



Wild red foxes (*Vulpes vulpes*) do not participate in SARS-CoV-2 circulation in Poland

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ARTICLE INFO

Keywords:

SARS-CoV-2

Red fox

Transmission

Spillover

Vulpes vulpes

Zoonoses, biomonitoring

ABSTRACT

Background: Biomonitoring is an essential activity for identifying possible vectors and reservoirs of pathogens and predicting potential outbreaks. Wild red foxes are present in both sylvatic and synanthropic environments, making them potential carriers of zoonotic pathogens. Experimental studies have shown that both coyotes and red foxes can transmit SARS-CoV-2. This study aimed to assess the prevalence and seroprevalence of SARS-CoV-2 in wild red foxes hunted in northern Poland. **Methods:** Oral swabs, blood clots or heat tissue samples were collected from 292 red foxes hunted in northern Poland. We used both molecular (RT-PCR) and serological (IFA) approaches to detect SARS-CoV-2 infections in the sampled animals. **Results:** We did not find any evidence of SARS-CoV-2 infection in the collected samples, using both molecular and serological methods. **Conclusions:** Despite foxes having frequent contact with humans, human waste, and other animals, they do not appear to participate in the circulation of the SARS-CoV-2 virus in our geographical region. Nevertheless, we believe that continuous biomonitoring should be performed to assess the SARS-CoV-2 epidemiological situation in the wild.

1. Introduction

There is now a great interest in searching for vertebrate species that may play a role as vectors or reservoirs of zoonotic agents, especially SARS-CoV-2 and avian influenza [1–4]. The emergence of SARS-CoV-2, the virus responsible for the COVID-19 pandemic, has brought to light the potential risks that zoonotic diseases pose to both human and wildlife populations. While the origin of SARS-CoV-2 is still uncertain, it is believed to have originated from bats and possibly passed through an intermediate host before spillover into humans [5]. However, the potential for the virus to infect and impact wildlife populations is also a cause for concern. Wildlife can serve as a reservoir for the virus, allowing it to persist and potentially mutate in animal populations [6]. This could lead to the emergence of new strains of the virus that could pose a threat to both human and animal health [7]. There have been reports of SARS-CoV-2 infections in a range of wild animal species,

including tigers, lions, minks, and gorillas, indicating that the virus can infect a variety of animals [8].

Wild red foxes are present in both sylvatic and synanthropic environments and they often contact humans [9,10]. These animals are often present around human settlements, farms, city parks and zoological gardens [11,12]. According to the Forest Yearbook in 2021, the population of wild red foxes in Poland was 198.8 thousand individuals [13]. While foxes are predators from the canid family, their primary diet in forests and fields consists of rodents, but they can hunt and eat anything they encounter. The main food for the red fox is small rodents, mainly voles and mice [14]. Additionally, the fox hunts hares and birds and is not averse to scavenging carrion. Sometimes, it visits garbage bins near forest parking lots, tourist trails, or on the outskirts of towns. They may also prey on poultry and other domestic animals if the opportunity arises. Moreover, in some countries, red foxes are shot by hunters and this activity increases human contact with fox carcasses, body fluids (i.e.

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<https://doi.org/10.1016/j.oneht.2024.100845>

Received 28 April 2024; Received in revised form 10 June 2024; Accepted 20 June 2024

Available online 22 June 2024

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blood, urine) or stool. Experimental studies showed that coyotes and red foxes can be infected with the SARS-CoV-2 virus [15].

In this study, we aimed to assess the prevalence and seroprevalence of the SARS-CoV-2 virus in a population of wild red foxes in Pomorskie Voivodeship in northern Poland. We believe that biomonitoring is a significant step in predicting possible disease outbreaks.

2. Materials and methods

2.1. Material collection and RNA isolation

Nasopharyngeal swabs were collected from 292 red foxes (*Vulpes vulpes*) shot by hunters in Pomorskie Voivodeship in northern Poland in February 2021 and February 2022 in three hunting regions – Lipiec, Rzucewo and Pazece (Fig. 1). Foxes were frozen at -20°C after shooting for further analysis. Since animal carcasses were stiff, we used a sterile plastic speculum to open the nasal cavity. Then using a thick swab we collected samples and preserved them in a virus deactivation buffer at $+4^{\circ}\text{C}$. Straight after a total of $150\ \mu\text{l}$ from each sample of a swab in inactivation buffer was added to $300\ \mu\text{l}$ of RLT lysis buffer (RNeasy Mini kit, Qiagen, Hilden, Germany). Samples were mixed by vortexing and incubated for 10 min at room temperature. After incubation, $400\ \mu\text{l}$ of 70% ethanol was added to each sample and mixed by pipetting. The lysate was transferred to an RNeasy Mini spin column with a collection tube and centrifuged for 1 min at 13,000 RPM. Columns were washed once with $700\ \mu\text{l}$ RW1 and twice with $500\ \mu\text{l}$ RPE. Between every wash, the columns were centrifuged and the flow-through was discarded. Elution was performed by adding $50\ \mu\text{l}$ of PCR-grade water to the

column and incubating for 2 min. Columns were placed into new tubes and centrifuged at 13000 RPM for 1 min. After isolation, the samples were stored for $<2\ \text{h}$ at 4°C . No human-origin samples were processed at the same time. (See Fig. 2.)

Serum samples were obtained from blood clots recovered during animal sections. Blood clots were centrifuged, and serum was collected for serological tests.

2.2. Real-time RT-PCR

For each sample, the reaction mixture was prepared using a TaqPath™ 1-Step RT-qPCR Master Mix (ThermoFisher Scientific), polymerase, DEPC-treated water (EURx), and primers and probes for the RdRp and E genes [17] in white 8-well q-PCR strips with optical clear caps. Positive control plasmids made in-house with the RdRp and E genes and a no template control (NTC) containing DEPC-treated water instead of template reactions were also prepared. Reactions were mixed and loaded into a Light Cycler 480 (Applied Biosystems, Foster City, California, United States). Cycling conditions were Uracil N-glycosylase (UNG) incubation for 2 min at 25°C , RT incubation for 15 min at 50°C , and enzyme activation for 2 min at 95°C , followed by 40 amplification cycles consisting of 3 s at 95°C and 30 s at 60°C . After each amplification cycle, the signal from each sample was measured in both the FAM (RdRp gene) and HEX (E gene) channels. Samples with $C_p < 35$ for any gene were considered positive for SARS-CoV-2.

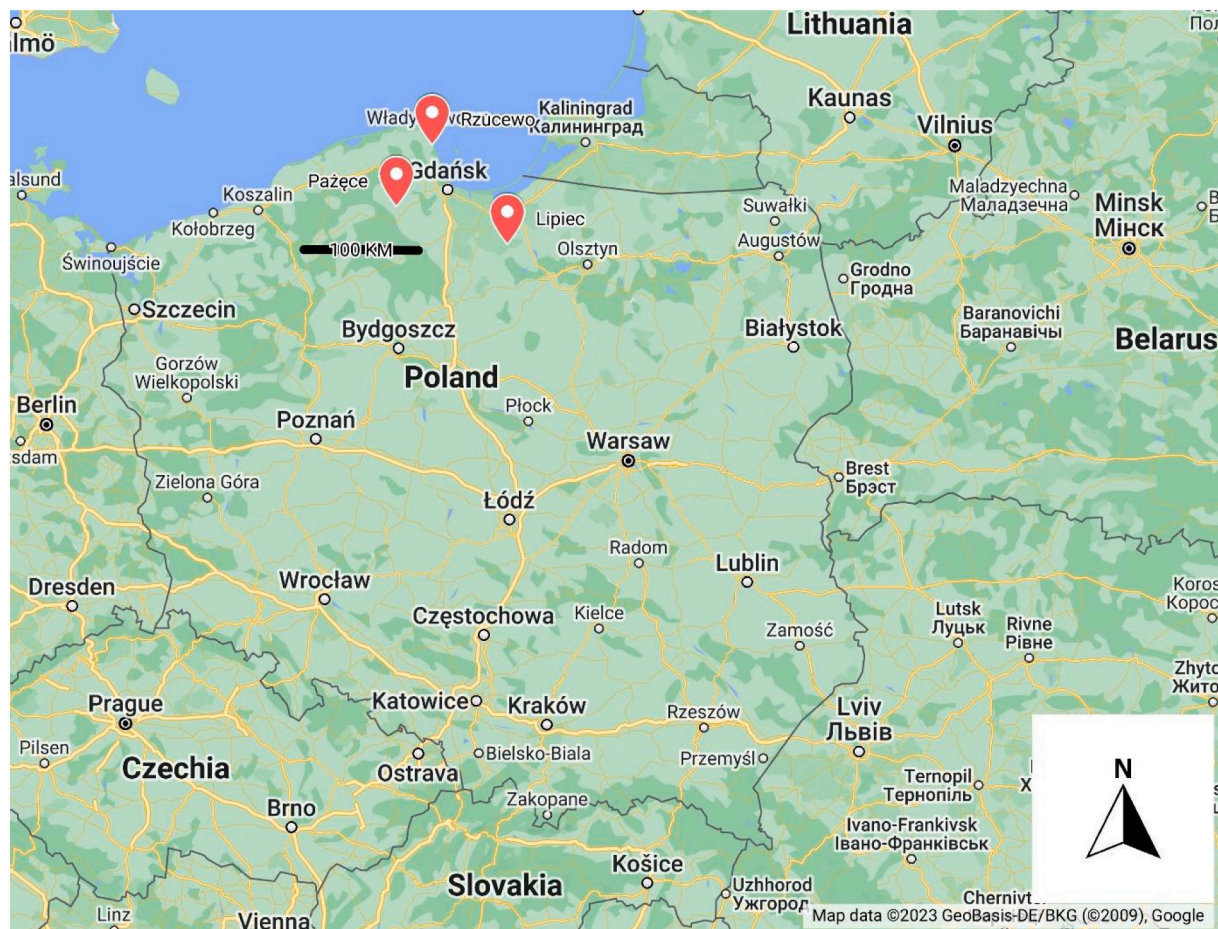


Fig. 1. Red marks indicate sampling sites. The black bar indicates a 100 km distance. Lipiec (53.92401 N, 19.4317 E), Rzucewo (54.68274 N, 18.44662 E), Pazece (54.21381 N, 17.9798 E). Map data 2023, Google Map [16].

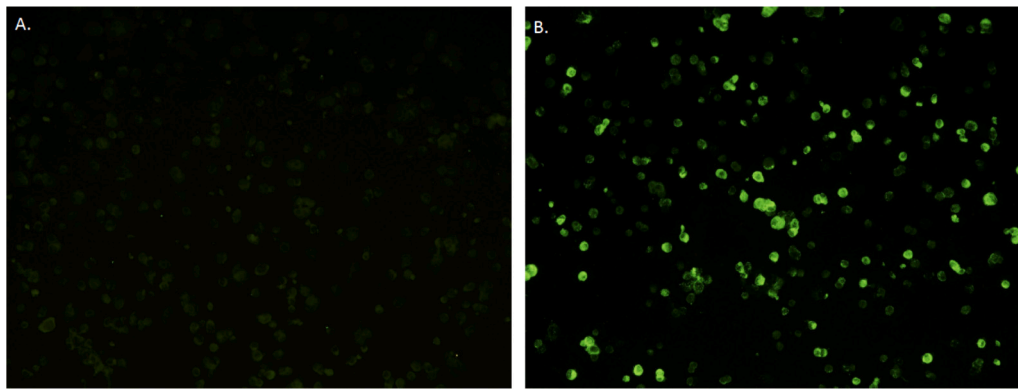


Fig. 2. Results of immunofluorescent assay for antibodies against SARS-CoV-2 detection; (A) SARS-CoV-2 negative sample; (B) SARS-CoV-2 positive control.

2.3. Immunofluorescent Assay (IFA) for Antibodies against SARS-CoV-2 Detection

Fox blood clots were analyzed using an immunofluorescence assay (IFA) with seropositive human serum as a positive control as previously described [3,18,19]. Blood clot samples were diluted 1:5 in PBS and 500 μ l of PBS was added to heart tissue samples. After adding PBS, heart samples were in a shaker +4 O/N. The reactivity of the samples to SARS-CoV-2 was tested with SARS-CoV-2-IFA. Infected Vero E6 cells were detached with trypsin, mixed with uninfected Vero E6 cells (in a ratio of 1:2), washed with PBS, spotted on IFA slides, air-dried, and fixed with acetone. The slides were stored at -70°C until use. We used rabbit anti-dog IgG fluorescein isothiocyanate labelled as a conjugate (Jackson ImmunoResearch, Ely, United Kingdom). The slides were read under a fluorescence microscope.

3. Results

We screened 292 wild red fox individuals and did not detect any SARS-CoV-2 infected red fox individuals using RT-PCR. Moreover, we did not detect any immunological signal using Immunofluorescent Assay against SARS-CoV-2 infection.

4. Discussion

Our study did not detect any SARS-CoV-2 signals in samples collected from hunted wild red foxes in Northern Poland. This finding aligns with other studies that have similarly investigated SARS-CoV-2 infections in wild red fox populations [20]. In contrast to the results of studies conducted in Croatia, ours did not include muscle or fecal samples, but nasopharyngeal swabs and heart tissue or blood clots. This may hinder the comparison of results. These results suggest that, despite their frequent presence in urban environments, red foxes may not have significant contact with SARS-CoV-2, nor do they appear to act as vectors or reservoirs for the virus. This indicates a lower likelihood of their contributing to the circulation of the pathogen. However, it's important to note that our study had limitations, one of which is the narrowing of the study area to the territory of three hunting clubs within one region - Pomorskie Voivodeship. Another one, for instance, is the collection of nasal swabs conducted on frozen fox carcasses. This method may not be as effective as collecting diagnostic material from freshly killed animals. As a result, some infections, especially low-level ones, may have remained undetected. Ideally, samples should be obtained immediately post-mortem, but this approach is impractical due to time and cost constraints. Another limitation of our study is that samples were collected twice (2021,2022) at a similar time of year, during winter. Perhaps animals examined at other times of the year would carry traces of SARS-CoV-2 infection due to seasonal variations in virus exposure, for example, differences in the intensity of contact between foxes and

humans or foxes and domestic animals. Therefore, conducting a year-round study may be necessary for a comprehensive description of the discussed issue.

The potential risks of SARS-CoV-2 in wildlife populations are substantial and warrant continued research and monitoring [21]. Understanding the impacts of the virus on wildlife, and its potential to persist and mutate in animal reservoirs, is crucial for assessing future pandemic risks [22–25]. Addressing the root causes of zoonotic disease emergence, such as habitat destruction and wildlife trade, is key to reducing these risks and protecting both human and animal health [26]. The movement and trade of wildlife pose additional challenges in containing the virus, as they can facilitate its spread between animal populations [27].

Ethics approval and consent to participate

This study was carried out with due regard for the European Union's principles and the Polish Law on Animal Protection. No permit from the Local Bioethical Committee for Animal Experimentation was obtained because animals were shot by hunters according to Polish hunting law (Polish Hunting Law-Act Dz. U. 1995 Nr 147 poz. 713 published on 13 October 1995). Samples were collected post-mortem.

Funding

We appreciate the support from the University of Gdańsk, the Medical University of Gdańsk, and the University of Helsinki. This research was funded through the 2018–2019 BiodivERsA joint call for research proposals, under the BiodivERsA3 ERA-Net COFUND program; the funding organizations ANR (France), DFG (Germany), EPA (Ireland), FWO (Belgium), and NCN (Poland). MG, AG, and JN were supported by the National Science Centre, Poland under the BiodivERsA3 program (2019/31/Z/NZ8/04028). MK was supported by the National Science Centre, Poland under the Preludium BIS programme (2020/39/O/NZ6/01777). AL was supported by the National Science Centre, Poland under the OPUS program (2020/37/B/NZ7/03934). LR was supported by the Ministry of Science and Higher Education (Decision No. 54/WFSN/2020. "Co-infections with SARS-CoV-2, Database of COVID-19 Accompanying Infections").

Consent for publication

All authors have reviewed the contents of the manuscript and consent to its publication in the OneHealth Journal. We confirm that the manuscript, or parts of it, has not been published elsewhere and is not under consideration for publication in another journal.

Authors' contributions

The study was conceived and designed by MG and AGoll. MG

supervised the biomonitoring process. AGoll, JN, MK, and AL conducted field sample collection. AGoll, MK, JN, AL, KB, AG, LR, TS and RK conducted the molecular analysis and laboratory work. MG and AGoll oversaw data handling and analysis. The initial draft of the manuscript was prepared by AGoll and MG, with contributions and input from all co-authors. MG and RK did a critical review and revision of the manuscript. All authors have read, provided feedback, and approved the final version of the manuscript.

CRedit authorship contribution statement

Martyna Krupińska: Methodology, Investigation. **Joanna Nowicka:** Investigation. **Karolina Baranowicz:** Methodology, Investigation. **Lukasz Rabalski:** Methodology, Investigation, Formal analysis. **Anna Lass:** Investigation. **Aleksandra Gorska:** Writing – original draft, Methodology, Investigation, Formal analysis. **Tarja Sironen:** Supervision, Investigation. **Ravi Kant:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maciej Grzybek:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

Acknowledgements

MG thanks Ewa Zieliniewicz for their assistance in the laboratory. All authors thank Helsinki University Library for financial support.

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