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ABSTRACT

Background: Our study explores the role of bats as reservoirs of coronaviruses.
Methods: We conducted virological screening of bats hibernating in military bunkers at the Natura 2000 site "Nietoperek" in Western Poland collecting oral and anal swab samples from 138 bats across six species to apply a combination of pan-coronavirus and SARS-CoV-2 specific PCR assays.
Results: Only one anal swab tested positive for coronavirus. No SARS-CoV-2 was detected in any of the samples. The low prevalence of coronavirus in the studied colony contrasts with higher rates found in other regions and may be influenced by hibernation.
Conclusions: Hibernating bats may show a low prevalence of coronavirus, potentially due to the hibernation process itself. This finding indicates that hibernating bats may not be the most optimal subjects for screening zoonotic pathogens. However, biomonitoring of bats for emerging and reemerging diseases is recommended for comprehensive epidemiological insights.

1. Introduction

During the last two decades, three major outbreaks of coronavirus-caused diseases occurred in the world. The first epidemic (caused by the Severe Acute Respiratory Syndrome Coronavirus, SARS-CoV) happened in China in 2002 and caused 744 deaths among 8096 patients [1]. After the outbreak of MERS-CoV disease in the Middle East in 2012 a total of 2298 cases were confirmed including 811 deaths [2]. When several cases of strange pneumonia were reported in the city of Wuhan (Hubei, China) in late 2019 [3], barely anyone could imagine that this would lead to a pandemic causing 7,031,216 deaths among 774,631,444 patients (as of 11th February 2024) [4].

Coronaviruses (among which is the SARS-CoV-2, the betacoronavirus responsible for the latest pandemic) can be hosted by multiple mammal species like bats, rats, mice, dogs, camels, calves, turkeys, rabbits, and pigs. Although they mainly cause respiratory disorders, some strains of coronaviruses may result in gastroenteritis, hepatitis, encephalitis or peritonitis in infected individuals [5,6]. In humans, coronavirus infections may vary from asymptomatic to severe pneumonia accompanied by fever, cough, or gastrointestinal irritation [7,8].

Among animal hosts, bats are of major epidemiological importance. This is because they have been reported to be a reservoir and carriers for many viruses [9,10]. Moreover, they form large colonies making it easier for the viruses to transfer between individuals. Finally, bats can travel vast distances spreading diseases both within their species and

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across other animal species [6,11]. Intermediate coronavirus hosts are crucial for animal-human transmission as people rarely get infected from the bats themselves [12]. Masked palm civets and pangolins are believed to be the direct source of SARS-CoV and SARS-CoV-2 infections for humans although they do not play the role of animal reservoirs. It contrasts with dromedary camels which are both intermediate hosts and reservoirs for MERS-CoV [13,14]. Searching for animal hosts and reservoirs and developing knowledge about relations between them and the possible consequences of their coexistence is vital for better understanding the dynamics of the diseases caused by coronaviruses. In this research, we focused on virological screening of hibernating bats residing in military bunkers in the Natura 2000 site PLH080003 "Nietoperek", western Poland. Besides virological screening itself, an important point of this study is to assess the quality of diagnostic material taken from hibernating animals.

2. Materials and methods

2.1. Bat sampling

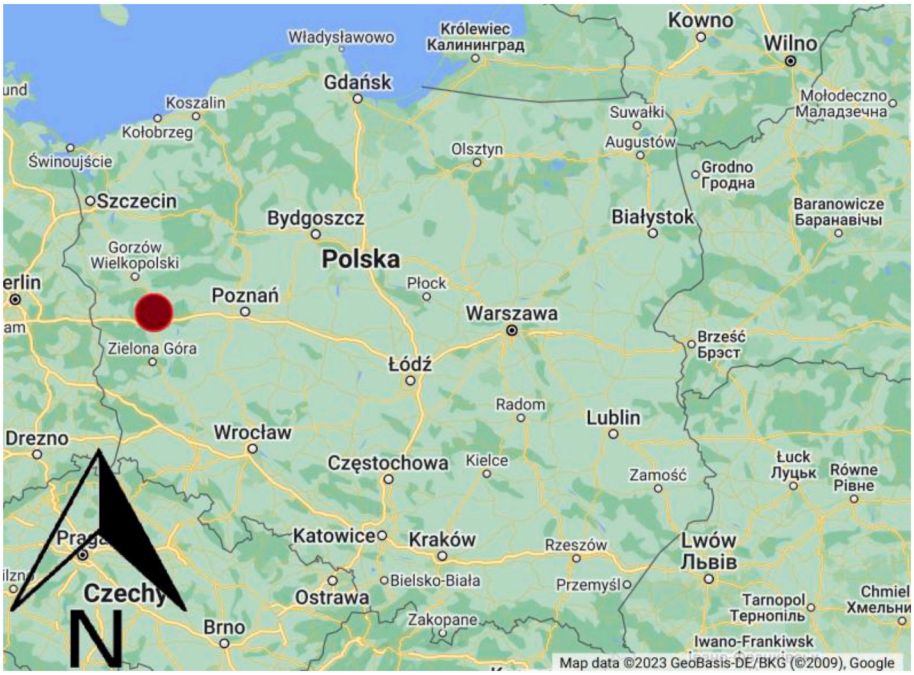
The samples were collected in section 7 of the central sector of Międzyrzecz Fortified Front (MFF) (Festungsfront Oder-Warthe-Bogen) (52°25′N, 15°32′ E) situated in Western Poland (Map. 1). The place is visited by tourists year-round, with potential human infection risk. Every year up to 40,000 bats of 13 species hibernate in both the main underground system of "Nietoperek" and the surrounding bunkers, making it one of the largest and most important bat hibernation sites in Europe [15–17]. Tracking data from the Dresden bat ringing center (Fledermausmarkierungszentrale, Germany) shows bats migrating to "Nietoperek" from the expansive Central European Lowlands. The furthest travel distances recorded to "Nietoperek" are 257 km for *Myotis daubentonii*, 226.7 km for *M. myotis*, and 242.1 km for *M. brandtii*. The minimal convex polygon (MCP) for large mouse-eared bats, based on recaptured individuals, spans at least 17,000 km^2 across significant parts of German territories: Brandenburg, Mecklemburg-Vorpommern, Sachsen-Anhalt, and western Poland (Lubuskie, Wielkopolskie, Zachodniopomorskie Voivodeships) [18]. Bats were individually collected from their colonies. Their species and sex were identified visually. For each bat, oral and anal swabs were taken using laboratory swabs. Swabs were then placed in Eppendorf tubes filled with a virus-inactivating medium and transported to the laboratory under cool conditions at +4 °C for RNA extraction. After sample collection and morphological measurements, the bats were carefully returned to their colony to minimize disturbance.

2.2. RNA extraction

The RNA was extracted from oral and anal swab samples and used in PCR assays to detect coronaviruses,using the QIAamp 96 Virus QIAcube HT Kit and QIAcube HT system (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Briefly, the swabs were transferred to tubes containing 800 µL of ATL buffer and 30 µL of proteinase K. The tubes were incubated for 1 h at 56 °C and homogenized by vortexing within intervals of 15 min. After this, 200 µL of lysate was transferred to a sample block and the extraction procedure was carried out with QIAcube, using onboard lysis. The final elution volume was set to 120 µL, and the extracted RNA was stored in −80 °C.

2.3. Pan-coronavirus molecular screening assay

Initially, the RNA extract obtained from oral and anal swabs were screened for the presence of coronaviruses using a broad range of one-step real-time RT-PCR assay. The reaction mix was prepared with qScript One-Step SYBR® Green RT-qPCR Kit (QUANTABIO, Beverly, MA, USA) and 600 nM of each primer, 11-FW (5'-TGATGATGSNGTTGTNTGYTAYAA-3′) and 13-RV (5'-GCATWGTRTGYTGNGARCARAATTC-3′) [19]. The reactions included 5 µL of template RNA and prepared to a final volume of 20 µL. The thermocycling conditions were as follows: first the reactions were incubated at 50 °C for 10 min, followed by initial denaturation at 95 °C for 5 min and then 45 cycles consisting of a denaturation step at 95 °C for 10 s, an annealing step at 50 °C for 20 s, and an elongation step at 72 °C for 30 s. Fluorescence was collected after each cycle. At the end, a melt curve was obtained by increasing the temperature by 0.5 °C /5 s, from 55 to 95 °C. The amplification was carried out using the AriaMx Real-time PCR System and the fluorescence was analysed using AriaMx Software (Agilent



Map 1. Lubrza "Nietoperek Reserve" is located in western Poland (52°23′25″N 15°31′02″E). Map Data, Google, 2023.

Technologies, Santa Clara, CA, USA). Samples selected based on their temperature melting curve were taken for further investigation. RNA extracted from SARS-CoV-2 was used as positive control in the screening and conventional PCR.

2.4. Pan-coronavirus confirmatory PCR assay

A broad-range, semi-nested PCR assay was used to confirm the presence of coronaviruses in the previously selected samples. Complementary DNA (cDNA) was produced from the extracted RNA using Maxima Reverse Transcriptase (Thermo Scientific, Waltham, Massachusetts, USA) and used in the confirmatory PCR assay. The RT reactions were prepared to a final volume of 20 μL. Initially, a reaction mix containing 5 μL of template RNA, 1 μL of random hexamers (50 μM), 1 dNTP's (10 mM each) and 7.5 μL of water was prepared and incubated at 65 °C for 5 min. Next, we added a second reaction mix containing RT Buffer to final concentration of 1×, 20 U of RiboLock RNase Inhibitor (Thermo Scientific™) and 200 U of Maxima Reverse Transcriptase. The final mix was initially incubated at 25 °C for 10 min, followed by 30 min at 50 °C and 5 min at 85 °C, after which it was stored at −20 °C to be used as template in the confirmatory PCR reaction.

To confirm the presence of coronaviruses in the selected samples we used the primers targeting the RdRp published by Holbrook and collaborators [20]. In this study, we used the DreamTaq DNA Polymerase kit (Thermo Scientific) and the reactions were modified as follows. Initial and semi-nested reactions were prepared with 1× buffer and 0.2 mM of dNTP's (each). In the first reaction, 2.5 μL of template cDNA was mixed with 2.5 U of polymerase, 4% of DMSO and 1 μM of each primer – F1 (5'-GGTGGGAYTAYCCHAARTGYGA-3′), R1 (5'-CCRTCATCAGAHARWATCAT-3′) and R2 (5'-CCRTCATCACTHARWATCAT-3′), to a 25 μL final volume reaction. The second reaction was prepared with 2 μL of template (PCR product of the first reaction), 1.25 U of polymerase and 400 nM of each primer - F2 (5'-GAYTAYCCHAARTGTGAYAGA-3′), F3 (5'-GAYTAYCCHAARTGTGAYMGH-3′), R1, R2), to a final volume of 50 μL. The thermocycling conditions for both reactions were: an initial denaturation step at 94 °C for 3 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s, and elongation at 72 °C for 40 s; and a final elongation step at 72 °C for 5 min. Both reactions' products were visualized in 2% agarose gel stained with ethidium bromide.

2.5. SARS-CoV-2 specific PCR assay

In parallel to the confirmatory PCR, the presence of SARS-CoV-2 was investigated with the Luna® SARS-CoV-2 RT-qPCR Multiplex Assay Kit (New England Biolabs, Ipswich, Massachusetts, USA), a SARS-CoV-2 specific qPCR assay, as per manufacturer's instructions. This assay was carried out on samples selected based on the initial screening assay.

2.6. Sequencing

The PCR products of reactions that amplified fragments of expected length were purified using the GeneJET PCR Purification Kit (Thermo Scientific) and sent for sequencing. The sequencing was carried out using Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit (Part No. 4336921) according to the manufacturer's instructions and further analysed using Applied Biosystems ABI3130XL Genetic Analyzer (16-capillaries).

3. Results

We sampled 138 individuals belonging to six bat species: greater mouse-eared bat *Myotis myotis*, Natterer's bat *M. nattereri*, western barbastelle *Barbastella barbastellus,* Daubenton's bat *M. daubentonii,* Brandt's bat *M. brandtii* and whiskered bat *M. mystacinus* (Table 1). All of the species involved in study, excluding *M. mystacinus*, have been previoiusly identified as hosts for various coronaviruses. [21,22]

Table 1
Number of bat individuals sampled in "Nietoperek" bat reserve.

	Males	Females	
M. myotis	68	49	117
M. nattereri	4	1	5
B. barbastellus	0	1	1
M. daubentonii	7	5	12
M. brandtii	1	0	1
M. mystacinus	1	1	2
Total	**81**	**57**	**138**

As a first screen, samples were sorted for the likelihood of having coronaviruses using a pan coronavirus qPCR. The criteria for selecting samples at this initial screening stage were adjusted to include samples with very low viral load and virus genetic diversity. Therefore, sample with both a threshold cutting value (Ct) lower than those of the no-template controls (NTC) and negative controls, and a melting temperature close to that of the positive control, were used for follow-up assays. A total of 83 individuals out of 138 had one or both (oral and anal) samples selected for a pan Coronavirus PCR confirmatory assay targeting the RdRp. In this assay, a single positive sample was detected, an anal swab sample from catchment 5 (A5). The amplicon purified and prepared for sanger sequencing. Unfortunately, no readable sequence could be obtained.

Finally, the presence of SARS-CoV-2 was investigated using a commercial diagnostic test. No SARS-CoV-2 positive samples were found.

4. Discussion

The prevalence of coronaviruses in bats exhibits significant spatial and temporal variation, however the mechanistic factors are not completely clear [23]. Factors like animal health, colony characteristics, and reproductive cycles can directly or indirectly influence prevalence. For instance, a study carried out in China, 2006, revealed an overall prevalence of 6.5%, with some colonies reaching rates as high as 55% [24]. In Northern Germany, the overall prevalence was 9.8%, varying between 5.2% and 25.4% among different bat species [25]. Additionally, a longitudinal study in Zimbabwe revealed varying prevalence of coronavirus in bats at the Chirundu farm site, ranging from 1.95% to 44.2% during pregnancy and weaning periods, respectively [26].

In our study, out of 138 individuals, only one anal swab tested positive in conventional PCR, and no readable sequence was obtained. The number contrasts with findings in northern Germany, relatively closer to our study area, where bats caught outside the shelter during their activity period showed higher coronavirus prevalence [25]. The low prevalence in the colony studied here might be due to unfavorable physiological and behavioral conditions during bats' hibernation period, including a drop in bat's heart rate from 200 to 300 beats per minute to as low as 10 beats per minute and suspension of breathing for several minutes at a time, which might affect viral replication and reduce bats overall mobility and interactions, thereby decreasing the probability of virus transmission [27]. Contrary to our results, Subudhi et al. (2017) found coronavirus persistence for up to 4 months in North American little brown bats during hibernation in laboratory settings [22]. Nonetheless, this data is limited to a specific host and to laboratory conditions and can be different to other host species in the natural environment, as our studied specimens. The hibernation factor may explain our unexpected results. We also acknowledge the limitations of our study in capturing the full spectrum of coronavirus prevalence among the region's bat populations due to the number of individuals tested and diversity of species involved. Obtaining the accurate picture requires further comprehensive study on both hibernating and active bats in this area. At this stage, we believe the risk of coronavirus transmission from bats to tourists visiting underground sites is negligible.

5. Conclusions

We did not find hibernating bats to be infected with SARS-CoV-2 virus. And the very low overall prevalence of coronavirus (1/138) might be caused by the hibernation process. Hibernating bats are not the most optimal source for screening of zoonotic pathogens. On the one hand they are easy to access, and material collection can be done with a relatively small disturbance to animals. On the other hand, hibernation causes significant decrease of metabolic processes in bat organisms. Therefore, hibernating bats produce very little amount of guano, and the bat metabolic changes might affect virus-host interactions. One potential limitation of this study is the possibility of occurring false negative results despite performing multiple PCRs and using different detection methods. Considering past reports on SARS-CoV-2 origins, and susceptibility of bats to carry highly zoonotic viruses we believe that bat populations should be constantly screened against emerging and reemerging diseases to provide the most accurate epidemiological image.

Author contributions

The study was conceived and designed by MG, RK, TK. Supervision of the biomonitoring by MG and TS. Sampling permits: TK. Samples were collected in the field by GA, TK, AR, AG and MG. Bats handling and measurements: GA, TK, AR. Molecular analysis and laboratory work were conducted by AG, JN, MG, VB, LD, ES, VV, RK. Data handling: LD, VB, MG. The manuscript was written by AG, LD, VB and MG in consultation with all co-authors. MG, RK, LD and VB revised the manuscript. All authors accepted the final manuscript version.

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Institutional Review Board Statement

This study was carried out in strict accordance with the recommendations in the Guidelines for the Care and Use of Laboratory Animals of the Polish National Ethics Committee for Animal Experimentation and according to the Polish national law for field work involving the trapping and culling of wild unprotected vertebrates for scientific purposes (Resolution No. 12/2022 of Polish National Ethics Committee for Animal Experiments, 11th March 2022). The study was performed according to the ARRIVE guidelines 2.0. Data collection was carried out under permission no. WPN-I.6205.24.2021.MG issued by the Regional Director for Environmental Protection in Gorzów Wielkopolski, Poland.

CRediT authorship contribution statement

Aleksander Goll: Data curation, Methodology, Writing – original draft. **Lara Dutra:** Data curation, Formal analysis, Investigation, Methodology. **Joanna Nowicka:** Formal analysis. **Elena Sgarabotto:** Investigation. **Vinaya Venkat:** Investigation. **Grzegorz Apoznański:** Data curation, Investigation, Methodology. **Tomasz Kokurewicz:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Alek Rachwald:** Data curation, Investigation, Methodology. **Lukasz Rabalski:** Formal analysis. **Hussein Alburkat:** Formal analysis, Methodology. **Jenni Virtanen:** Investigation, Methodology. **Tarja Sironen:** Data curation, Resources, Supervision. **Ravi Kant:** Data curation, Investigation, Methodology, Supervision. **Vincent Bourret:** Data curation, Investigation, Methodology, Resources. **Maciej Grzybek:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Maciej Grzybek reports financial support was provided by National Science Centre Poland. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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