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

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Short Report

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Abstract

(1) **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.

(2) **Methods**: Oral swabs and blood clots 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.

(3) **Results:** We did not find any evidence of SARS-CoV-2 infection in the collected samples, using both molecular and serological methods.

(4) **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

It is now a great interest in searching for vertebrate species that may play a role as vectors or reservoirs of zoonotic agents and specially 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 synantropic 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 fox 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 increase human contact with fox carcasses, body fluids (i.e. 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 SARS-CoV-2 virus in a population of wild red foxes in northern Poland. We believe that biomonitoring is a significant step in predicting possible disease outbreaks.

2. Materials and Methods

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 (Map 1). Foxes were frozen at -20°C after shooting for further analyses. Since animal carcass were stiff, we used sterile plastic speculum to open nasal cavity. Then using thick swab we collected samples and preserved them in virus deactivation buffer at + 4°C (Movie 1). Straight after a total of 150 µl from each sample of a swab in inactivation buffer was added to 300 µl of RLT lysis buffer (RNeasy Mini kit, Qiagen, Hilden, Germany). Samples were mixed by vortexing and incubated for 10 minutes at room temperature. After incubation, 400 µl of 70% ethanol was added to each sample and mixed by pipetting. The lysate was transferred to an RNeasy Mini spin column with collection tube and centrifuged for 1 minute at 13 000 RPM. Columns were washed once with 700 µl RW1 and twice with 500 µl RPE. Between every wash, the columns were centrifuged and the flow-through discarded. Elution was performed by adding 50 µl of PCR-grade water to the column and incubating for 2 minutes. Columns were placed into new tubes and centrifuged at 13 000 RPM for 1 minute. After isolation, the samples were stored for less than 2 hours at 4°C. No human origin samples were processed at the same time.

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

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 minutes at 25°C, RT incubation for 15 minutes at 50°C, and enzyme activation for 2 minutes at 95°C, followed by 40 amplification cycles consisting of 3 seconds at 95°C and 30 seconds 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 Cp < 35 for any gene were considered positive for SARS-CoV-2.

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

Fox blood clots and heart samples 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 µl 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 labeled 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 individual 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]. 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. For instance, the collection of nasal swabs was conducted on frozen fox carcasses. This method may not be as effective as collecting diagnostic material from freshly killed animals. Ideally, samples should be obtained immediately post-mortem, but this approach is impractical due to time and cost constraints.

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 [22–24]. 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 [25]. The movement and trade of wildlife pose additional challenges in containing the virus, as they can facilitate its spread between animal populations[26].

Declarations

Consent for publication: All authors have reviewed the contents of the manuscript and consent to its publication in the Virology 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.

Availability of data and materials: All data generated or analyzed during this study are included in this published article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions: The study was conceived and designed by MG and AGoll. MG supervised the biomonitoring process. Field sample collection was conducted by AGoll, JN, MK, and AL. The molecular analysis and laboratory work were carried out by AGoll, MK, JN, AL, KB, AG, LR, and RK. Data handling and analysis were overseen by MG and AGoll. The initial draft of the manuscript was prepared by AGoll and MG, with contributions and input from all co-authors. Critical review and revision of the manuscript were done by MG and RK. All authors have read, provided feedback, and approved the final version of the manuscript.

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Map

Map 1 is available in the Supplementary Files section.

Figures
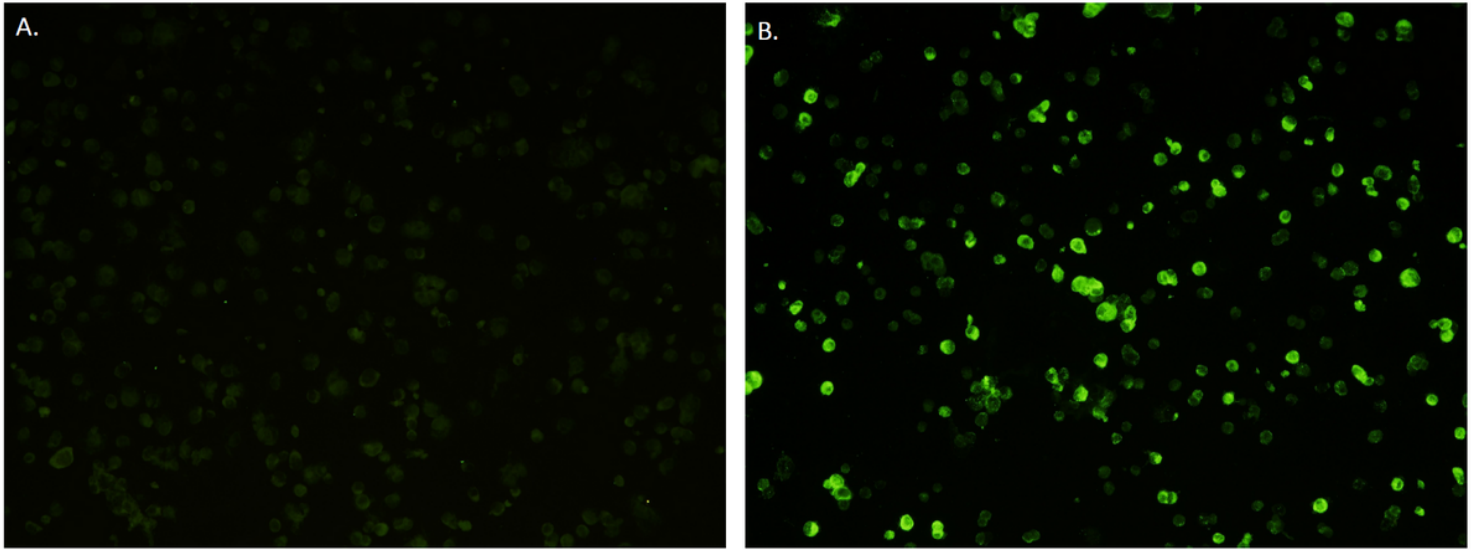
Figure 1

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

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- PolishFoxesresults26122023.xls
- Map1.jpg