: APAFIS #32431–2021071514369893 v2 for the plasma collection experiment and APAFIS #33544–2021102114466426 v2 for the heart collection experiment).

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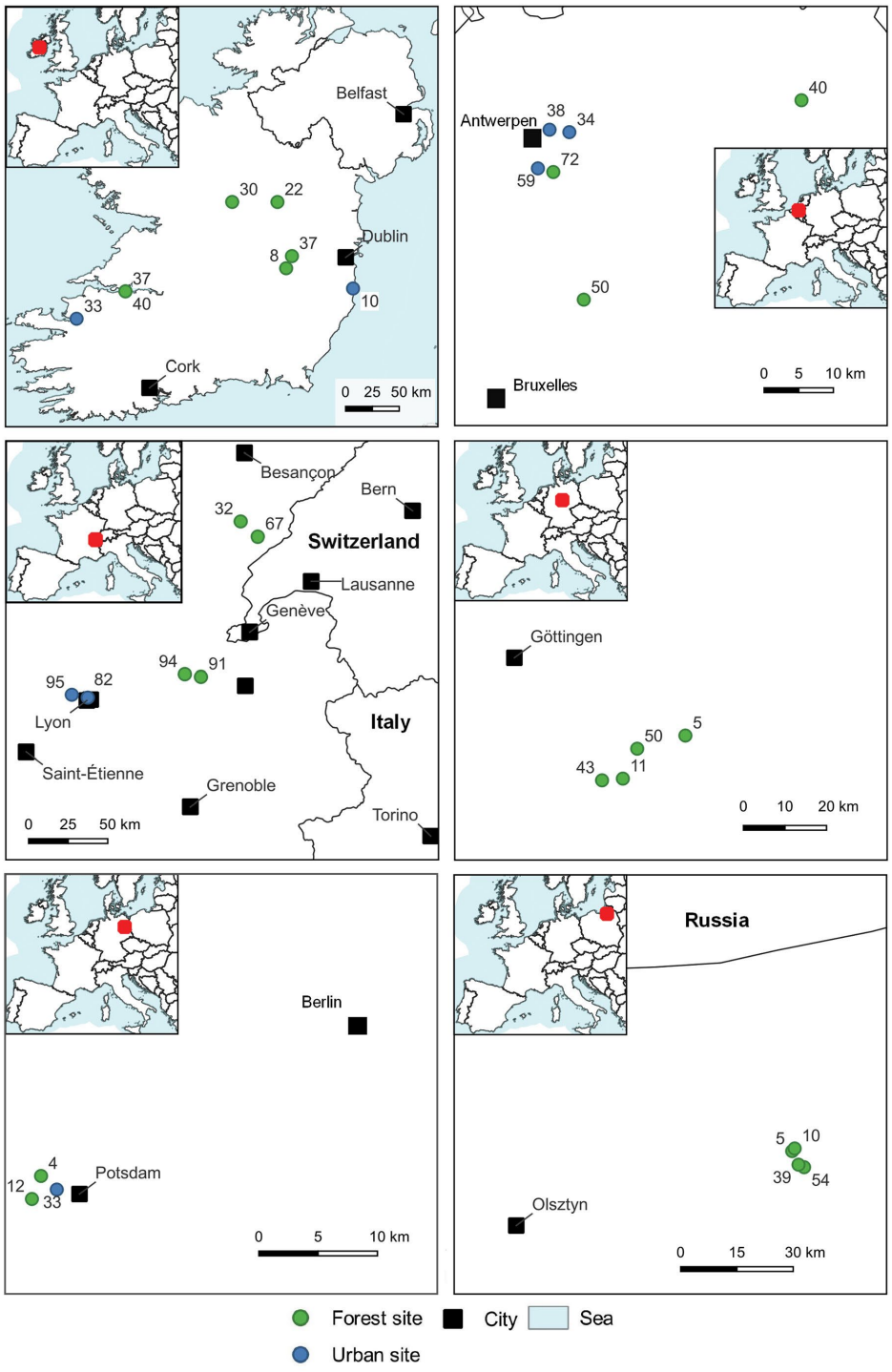
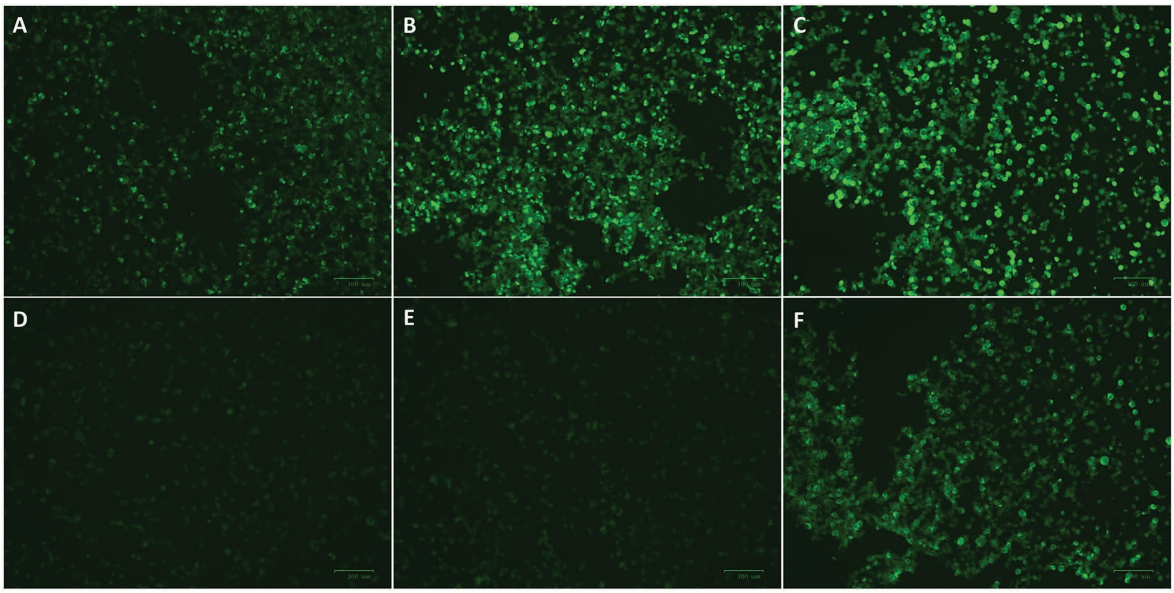
Appendix Table. Characteristics of hamster positive controls used to assess the ability of the immunofluorescent assay to detect rodents seropositive for SARS-CoV-2.

Sample name	Immunization method	Days post immunization	Sample type	Secondary conjugate	IFA result
pos176	Vaccination	14	Heart in PBS	Anti-mouse	+
pos177	Vaccination	14	Heart in PBS	Anti-mouse	+
pos178	Vaccination	14	Heart in PBS	Anti-mouse	+
pos176	Vaccination	14	Heart in PBS	Anti-hamster	+
pos177	Vaccination	14	Heart in PBS	Anti-hamster	+
pos178	Vaccination	14	Heart in PBS	Anti-hamster	+
H1242	Infection	14	Plasma	Anti-mouse	+
H1243	Infection	14	Plasma	Anti-mouse	+
H1244	Infection	14	Plasma	Anti-mouse	+
H1245	Infection	14	Plasma	Anti-mouse	+
H1246	Infection	14	Plasma	Anti-mouse	+
H1247	Infection	14	Plasma	Anti-mouse	+
H1242	Infection	14	Plasma	Anti-hamster	+
H1243	Infection	14	Plasma	Anti-hamster	+
H1244	Infection	14	Plasma	Anti-hamster	+
H1245	Infection	14	Plasma	Anti-hamster	+
H1246	Infection	14	Plasma	Anti-hamster	+
H1247	Infection	14	Plasma	Anti-hamster	+
H4891	Infection	15	Heart in PBS	Anti-mouse	+
H4892	Infection	15	Heart in PBS	Anti-mouse	+
H4893	Infection	15	Heart in PBS	Anti-mouse	+
H4894	Infection	15	Heart in PBS	Anti-mouse	+
H4895	Infection	15	Heart in PBS	Anti-mouse	+
H4896	Infection	15	Heart in PBS	Anti-mouse	+

*IFA, immunofluorescent assay; PBS, phosphate-buffered saline; +, positive



Appendix Figure 1. Sampling sites within A) Ireland, B) Belgium, C) France, D) Germany (central), E) Germany (east), and F) Poland. Insert in each panel indicates the location of the area in that panel (red dot) in Europe. Numbers near each sampling site indicate the number of rodents sampled.



Appendix Figure 2. Representative images of SARS-CoV-2 immunofluorescent assays (IFA) in rodents. Top row: positive controls A) vaccinated hamster, heart in PBS; B) challenged hamster, plasma, anti-mouse secondary conjugate; C) challenged hamster, plasma, anti-hamster secondary conjugate). Bottom row: field samples D) negative *Myodes glareolus*; E) negative *Apodemus sylvaticus*; F) positive *Apodemus sylvaticus*). Scale bar at the bottom right of each photograph represents 100 µm. Note the presence of both positive (brighter green) and negative (dim) cells in comparable proportions in positive tests (A, B, C, F), while the negative tests show exclusively negative cells (D, E).