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SHORT COMMUNICATION

# SARS-CoV-2 surveillance in Norway rats (*Rattus norvegicus*) from Antwerp sewer system, Belgium

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## Abstract

SARS-CoV-2 human-to-animal transmission can lead to the establishment of novel reservoirs and the evolution of new variants with the potential to start new outbreaks in humans. We tested Norway rats inhabiting the sewer system of Antwerp, Belgium, for the presence of SARS-CoV-2 following a local COVID-19 epidemic peak. In addition, we discuss the use and interpretation of SARS-CoV-2 serological tests on non-human samples. Between November and December 2020, Norway rat oral swabs, faeces and tissues from the sewer system of Antwerp were collected to be tested by RT-qPCR for the presence of SARS-CoV-2. Serum samples were screened for the presence of anti-SARS-CoV-2 IgG antibodies using a Luminex microsphere immunoassay (MIA). Samples considered positive were then checked for neutralizing antibodies using a conventional viral neutralization test (cVNT). The serum of 35 rats was tested by MIA showing three potentially positive sera that were later negative by cVNT. All tissue samples of 39 rats analysed tested negative for SARS-CoV-2 RNA. This is the first study that evaluates SARS-CoV-2 infection in urban rats. We can conclude that the sample of rats analysed had never been infected with SARS-CoV-2. However, monitoring activities should continue due to the emergence of new variants prone to infect Muridae rodents.

KEYWORDS
Belgium, disease outbreaks, Muridae, rats, SARS-CoV-2

## 1 | INTRODUCTION

Emerging infectious diseases have been in the spotlight of scientific research in recent years. Most studies have focused mainly on the role of domestic and wild animals as zoonotic virus reservoirs and the phenomena that drive animal-to-human transmission in order to explain outbreak processes and spillover dynamics (e.g. Han et al., 2016). However, the possibility of human-to-animal viral transmission raised concern during the Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) pandemic in 2020, when an asymptomatic dog from Hong Kong, whose owner was a coronavirus disease (COVID-19) patient, tested positive for the virus (Sit et al., 2020). Since then, similar human-to-animal transmission events have been reported worldwide in domestic dogs (Sit et al., 2020), cats (Garigliany et al., 2020), farmed mink (e.g. Hammer et al., 2021; Oude Munnink et al., 2021) and numerous zoo animals (World Organization for Animal Health (OIE), 2021). These events stimulated the scientific and public health community to better understand the implications and origins of this phenomenon.

  

The probability of human-borne SARS-CoV-2 emerging in animal populations differs between animal species through genetic and ecological differences (Gryseels et al., 2021). Susceptibility firstly depends on the ability of SARS-CoV-2 to enter host cells, which is determined by the affinity between the SARS-CoV-2 receptor-binding domain (RBD) in the spike (S) protein and its binding receptor in host cells, angiotensin-converting enzyme II (ACE2) protein (Qiu et al., 2020). Whether the virus, after entering a host cell, can be transmitted persistently depends on individual characteristics, infection dynamics and ecological characteristics of the population. The longer the virus is shed from infected animals and/or the higher the contact frequency between animals, the likelier it can initiate a successful transmission chain. A good example of a hazardous situation can be found in fur farms of mink, which present a highly susceptible species (American mink *Neovison vison*) housed indoors in extreme high densities, as evidenced by SARS-CoV-2 outbreaks reported in hundreds of such farms worldwide (World Organization for Animal Health (OIE), 2021). In nature, some mammals may also live in such high-density settings, particularly gregarious bats and fast-reproducing rodents. House mice (*Mus musculus*), Norway or brown rat (*Rattus norvegicus*) and the black or roof rat (*Rattus rattus*) are among the most ubiquitous rodents in the world (Feng & Himsworth, 2014). They are a source of a wide range of viral, bacterial and parasitic zoonoses and often live commensally or in close proximity to humans, increasing the risk of pathogen transmission (Himsworth et al., 2013). In Europe, Norway rats are well adapted to a synanthropic lifestyle and thrive in urban environments, including city sewer systems, where they find food, water and shelter (Pascual et al., 2020). Considering that many studies have detected SARS-CoV-2 in wastewater from the sewage system globally (e.g. Medema et al., 2020; Randazzo et al., 2020), as well as in Antwerp, Belgium (Boogaerts et al., 2021), these below-ground rodent populations can be exposed to SARS-CoV-2.

To date, only non-zoonotic betacoronaviruses were detected in Norway rats like Rat Coronavirus (RCov), China Rattus coronavirus HKU24 (ChRCoV HKU24) and Longquan RI rat coronavirus (LRLV) (Decaro & Lorusso, 2020), though some human endemic coronaviruses (OC43 and NL63) may have originated from a rodent reservoir (Corman et al., 2018). Furthermore, SARS-CoV-2 has been shown to efficiently infect and replicate in Cricetid rodent species like the golden Syrian hamster, *Mesocricetus auratus* (Boudewijns et al., 2020; Sia et al., 2020), the deer mouse (*Peromyscus maniculatus*) and the bushy-tailed woodrat (*Neotoma cinerea*) (Bosco-Lauth et al., 2021). However, rodent species of the Muridae family, like house mice (*Mus musculus*) (Bosco-Lauth et al., 2021) and Norway rats (Cohen, 2020), were found not susceptible to infection by the 'wild-type' (WT) Wuhan SARS-CoV-2 strain. Their ACE2 receptor does not bind to this strain's spike RBD *in vitro*. However, after serial passage in laboratory mice, SARS-CoV-2 evolves the ability to replicate efficiently in this host, thanks to a single substitution in the RBD, that is N501Y (Gu et al., 2020). Remarkably, the N501Y substitution has arisen repeatedly in SARS-CoV-2 lineages circulating in humans, most notable the variants of concern like B. 1.1.7, B.1.351 and P.1 (Yao et al., 2021). This suggests that (1) SARS-CoV-2 can evolve relatively easily to infect a previous resistant species, and (2) several SARS-CoV-2 variants currently circulating have the inherent ability to infect *M. musculus* and potentially other species of the Muridae family.

For these reasons, in the present study, we tested Norway rats inhabiting the sewer system of Antwerp, Belgium, for the presence of SARS-CoV-2 in November and December 2020, following a local COVID-19 epidemic peak by viruses mostly not carrying the N501Y substitution. In addition, we discuss the use and interpretation of SARS-CoV-2 serological tests on non-human samples.

## 2 | MATERIALS AND METHODS

### 2.1 | Study area

The study was conducted in the sewage system of the city of Antwerp (the Ruien) (51°13′16.6"N 4°23′50.2"E), Belgium, for 2 weeks during November–December 2020. The Ruien is an old network of small-scale waterways covered in 1882 that nowadays receives and directs the wastewater and the rainwater of the city of Antwerp to a water treatment plant (Marin & De Meulder, 2016).

### 2.2 | Data collection

To test for the presence of SARS-CoV-2 in the sewage water at the exact location where Norway rats were trapped; eight water samples of 150 ml each were taken from flowing household sewage water in open parts of the sewage pipes on two different days during the rat trapping sessions. Samples were stored in individual tubes at 4°C and processed the next day.

Up to 30 rat-live-traps baited with fish boilies (Decathlon – 'taste') were set out and checked every morning during 2 weeks. The aim was to trap at least 32 rats in order to have an 80% probability of detecting one rat or more positive to SARS-CoV-2, assuming a prevalence of 5%. Trapped rats were transported to a BSL-$2^+$ laboratory at the Central Animal Facility, Campus Drie Eiken, University of Antwerp. Rats were euthanized with an overdose of isoflurane, and then weighed, measured and data of their species, sex and reproductive status were registered. Blood samples were collected in tubes without anticoagulant; serum was separated and stored at −20°C. Tissue samples of the kidney, lung, liver and a 5 mm piece of colon were stored at −80°C. Oral swabs in PBS and faeces samples in RNA later were also collected and stored at −80°C.

### 2.3 | Detection of SARS-CoV-2 RNA in wastewater

Wastewater samples were analysed essentially as described in Boogaerts et al. (2021). Wastewater was first centrifuged at 4625 g for 30 min at 4°C in an Eppendorf 5910R Centrifuge (Aarschot, BE). The supernatant (40 ml) was transferred to a Macrosep Advance Centrifugal devices with Omega Membrane (100 kDa; Pall, NY, USA). The

concentrate was standardized to 1.5 ml with UltraPureTM DEPC-Treated Water (ThermoFisher Scientific). RNA extraction was performed from 200 $\mu$l of the concentrate with the automated Maxwell PureFood GMO and the Authentication RNA extraction kit in the Maxwell® RSC Instrument (Promega). Reverse transcription and amplification of the N1, N2 and E genes was performed by qPCR in duplicate in 20 $\mu$l reaction mixtures using a 2x SensiFAST™ Probe No-ROX One-Step kit following (Boogaerts et al., 2021). A six-point calibration curve with a known concentration of $10^5$–$10^0$ copies/$\mu$l was constructed in ultrapure DEPC-treated water for the quantification of the different genes of interest. The EURM-019 reference standard for the construction of the calibration curve was obtained from the Joint Research Centre (JRC, European Commission). The lower limit of quantification (LLOQ) was defined as the concentration in the lowest point of the calibration curve and was $10^0$ copies/$\mu$l. The LLOQ of the N1, N2 and E qPCR corresponded with Ct-levels of 36.1, 36.4 and 36.6, respectively.

## 2.4 | Serology

To test SARS-CoV-2 exposure in sewer rats, serum samples were first screened for the presence of anti-SARS-CoV-2 IgG antibodies, using an in-house Luminex microsphere immunoassay (MIA) (Mariën et al., 2021). The MIA is a high-throughput test that allows the simultaneous detection of binding antibodies against different antigens of the same pathogen, increasing significantly the specificity of the test.

Considering that laboratory rats, as well as WT immunocompetent mice, are barely susceptible to WT SARS-CoV-2 (Wuhan) strain infection (Cohen, 2020; Gu et al., 2020; Qiu et al., 2020), positive control sera ($n$ = 10) were obtained from laboratory mice knockout for type I interferon receptors (ifnar−/−) inoculated with a recombinant live-attenuated yellow fever virus that expressed the spike unit of SARS-CoV-2 (Sanchez-Felipe et al., 2020). In addition, ifnar−/− mice lack the type-I interferon receptor function, which results in a reduced immune response and an increased susceptibility to viral infection.

The prediction performance of the MIA depends on the possibility to estimate correctly the cut-off values of the negative controls. Considering that the overall background noise (due to IgG antibodies developed against other pathogens) will depend on the exposure rate to other pathogens, the control panel included serum from susceptible laboratory rodents (less likely to be in contact with other pathogens) as well as serum from wild rats (more likely to be in contact with other pathogens). Since serum samples from sewer rats captured before the SARS-CoV-2 outbreak were not available, we used as negative controls serum from wild rats ($n$ = 7) trapped in forest and parks from Antwerp, outside the sewer system, as we considered that they were less likely to be exposed to SARS-CoV-2 (albeit with a similar exposure rate to other pathogens in the wild). Also, naïve laboratory mice ($n$ = 8) samples were used as negative controls.

The MIA was run with two different beads coated with the virus' nucleocapsid and spike antigens (Ayouba et al., 2020). A biotin-labelled goat anti-mouse IgG Y-chain specific conjugate (Sigma, B7022, 1/300 dilution) was used for visualization of the primary antibodies. Samples were considered positive if crude median fluorescence intensity values (MFI) were higher than 3x standard deviation (SD) of the negative control samples for both nucleocapsid and spike antigen-coated bead sets, increasing the specificity of the test. All samples that were considered positive on the MIA ($n$ = 7) were checked for neutralizing antibodies using a conventional viral neutralization test (cVNT) with the WT Wuhan strain (2019-nCoV-Italy-INMI1, reference 008V-03893) as antigen (Mariën et al., 2021). We only considered a sample seropositive if antibodies were detected on both the MIA and cVNT.

## 2.5 | RT-PCR analysis of tissue samples

Viral RNA was extracted from 140 $\mu$l of oral swab samples in PBS and from 1 $cm^2$ of faeces using the QIAamp Viral RNA mini kit (QIAGEN, Valencia, CA, USA) and from 30 mg kidney, lung, liver and colon samples using the NucleoSpin RNA mini kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. We tested for the presence of SARS-CoV-2 RNA via the CDC 2019-nCoV real-time RT-PCR protocol targeted to two regions of the nucleocapsid protein (N) gene, N1 and N2 (Lu et al., 2020), performed on 5 $\mu$l of RNA using the SARS-CoV-2 (2019-nCoV) CDC RUO kit (IDT Cat. No. 10006713). The positive control used was a SARS-CoV-2 N synthetic probe (IDT, USA) designed for the present study. To monitor RNA extraction, we ran simultaneously a beta-actin (ACTB) assay as internal control (Borremans et al., 2015) in a duplex assay N1/ACTB designed following Vogels et al. (2020). N1/ACTB and N2 PCRs were performed separately for each sample with the Luna Universal qPCR Master Mix (New England Biolabs) on an Applied Biosystems StepOne Real-Time PCR Instrument (Thermo Fisher Scientific) under the following thermal conditions: 52°C for 10 min, 95°C for 2 min, 44 cycles with 95°C for 10 s and 55°C for 30 s.

# 3 | RESULTS

Of the eight water samples tested, four samples had detectable Ct values for SARS-CoV-2. Two of them were positive to SARS-CoV-2 PCR but below the LLOQ. On the other hand, two water samples had Ct values above the LLOQ for at least two of the gene targets (Ct ranging between 35.5 and 36.4), which would correspond to approximately the concentration of seven copies of the target SARS-CoV-2 RNA per ml of wastewater.

Serum samples of 35 sewer rats were analysed by MIA. Three had MFI values higher than the cut-off values of the negative controls for both nucleocapsid and spike-reactive IgG antibodies, but all remained lower than the MFI values of the positive control samples (Figure 1). The three potentially positive sera and four other sera with high MFI values were subsequently checked for neutralizing antibodies by cVNT. All samples were seronegative for neutralizing antibodies, suggesting that the captured sewer rats had not experienced SARS-CoV-2 infection in their life.

Regarding the tissue samples analysed, oral swabs, faeces, colon, lung, liver and kidney samples of 39 sewer rats tested for the presence of SARS-CoV-2 by qRT-PCR were all considered negative.

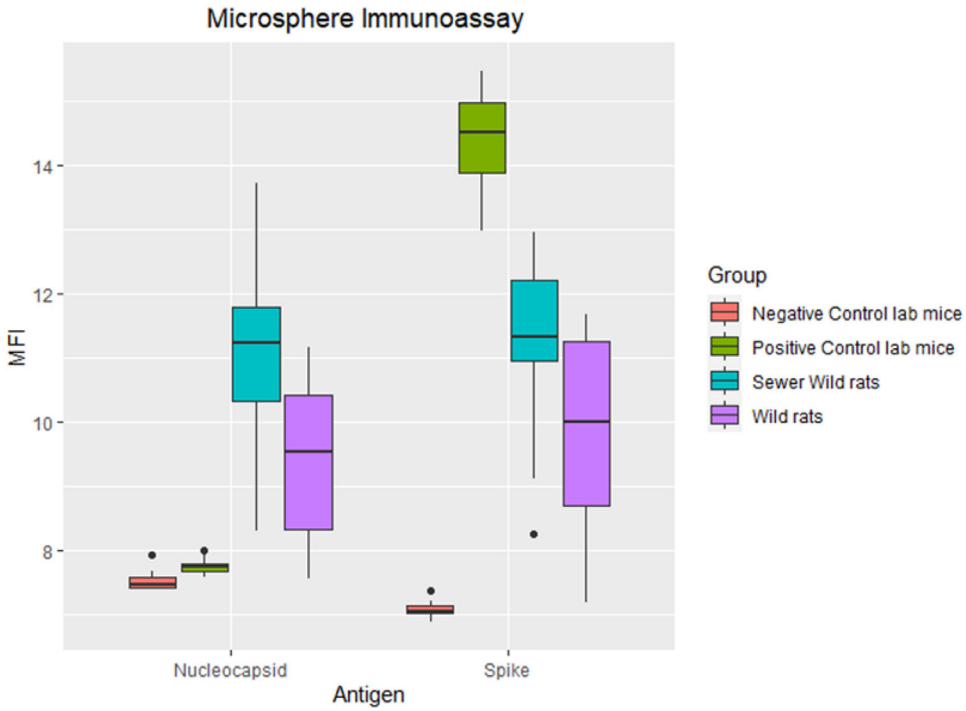


**FIGURE 1** Boxplot showing the variation in log(MFI) values (median fluorescent intensities) for the different categories of mice/rats serum samples analysed in the microsphere immunoassay using the SARS-CoV-2 nucleocapsid and spike antigens in Antwerp, Belgium

## 4 | DISCUSSION

To our knowledge, this is the first study that evaluates SARS-CoV-2 infection in urban Norway rats exposed to an environment contaminated with the virus, the sewer wastewater. According to the negative results obtained in both serology and PCR tests, we can conclude that the rodents studied had never been infected with SARS-CoV-2 despite continuous detection of viral RNA in the Antwerp sewer water (Boogaerts et al., 2021), including sewer water collected at the exact location where the rats were captured.

Regarding the observed discrepancy between the results of the MIA and cVNT, we think it is worth mentioning that the interpretation of SARS-CoV-2 binding antibody tests (MIA or Elisa) should be made with care when used on different types of samples than what the assays were validated for. Indeed, although our MIA was clearly able to differentiate negative from positive control cases in laboratory mice (Figure 1), it falsely categorized three WT rats as positive when we estimated cut-off values based on serum from wild rats that were trapped outside of the sewers. The misclassification is explained by the fact that sewer rats had overall higher MFI values than rats trapped outside of the sewers (Figure 1). This difference is likely caused by the higher exposure rate to many other pathogens (potentially including other betacoronaviruses) in the sewer systems (dirtier conditions and higher population densities), which stimulates the adaptive immune system and results in overall higher binding antibody levels. Therefore, to confirm exposure to SARS-CoV-2 in a particular wildlife population based on serological data, VNTs are a better alternative.

Studies to elucidate the animal species susceptible to SARS-CoV-2 have demonstrated the ability of the virus to spillover to several distantly related mammalian species (e.g. Garigliany et al., 2020; Hammer et al., 2021; Sit et al., 2020; World Organization for Animal Health (OIE), 2021), with the potential to stimulate the evolution of new variants with different antigenic properties (van Dorp et al., 2020). This phenomenon can lead to various consequences, such as putting species conservation actions at risk if the virus affects endangered species, the establishment of novel reservoirs with the potential to start new outbreaks in humans, and the evolution of novel variants that may evade antibodies generated in humans, forcing the development of new antiviral therapies (Gryseels et al., 2021; Hammer et al., 2021; Mercatelli & Giorgi, 2020; Oude Munnink et al., 2021). Since the beginning of the SARS-CoV-2 pandemic, many new variants have been evolved in humans and non-human animal hosts (Leung et al., 2021; Mercatelli & Giorgi, 2020; van Dorp et al., 2020). Some of the currently most widespread variants, like B.1.1.7/501Y.V1, B.1.351/501Y.V2 and P.1/501Y.V3 that emerged from the UK, South Africa and Brazil, are potentially able to infect previous resistant species, such as Muridae rodents, thanks to the N501Y substitution in the RBD (Gu et al., 2020; Yao et al., 2021).

The absence of SARS-CoV-2 in our sample of Norway rats may be due to different reasons. The first cases of SARS-CoV-2 lineages with the spike N501Y substitution in humans were detected in Belgium in December 2020 (Risk Assessment Group & Sciensano, 2020), after the sewer rats sampling period. This could have represented a low risk of exposure of the rats to variants able to infect them. On the other hand, the infectivity potential of SARS-CoV-2 in wastewater is still uncertain.

While many studies failed to recover infectious virus from wastewater, others have shown the potential of the virus to remain infective in human excrements (Giacobbo et al., 2021; Jones et al., 2020). Studies performed on different species of coronavirus showed the capacity of the virus to remain infective on different water sources (Giacobbo et al., 2021). However, none was able to demonstrate the infectivity of SARS-CoV-2 in wastewater, although the authors suggest that there is insufficient evidence to rule out this possibility. This evidence suggests that the absence of infection in the sewer rats tested on the present study could also be linked to the exposition of the rats to low doses of SARS-CoV-2 or to non-infective viral particles.

The presence of new variants in human populations prone to infect Muridae rodents with the potential to remain infective in water sources, in conjunction with the synanthropic habits of several Muridae rodents and their ability to develop high-density populations, creates the ideal conditions for the spread of new epidemics. As such, despite the negative results found in Norway rats in the present study, we emphasize the need to carry out regular monitoring activities for the presence of SARS-CoV-2 in Muridae rodents, as well as other mammals exposed to humans, in order to detect human-to-animal transmission events and prevent future outbreaks emerging from new animal reservoirs.

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## CONFLICT OF INTEREST

None declared.

## ETHICAL STATEMENT

The authors confirm that the ethical policies of the *Transboundary and Emerging Diseases Journal* have been adhered to and that all procedures with rodents for this study were carried out under the approval of the University of Antwerp Ethical Committee for Animal Experiments (ECD code 2020–21). The Directive 2010/63/EU was followed.

## AUTHOR CONTRIBUTIONS

VCC, VS, JM, HL and SG developed the research methodology, collected samples for this study and drafted the article. BVB, WL, AI and JE collected rodent and water samples and reviewed the article. JM, LH and KA developed the serological tests and reviewed the article. NVH, MH, HG, VCC and SG developed the tissue collections, SARS-CoV-2 RNA tests and reviewed the article. LJ, PD and NDR developed the water samples tests and drafted the article. All authors are responsible for the approval of the final manuscript and its submission for publication.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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