

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

## 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  $-20^{\circ}\text{C}$  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  $-20^{\circ}\text{C}$ .

#### 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 Janvier'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  $\mu\text{L}$  containing  $10^4$  TCID<sub>50</sub> of SARS-CoV-2 virus (20  $\mu\text{L}$  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  $-16^{\circ}\text{C}$  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  $\mu\text{L}$  containing  $10^5$  TCID<sub>50</sub> of virus (20  $\mu\text{L}$  in each nostril). At fifteen days post-infection, after exsanguination, the hearts were collected in vials containing 500  $\mu\text{L}$  of sterile PBS. The samples were then stored at  $-16^{\circ}\text{C}$  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  $\mu$ L 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<sub>50</sub> (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<sub>2</sub> 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  $\mu$ L of the virus + serially diluted sample (or control) mixes. The microplates were then incubated at 37°C in a humid chamber containing 5% CO<sub>2</sub> 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  $\mu$ L ATL buffer + 20  $\mu$ L 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  $\times$  2 min using a TissueLyser (Qiagen). Lysates were then spun at 500 g for 5 minutes, and 200  $\mu$ L 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  $\mu$ L. 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 6<sup>th</sup>, 2001 and Directive 2010/63/EU issued on September 22<sup>nd</sup>, 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).

### **References**

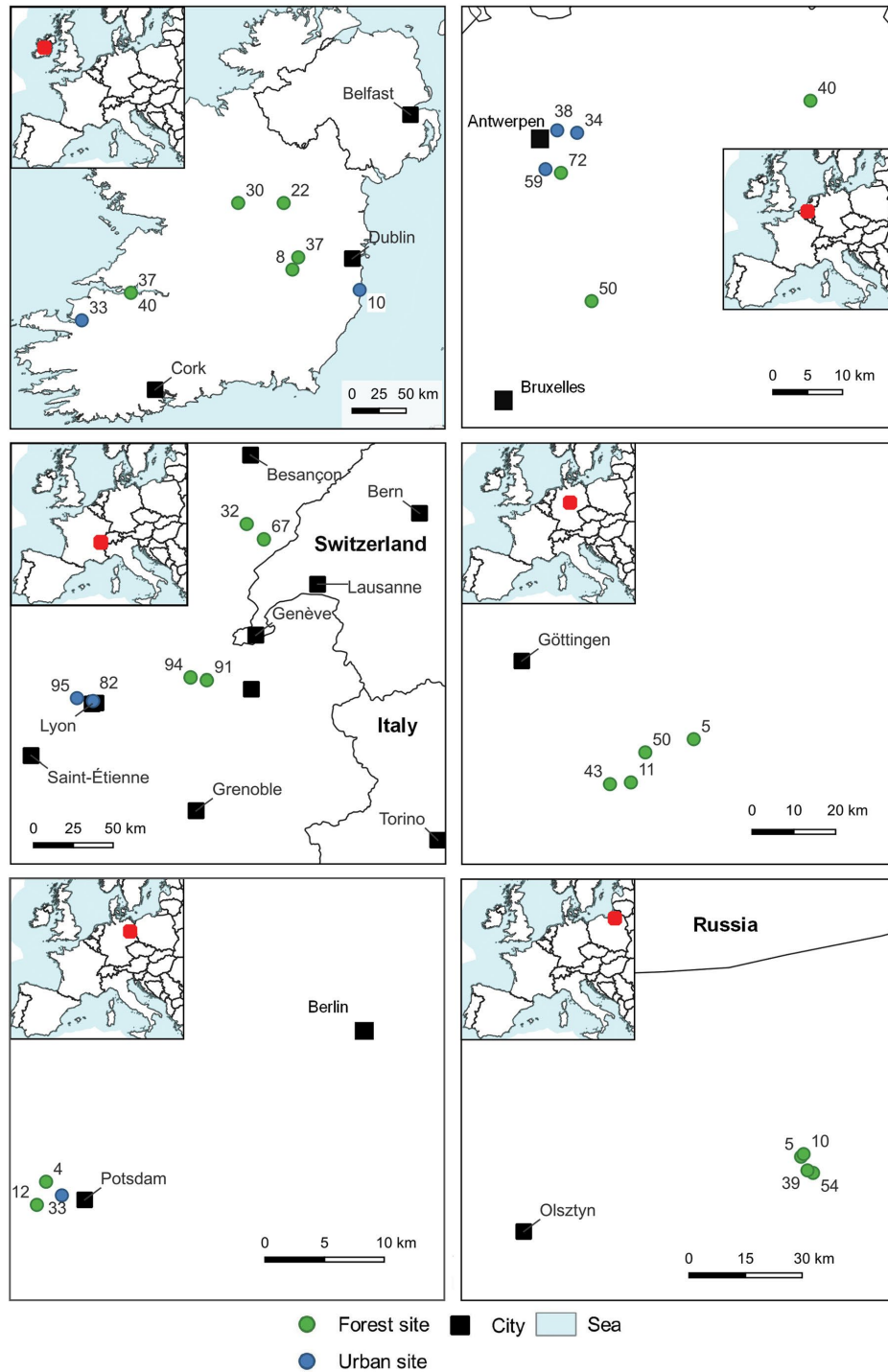
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**Appendix Table.** Characteristics of hamster positive controls used to assess the ability of the immunofluorescent assay to detect rodents seropositive for SARS-CoV-2.

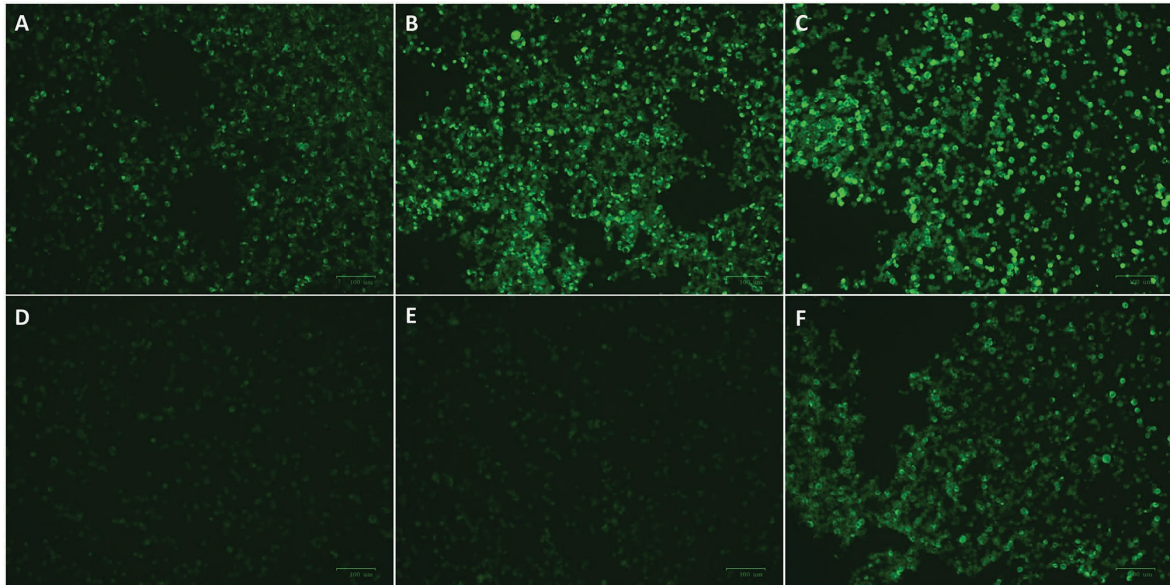
Sample name	Immunization method	Days post immunization	Sample type	Secondary conjugate	IFA result
pos176	Vaccination	14	Heart in PBS	Anti-mouse	+
pos177	Vaccination	14	Heart in PBS	Anti-mouse	+
pos178	Vaccination	14	Heart in PBS	Anti-mouse	+
pos176	Vaccination	14	Heart in PBS	Anti-hamster	+
pos177	Vaccination	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  $\mu\text{m}$ . 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).