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SARS-CoV-2 in zoo-kept and wild-living animals

Ph.D. DISSERTATION THESIS

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ZADÁNÍ DISERTAČNÍ PRÁCE

Jignesh Italiya

Tropical Agrobiology and Bioresources Management

Název práce

SARS-CoV-2 in zoo-kept and wild-living animals

Název anglicky

SARS-CoV-2 in zoo-kept and wild-living animals

Cíle práce

SARS-CoV-2, a coronavirus causing COVID-19, is able to infect relatively wide range of animal species. Animals from some of these species can serve as virus reservoirs from which SARS-CoV-2 can again reinfect humans. Moreover, new genetic variants of SARS-CoV-2 can develop during it passaging on these animal hosts which can lead to dangerous phenotype after the virus reenter human population (e.g., increased infectivity, virulence, or vaccine resistance).
Despite an enormous effort was given in understanding how SARS-CoV-2 infects humans beings, we still do not understand its biology in different animal species. It was already proven that it can infect numerous species such as dogs, cats, minks, large cats, monkeys, hamsters, rabbits etc. Some other animals species were suggested to be susceptible to infection based on bioinformatics analyses.
The goal of this dissertation thesis is to increase our understanding in which animal species are permissive to SARS-CoV-2 infection.

Metodika

After comprehensive literature search which will detect animal species potentially permissive for SARS--CoV-2, the author will focus on analyses of sera from animals of various species which in past came into close contact with humans infected by SARS-CoV-2. ELISA and virus inhibition assay will be used to detect antibodies against SARS-CoV-2 in sera of these animals. Further, other samples taken from these animals will be tested using RT-qPCR or corresponding method to detect presence of viral RNA. If positive, virus will be sequenced to detect genetic changes associated with infection of these animals.

Doporučený rozsah práce

40-60 normal pages

Klíčová slova

SAR-CoV-2, COVID-19, animals, zoo, wild-living, domestic, mutation

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Doporučené zdroje informací

Boklund et al: SARS-CoV-2 in Danish Mink Farms: Course of the Epidemic and a Descriptive Analysis of the Outbreaks in 2020, Animals (Basel). 2021 Jan 12;11(1):E164. doi: 10.3390/ani11010164.

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DECLARATION

I, Jignesh Italiya, hereby declare that I have written the enclosed PhD thesis entitled "SARS-CoV-2 in zoo-kept and wild-living animals" independently and in collaboration with co-authors in the respective scientific articles related to this work. All the texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to the Citation rules of the FTA. I state that the work has not been submitted for any other degree to this or any other university within and outside the Czech Republic.

In Prague,……

M.V.Sc. Jignesh Italiya

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ABSTRACT

Coronaviruses, prominent in veterinary medicine for causing various animal diseases, were largely overlooked in human medicine until significant outbreaks occurred in the past two decades. On December 31, 2019, the WHO China country office received notifications about pneumonia cases of unknown etiology in Wuhan, Hubei Province, China. The disease, later named COVID-19, was caused by a novel coronavirus (SARS-CoV-2) identified on January 7, 2020. SARS-CoV-2 caused a broad transmission of diseases between humans and animals, known as zooanthroponosis, which carries the danger of virus mutation and the possibility of re-emerging in human populations. This requires extensive animal testing to minimize future hazards, yet detecting diseases in wildlife on a large scale is difficult. In this context, this study evaluates the assessment and strategy development for SARS-CoV-2 screening in wildlife by using in silico predictions, experimental studies, and documented natural infections and further we implemented in our following research. SARS-CoV-2, which led to widespread zooanthroponosis, posing risks for virus mutation and potential re-emergence into human populations. This necessitates widespread animal screening to mitigate future risks, although detection in wildlife is challenging.

Three research processes led to the entirety of the present study, the first being a first detection of SARS-CoV-2 in white rhinoceros during a small-scale coronavirus surveillance in the Bandia reserve, Senegal. The COVID-19 pandemic spurred interest in monitoring coronaviruses in wildlife, revealing critical information about viral reservoirs, transmission, and pathogenesis. This study presents molecular surveillance results from Senegalese wildlife, screening fecal samples from various species in the Bandia Reserve and urban African four-toed hedgehogs in Ngaparou. Most samples tested negative, but one white rhinoceros was positive for SARS-CoV-2, marking the first documented instance of this virus in white rhinoceros and expanding knowledge on potential SARS-CoV-2 hosts. The second study was on serological screening carried out in several mammalian species in Wilhelma Zoo, Stuttgart, Germany. Between July 2022 and January 2023, blood samples from twelve animal species at Wilhelma Zoo, Germany, were analyzed for SARS-CoV-2 antibodies. Two gorillas exhibited antibodies specific to the nucleocapsid protein of SARS-CoV-2, suggesting previous infection. Symptoms observed in these gorillas were not typically associated with COVID-19, highlighting the need for ongoing screening to understand the virus's spread among different species. The third study was on the development of multi-species protein A-ELISA assay for detection of SARS-CoV-2 antibodies in zoo animals as a proof of concept for wildlife surveillance. COVID-19, originating in Wuhan in 2019, has infected various wild animals, necessitating further research. IgG concentration is a valuable diagnostic parameter for wild animals, and a Protein A-based indirect ELISA was developed for detecting IgG antibodies against SARS-CoV-2. This assay, using serum samples from 44 animal species, identified antibodies in 16 animals. Virus neutralization assays confirmed SARS-CoV-2 neutralizing antibodies in two white rhinoceros and one Persian leopard, enhancing understanding of the virus's host range and interactions with various animal species. This study underscores the importance of surveillance to understand SARS-CoV-2's epidemiological landscape and its potential for cross-species transmission, contributing to comprehensive wildlife disease surveillance programs to mitigate future zoonotic risks.

Keywords: *Covid-19 in animals; wildlife surveillance; molecular detection; serological surveillance; western lowland gorillas; white rhinoceros*

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LIST OF ABBREVIATIONS

ACE2: Angiotensin-Converting Enzyme 2

ADAM-17: A Disintegrin and Metalloprotease Domain 17

BCoV: Bovine Coronavirus

BLAST: Basic Local Alignment Search Tool

BSA: Bovine Serum Albumin

COVID-19: Coronavirus Disease 2019

CoV: Coronavirus

CoVs: Coronaviruses

CRISPR/Cas: Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-associated Protein

CTS: Cathepsin

CTSL: Cathepsin L

DMEM: Dulbecco's Modified Eagle Medium

E: Envelope (protein)

EIDs: Emerging Infectious Diseases

ELISA: Enzyme-Linked Immunosorbent Assay

GIT: Gastrointestinal Tract

H2SO4: Sulfuric Acid

HCoV229E: Human Coronavirus 229E

HCoV-NL63: Human Coronavirus NL63

HCoV-OC43: Human Coronavirus OC43

HRP: Horseradish Peroxidase

LAMP: Loop-Mediated Isothermal Amplification

M: Membrane (protein)

MERS-CoV: Middle East Respiratory Syndrome Coronavirus

nCoV-2019: Novel Coronavirus 2019

NGS: Next-Generation Sequencing

N: Nucleocapsid (protein)

NRP1: Neuropilin-1

ORF1ab: Open Reading Frame 1ab

PBS: Phosphate-Buffered Saline

PFU: Plaque-Forming Units

RPA: Recombinase Polymerase Amplification

RT-PCR: Reverse Transcription Polymerase Chain Reaction

RT-qPCR: Real-Time Reverse Transcription-Quantitative Polymerase Chain Reaction

S: Spike (protein)

SADS-CoV: Swine Acute Diarrhea Syndrome Coronavirus

sVNT: Surrogate Virus Neutralization Test

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2

TMB: Tetramethylbenzidine

TMPRSS2: Transmembrane Protease Serine 2

VNT: Virus Neutralization Test

WHO: World Health Organization

CHAPTER 1

Introduction

1.1 Introduction

On December 31, 2019, the WHO China Country Office received notification, regarding instances of pneumonia with an unknown etiology that were identified in Wuhan, located in the Hubei Province of China (Zhou et al., 2020). The disease demonstrated a respiratory ailment that varied in intensity, encompassing minor symptoms in the upper respiratory tract, as well as acute respiratory distress syndrome and severe interstitial pneumonia. The medical manifestations encompass elevated body temperature, shivering, coughing, and dyspnea or respiratory distress (Petrosillo et al., 2020) (Pal et al., 2020). On January 7th, 2020, a novel coronavirus (nCoV-2019) has been isolated and identified as the causative pathogen of the diseases named as coronavirus disease 2019 (COVID-19) (Zhou et al., 2020). Following this, the virus was officially classified as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses. The World Health Organization officially declared COVID-19 as a pandemic in March 2020, following the widespread transmission of the virus across all continents. Throughout the course of the pandemic, there has been notable advancement in comprehending the effects of SARS-CoV-2 on both humans and animals.

Research indicates that SARS-CoV-2 may be transmitted across different animal species, which highlights the need for enhanced surveillance efforts. To avoid the spread of the virus during the early stages, precautionary measures were implemented. Efforts to prevent the spread of the pandemic, such as the use of masks, maintaining physical distance, conducting tests, and identifying contacts, have shown only moderate effectiveness in reducing the transmission of the virus (Chu et al., 2020). Consequently, several scientific organizations and pharmaceutical companies worldwide have implemented a rapid vaccine development drive. This facilitated the global availability of vaccines. In addition to vaccination, many treatment approaches including as immunotherapy and antiviral drugs have been and continue to be used as measures to prevent infection and effectively control the spread of the virus (Miteva et al., 2023). The combined endeavors have facilitated the end of the COVID-19 pandemic and on

May 5, 2023, the World Health Organization (WHO) formally declared the pandemic to have ended.

Coronaviruses (CoV) are a class of positive-stranded RNA viruses within the family Coronaviridae, subfamily Orthocoronavirinae, and order Nidovirales. They have the potential to induce neurological, enteric, respiratory, and hepatic disorders in numerous animal species. The nomenclature of this virus is derived from its distinctive morphology, characterized by the presence of a crown-like structure formed by prominent peplomers that extend from the surface (Pal et al., 2020). The S (spike) and E (envelope) proteins are considered to be two of the most significant peplomers. The virus tropism is determined by the receptor binding properties of S peplomers, which are composed of two subunits (S1 and S2) (Belouzard et al., 2012). The frequent host-shifts of coronaviruses between mammals necessitate a more comprehensive understanding of the origins of non-human animal coronaviruses that infect people, as well as mammals that may serve as natural reservoirs for human and veterinary diseases (Gunasekara et al., 2022). Prior to the emergence of SARS-CoV-2, several well studied coronaviruses (Figure 1.1) in animals have provided valuable knowledge about this particular virus type. Coronaviruses (CoVs) encompass four distinct genera, namely *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (Fan et al., 2019).

Betacoronaviruses have been responsible for significant zoonotic epidemics in recent years, including SARS-CoV, MERS-CoV, and SARS-CoV-2. The SARS outbreak in 2002-2003 was caused by the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV). The origin of SARS-CoV is thought to be bats, with its transmission to humans occurring through intermediate hosts, such as civet cats, in live animal markets (Song et al., 2005). MERS-CoV, a member of the beta coronavirus genus, originated in Saudi Arabia in 2012. The transmission of MERS-CoV to humans is predominantly facilitated by dromedary camels, resulting in a severe respiratory infection that exhibits a greater mortality rate (case-fatality ratio of 36%) in comparison to SARS-CoV-2 (Durai et al., 2015).

SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus with a genome length of 29.9 kB. The genome structure of Sars cov-2 exhibited gene characteristics that correspond to known CoVs. Specifically, over two-thirds of the genome is composed of ORF1ab gene, which encodes ORF1a and ORF1ab polyproteins. The remaining one-third of the genome is composed of genes that encode structural proteins, such as nucleocapsid N, envelope (E), membrane (M), and surface (S), (Khailany et al., 2020). The non-structural proteins (nsps) of

SARS-CoV-2 play a vital role in the replication and transcription of the virus. The viral genome's ORF1ab region encodes many proteins, such as proteases, RNA-dependent RNA polymerase (RdRp), and helicase. They have functions in the processing of viral polyproteins, evading the immunological responses of the host, and altering the machinery of host cells to help the virus multiply (Jahirul Islam et al., 2023).

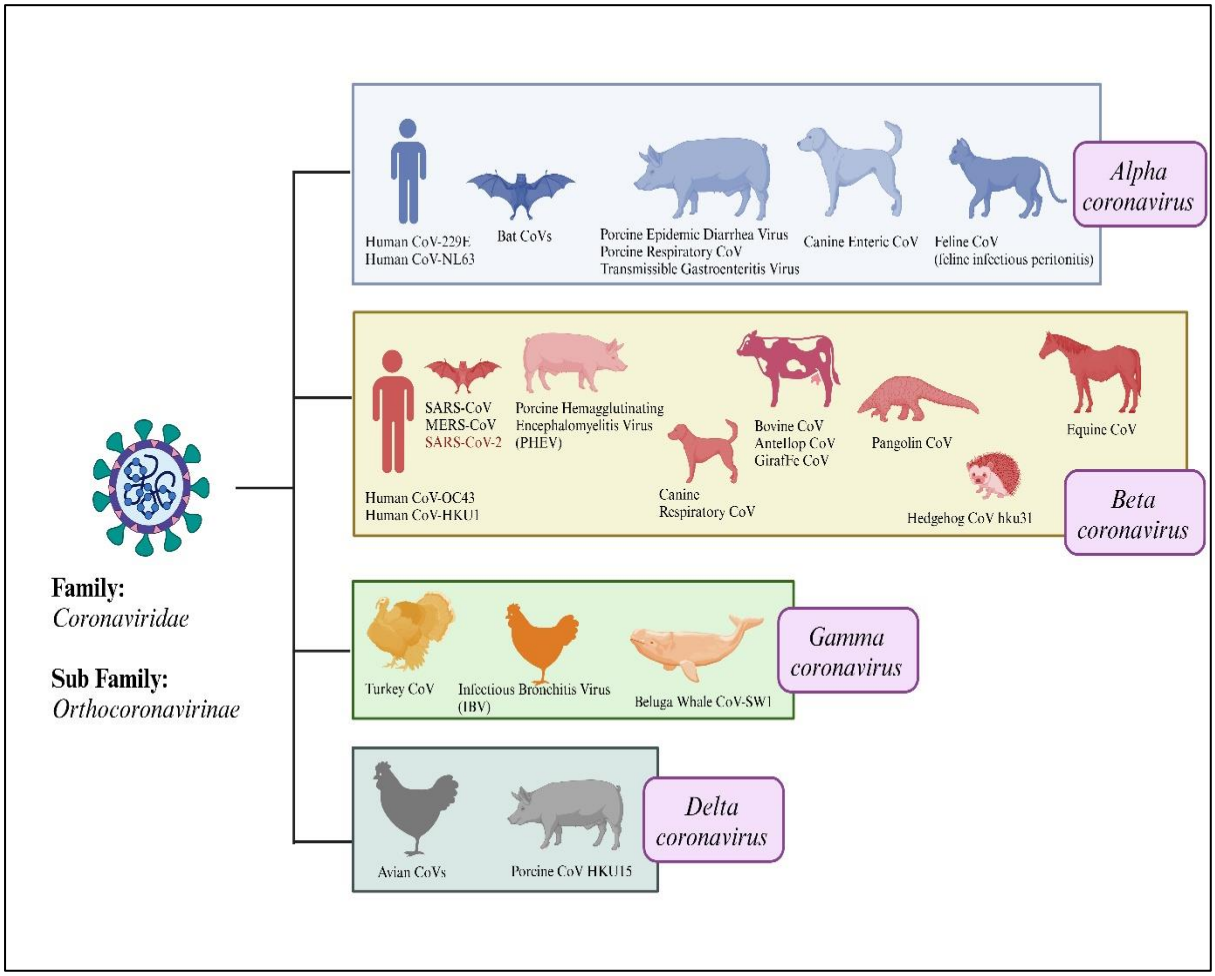

Figure 1.1 Diversity of coronaviruses and their mammalian hosts

The nucleocapsid (N) protein participates in the packaging of RNA, adopting a configuration reminiscent of beads on a string. In addition, apart from its involvement in genome organization, the N protein plays a crucial role in promoting virion assembly and enhancing the efficacy of viral transcription, among various other tasks (Yadav et al., 2021). The envelope protein, with a size ranging from 8 to 12 kilodaltons (kDa), plays a crucial role in pathogenesis, viral assembly, and release (Santos & Mendoza, 2023).

The membrane (M) protein, which is approximately 25–30 kDa and is predominantly O-linked glycosylated, aids in the assembly of the virus through its interaction with other structural

proteins, including the nucleocapsid. This interaction potentially impacting pathogenesis and exhibiting conserved structural integrity across different coronavirus genera (Yadav et al., 2021).

The spike glycoprotein (S) is classified as a type I membrane protein (Zhang et al., 2021). It is composed of two distinct cleavage sites, namely S1/S2 and S2′. Notably, the S1/S2 site exhibits a distinctive cleavage pattern for furin protease, which arises from a unique insertion of four amino acids. This particular motif serves as a distinguishing characteristic of SARS-CoV-2 (Takeda et al., 2022).

After the viral particle is integrated, the S protein, which has already been cleaved by furin at the S1/S2 site, then undergoes further cleavage at the S2′ site. The fusing of the plasma membrane is facilitated by the type II transmembrane serine protease, transmembrane protease serine 2 (TMPRSS2), when it binds to the angiotensin-converting enzyme 2 (ACE2) receptor (Matsuyama et al., 2020) which is required in original strain of virus. Aside from direct fusion with host ACE2 cell receptors, the S protein of SARS-CoV-2 utilizes alternative entry pathways including cathepsin L-mediated endocytosis and Neuropilin-1 (NRP1) facilitated entry. Endocytosis involves the uptake of the virus through the vesicles of the host cell membrane. Within these vesicles, proteases such as cathepsin L cleave the S protein, which then permits the virus to fusion with the endosomal membrane and release its genome into the cytoplasm of the host cell (Bayati et al., 2021). Through its function as a co-receptor, NRP1 facilitates cellular infection and viral absorption by encouraging the S protein to interact efficiently with host cells (Cantuti-Castelvetri et al., 2020).

The emergence of the SARS-CoV-2 outbreak has prompted concerns regarding the potential for reverse transmission within animal populations, with subsequent mutations posing risks not only to humans but also to wild animal species. The first instances of infection were documented in domesticated dogs and cats as a result of the close proximity between infected individuals and their pet animals (Bosco-Lauth et al., 2020; Sit et al., 2020). The first natural SARS-CoV-2 infection in lions and tigers were reported at the Bronx Zoo in the United States in March 2020 providing the first evidence for transmission of the virus from humans to wild animals (captive-kept) (McAloose et al., 2020). Subsequently, a number of instances of natural SARS-CoV-2 infection were documented in zoos, wildlife, and domesticated animals. Figure 2 illustrates the temporal distribution of animal hosts susceptible to SARS-CoV-2, referred to by natural or experimental infection, along with the corresponding dates of detection or

publicized. Some animal species infected by SARS-COV-2 have the ability to transfer the infection to human populations. During a sequence analysis of affected individuals working in mink farms in the Netherlands, it was discovered that individual been infected with strains exhibiting sequence signature similar to SARS-CoV-2 infected animals in the farm. This finding serves as proof of the transmission of SARS-CoV-2 from animals to humans inside mink farms (Oude Munnink et al., 2021). Likewise, a study unveiled an epidemiologically connected human infection, providing proof of the continuous evolution of SARS-CoV-2 within white-tailed deer populations and subsequent transmission from deer to humans (Pickering et al., 2022a).

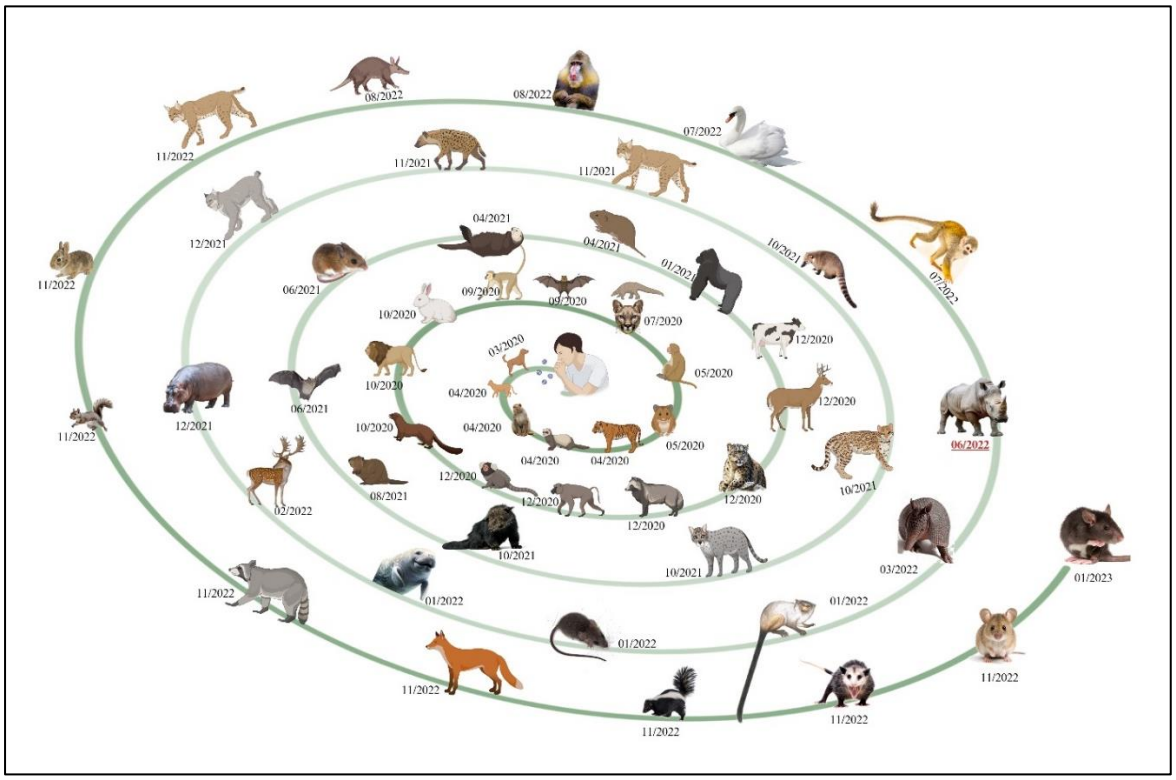

Figure 1.2. Temporal Distribution of SARS-CoV-2 Infections Across Animal Species: Dates of Detection or publicized.

Surveillance efforts during disease outbreaks are crucial for elucidating the epidemiological landscape and the pathogen's propensity to cross species barriers. During the SARS-CoV-2 pandemic, worldwide surveillance was conducted on domestic animals, wildlife, and companion animals in an effort to determine the pathogen's potential to cross the interspecies barriers and infect new animal species (Qiu et al., 2023). This surveillance encompasses a wide array of settings, including pet animals, domestic livestock, zoological facilities, and wildlife habitats. In order to understand possible cases of SARS-CoV-2 transmission from animals to

humans, many surveillance methods can be used, including identifying the pathogen, detecting antibodies in the blood, and conducting clinical investigations (Sparrer et al., 2024). Molecular techniques such as RT-PCR the gold standard for early phase virus detection, loop-mediated isothermal amplification (LAMP), based on recombinase polymerase amplification (RPA) and CRISPR/Cas, and genome sequencing have been extensively utilized for pathogen detection and characterization, facilitating comprehensive surveillance efforts, and enhancing our understanding of zoonotic transmission dynamics (Liang et al., 2023; Y. Zhang et al., 2023).

Serological surveillance offers a valuable approach to elucidate prior spillover incidents in animals through the detection of antibodies against SARS-CoV-2 in blood samples (Tan et al., 2023). Various serological techniques can be employed to identify the presence of antibodies in animal specimens. For instance, the Enzyme-Linked Immunosorbent Assay (ELISA) represents a widely utilized method. Additionally, surrogate virus neutralization test (sVNT) the Virus Neutralization Test (VNT) is commonly employed and regarded as the gold standard assay (Vilibic-Cavlek et al., 2023).

Continued surveillance and monitoring are imperative for achieving a comprehensive understanding of SARS-CoV-2 biology across diverse animal species. Our studies explore the feasibility of employing mobile molecular biology laboratories for pathogen detection in semi-free living wildlife environments, circumventing the need for traditional laboratory facilities. Furthermore, we investigate the potential utility of protein-A in developing an indirect ELISA for detecting antibodies against SARS-CoV-2 across a wide range of animal species, facilitating its application in wildlife surveillance. The utilization of mobile laboratories for wildlife pathogen detection, alongside the development of a Multispecies protein-A ELISA for SARS-CoV-2 antibody detection, serves as a proof of concept for its' potential use for routine surveillance efforts.

1.2 The aims of the thesis

The aim of this study was to undertake SARS-CoV-2 surveillance within wildlife settings employing a mobile laboratory. In addition, the project aimed to develop a multi-species enzyme-linked immunosorbent test (ELISA) using protein A to detect antibodies against SARS-CoV-2, with the intended application for surveillance in zoo animals (and latter potentially also in wild-living animals). In order to accomplish these aims, the following objectives were pursued:

- To carry out assessment and strategy development for SARS-CoV-2 screening in wildlife.
  (This objective was accomplished through the publication of the review article "Assessment and Strategy Development for SARS-CoV-2 Screening in Wildlife: A Review.")

- To identify animal species that are permissive to SARS-CoV-2 natural infection.
  (This objective was met through pathogen surveillance conducted in Senegal, as detailed in the publication "First Detection of SARS-CoV-2 in White Rhinoceros during a Small-Scale Coronavirus Surveillance in the Bandia Reserve, Senegal.")

- To carry out serological surveillance within zoo animals.
  (This objective was achieved through two publications: “Serological Screening of SARS-CoV-2 Infection in Several Mammalian Species in Wilhelma Zoo, Stuttgart, Germany” and “Wildlife Sentinel: Development of Multi-Species Protein A-ELISA Assay for Detection of SARS-CoV-2 Antibodies in Zoo Animals as a Proof of Concept for Wildlife Surveillance.”)

CHAPTER 2: Literature Review

Assessment and strategy development for SARS-CoV-2 screening in wildlife: A review

Adapted from: Italiya, J., Bhavsar, T., & Černý, J. (2023). Assessment and strategy development for SARS-CoV-2 screening in wildlife: A review. Veterinary World, 16(6), www.doi.org/10.14202/vetworld.2023.1193-1200

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Assessment and strategy development for SARS-CoV-2 screening in wildlife: A review

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2.1 Abstract

Coronaviruses (members of the Coronaviridae family) are prominent in veterinary medicine, with several known infectious agents commonly reported. In contrast, human medicine has disregarded coronaviruses for an extended period. Within the past two decades, coronaviruses have caused three major outbreaks. One such outbreak was the coronavirus disease 2019 (COVID-19) caused by the coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Over the 3-year COVID-19 outbreak, several instances of zooanthroponosis have been documented, which pose risks for virus modifications and possible re-emergence of the virus into the human population, causing a new epidemic, as well as possible threats for vaccination or treatment failure. Therefore, widespread screening of animals is an essential technique for mitigating future risks and repercussions. However, mass detection of SARS-CoV-2 in wild animals might be challenging. In silico prediction modeling, experimental studies conducted on various animal species, and natural infection episodes recorded in various species might provide information on the potential threats to wildlife and may be useful for diagnostic and mass screening purposes. In this review, the potential methods of wildlife screening, based on experimental data and environmental elements that might play a crucial role in its effective implementation, are reviewed.

Keywords: *angiotensin-converting enzyme 2, coronavirus disease 2019 in animals, severe acute respiratory syndrome coronavirus 2, wildlife surveillance.*

2.2 Introduction

With the increasing human population, climate change, and human interference in wildlife ecosystems over the past few decades, many emerging infectious diseases (EIDs) have developed. The ongoing coronavirus disease 2019 (COVID-19) pandemic is one of them. The novel zoonotic coronavirus, namely, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), belongs to the order Nidovirales, suborder Cornidovirineae, family Coronaviridae, subfamily Orthocoronavirinae, genus Betacoronavirus, and subgenus Sarbecovirus [1]. The causative agent of the ongoing pandemic in humans has also demonstrated the ability to infect different animal species [2].

Over the past two decades, three major epidemic and pandemic outbreaks have been reported as a result of viruses from this family, especially from the Betacoronavirus genus [3]. The first epidemic of SARS-CoV-1 was reported in Foshan, Guangdong, China, in 2001. Horseshoe bats, from the genus Rhinolophus, and palm civets have been identified as natural reservoirs for SARS-CoV-1 [4]. In 2012, a second outbreak in the Middle East was reported, caused by the Middle East respiratory syndrome coronavirus (MERS-CoV). According to the latest WHO report, it resulted in 2519 cases with 866 deaths [5]. Dromedary camels were identified as reservoirs for MERS-CoV [6]. In addition, one outbreak on pig farms was reported; swine enteric alphacoronavirus, or swine acute diarrhea syndrome coronavirus (SADS-CoV), was discovered in pig farms within Guangdong province, China, in 2017. It initially appeared as outbreaks of severe diarrhea in suckling piglets within four swine herds in a mountainous area of northern Guangdong [7]. Later, it reemerged in pig herds in Guangdong, starting in February 2019, and caused the mortality of about 2000 pigs [8]. Swine acute diarrhea syndrome coronavirus originated in bats, like other zoonotic viruses, including SARS-CoV and MERS-CoV [9].

Humans, domestic animals, wildlife, and the environment are linked by their different roles in transmitting and maintaining infectious agents [10]. Recent coronavirus outbreaks have increased the focus on disease surveillance and identification of other pathogenic organisms in wild animals. Wildlife disease surveillance will bring benefits to conservation efforts and the

monitoring, prevention, and control of zoonotic diseases. Increased wildlife disease surveillance and disease ecology modeling data were generated through the widespread application of molecular tools to expand the knowledge on different infectious agents and possible future EIDs. The concept of wildlife disease surveillance is similar to domestic animal health surveillance [11].

In this review, the available information on SARS-CoV-2 in wild animals was analyzed, as well as its implementation in the planning and preparing wildlife health surveillance efforts and specific pathogen surveillance.

2.3 Risk Assessment of SARS-CoV-2 Exposure in Free-ranging Wild Animals

Risk assessment of wildlife health includes assessing the hazard release from the source, the hazard exposure, and its consequences [12].

2.3.1 Source of SARS-CoV-2

Infectious SARS-CoV-2 is present in the respiratory secretions of infected humans, pet animals, captive wild animals, and production animals (e.g., minks). Humans could be a potential source of infection for free-ranging wild animals due to the high infection rates of SARS-CoV-2 in humans [13]. Severe acute respiratory syndrome coronavirus-2 was also discovered in the feces and urine of infected human patients [14, 15]. It has been observed that SARS-CoV-2 can survive on non-living substances such as plastic waste and masks. For instance, SARS-CoV-2 can survive for 21 days on plastic, 14 days on stainless steel, 7 days on nitrile gloves, and 4 days on chemical-resistant gloves [16]. A recent study reported multiple spillovers from humans and onward transmission of SARS-CoV-2 in white-tailed deer, which highlights an urgent need for a robust and responsive “One Health” approach to obtain an enhanced understanding of the ecology, molecular evolution, and dissemination of SARS-CoV-2 [17].

2.3.2 Exposure to SARS-CoV-2

The transmission of SARS-CoV-2 primarily occurs through respiratory droplets and airborne aerosols [18]. When in close contact with humans, cases of animal infection have been reported among pet animals, and zoo-kept wildlife [13]. Human waste can be the source of infections for wild animals, and free-living animals in the human population could be the potential linkage between humans and wild animals for SARS-CoV-2 infection. Handling, keeping, caring for,

and releasing wild animals may expose them to infections transmitted by infected handlers. Biologists, wildlife veterinarians, forest workers, and people living near protected areas could be the source of infections for animals.

2.3.3 Consequences of SARS-CoV-2 infection

The occurrence of SARS-CoV-2 infections in wild animals has an impact on animal, as well as human, health. Severe acute respiratory syndrome coronavirus-2 infections in wild animals impact the welfare and conservation of wild [19, 20]. In addition, it also impacts virus mutation once it crosses the species barrier [21]. Such mutations have been observed in mink infection cases [22]. There have been several cases reported worldwide of SARS-CoV-2 transfer from humans to minks. During the natural passage of this virus in minks, several mutations have been observed, mostly in spike protein S, the most important SARS-CoV-2 structural protein. These include Y453F, F486L, and N501T [23]. N501T has shown a greater ability to bind to mink angiotensin-converting enzyme 2 (ACE2), the SARS-CoV-2 receptor, and therefore leads to more effective use of mink ACE2 receptors for SARS-CoV-2 entrance [24]. The mutation Y486F occurred early in various mink outbreaks, and the mutations F486L and Q314K may co-occur, according to research [25]. This demonstrates that SARS-CoV-2 experiences a transient, but significant, increase in evolutionary pace in response to increased selection pressures during species jumps, which may result in mink-specific mutations [25]. Recent studies reveal the existence of five mutation sites typical of all early human-isolated SARS-CoV-2 Omicron variants. These mutations adapted the virus to infect mice, indicating that Omicron may have evolved in a mouse host [26].

2.4 Role of Surveillance in the Investigation of EIDs

The majority of EIDs originate from wildlife; they pose a zoonotic threat and often have a considerable impact on society [27]. To avoid future zoonotic outbreaks, it is essential to maintain the integrity of ecosystems together with other crucial measures, such as critical measures on wildlife trade and building proper surveillance systems around this trade. Monitoring and surveillance are important to the understanding of emerging epidemiological situations and should not only be used in response to disease threats and outbreaks but also when considering the risk of wild animal translocations. In the context of animal health, wildlife disease surveillance provides information about disease pattern, epidemiology, and

intensity, identifies changes in patterns of disease occurrence over time, and assists in the early detection of potential outbreaks, according to the World organization for Animal Health [28].

Over the past two decades, the growing frequency of outbreaks from the Coronaviridae family has increased pathogen-specific surveillance, which has resulted in the identification of some new viruses with zoonotic potential. The implication that bats could act as possible progenitors of emerging coronaviruses prompted global surveillance activities and resulted in the identification of different bat coronaviruses from other bat species with cross-species transmission events [29]. Moreover, after the SARS-CoV-1 outbreak, several animal coronaviruses related to HCoV229E, HCoVNL63, MERS-CoV, and SARS-CoV were found in different African countries [30].

Similarly, a 5-year surveillance program (from 2011 to 2015) carried out in a single cave inhabited by multiple species of horseshoe bats in Yunnan Province, China, revealed 15 severe acute respiratory syndrome-related coronavirus strains (11 novel ones and four that are known from the previous studies) [31]

2.5 Different Surveillance Strategies and their Implementation in the Current Pandemic

The World Organization for Animal Health defined surveillance in an epidemiological sense as the ongoing recording of disease in animal populations from the disease management perspective [11]. The first step of any disease surveillance program is to identify the goal(s). Once the system is established, it may vary depending on the desired outcome. Surveillance output can include the detection of new diseases, declaring a population free of specific diseases or infections, or identifying disease levels and distributions in the population [32].

Surveillance is mainly divided into two categories: active surveillance and passive surveillance. Active surveillance includes actively searching for particular diseases, while passive surveillance involves continuously searching for diseases on an ad hoc basis [33]. Passive surveillance includes vector surveillance, sentinel surveillance, serological surveillance, pathogen surveillance, and participatory surveillance. In comparison, active surveillance includes clinical investigation, syndromic surveillance, mortality investigation, and parameter monitoring [34]. Among these different surveillance modalities, described in **Table 2.1**, some have been found to be valuable for the current SARS-CoV-2 pandemic. During the current

pandemic situation, pathogen detection, or target surveillance, and serological surveillance are commonly implemented.

Clinical investigation can be conducted by observing clinical signs reported in natural infection cases and experimental infection demonstrations. Several clinical signs have been observed in different animals infected with SARS-CoV-2, summarized in **Table 2.2** [28, 35-47].

Table 2.1: Different surveillance modalities that can be useful for the current pandemic.

S. No.	Specific category	Description
1.	Pathogen determination	Search for a specific pathogen (or its antigens or nucleic acids)
2.	Serological determination	Search for antibodies against a specific pathogen
3.	Clinical investigation	Monitoring the clinical signs compatible with the disease(s)
4.	Parameter monitoring	Screening of biological indicators (e.g., food intake, fecal output, body weight, and animal activity)

2.6 Fundamental Challenges and Strategy Development for SARS-CoV-2 Mass Screening in Wild Animals

Mass screening could be implemented using different surveillance modalities such as pathogen determination, serological determination, clinical investigation, and parameter monitoring. However, with current pandemic situations and considerations, target pathogen detection and serological surveillance could be essential tools to use. For example, Jemeršić *et al.* [48] carried out serological surveillance and pathogen detection in free-living and captive animals during the first wave of COVID-19 in Croatia.

The mass screening of wild animals for SARS-CoV-2 is quite challenging in terms of budget, planning, preparation, and implementation of the strategy, and meeting the desired goals. In general, there are several challenges listed for wildlife surveillance. The unique challenges regarding wildlife disease surveillance are the detection of disease and pathogens in these animals. In wild animals, the signs of illness are often not obvious when diseased, especially

subclinical infections, and observation and/or access to dead animals are difficult due to the rapid removal by predators and scavengers [49]. In addition, the cost implications are also a big challenge for surveillance programs. Thus, it is important to regularly evaluate large-scale active surveillance programs to ensure that goals are being met. Figure 2.1 depicts the fundamental challenges of SARS-CoV-2 mass screening in wildlife, including sampling strategies, access to the investigatory material, laboratory analysis, and data interpretation.

Table 2.2: Common clinical signs observed in different species with SARS-CoV-2 infection based on data from the world organization for animal health.

Animal species	**Observed clinical signs**	**References**
Cat	Anorexia, sneezing, acute dyspnea, rattle, snoring, nasal secretion, severe respiratory failure, lethargy, breathing difficulties, and digestive signs	[28, 35]
Dog	Conjunctivitis, cough, rhinitis, dyspnea and weakening, high respiratory distress and apathy, nasal discharge and fever, febrile peaks, anorexia, abnormal lung sounds, pharyngitis, bronchitis, lymphadenomegaly, and positive palmopercussion	[36, 37]
Mink	Respiratory symptoms, high mortality & anorexia	[38]
Lion	Mild-to-moderate symptoms in the upper respiratory tract (serous nasal discharge, sneezing, and coughing	[28, 39]
Puma	Anorexia	[40]
Hyenas	Extremely mild symptoms, including slight lethargy, some nasal discharge, and occasional coughs	[28]
Ferret	Clinical signs of gastrointestinal tract (GIT)	[41]

Snow leopard	Coughing and some wheezing	[42]
Gorilla	Tiredness, dry cough, and loss of appetite	[43]
Amur leopard cat	Serous and bloody nasal discharge and rhinitis	[28,44]
Malayan tigers	Growl and wheeze, followed by coughing, nasal discharge, lethargy, and loss of appetite	[28,45]
Sumatran tiger	Growl and wheeze, followed by coughing, nasal discharge, lethargy, and loss of appetite	[28, 46]
Hippopotamus	Mild symptom like nasal discharge	[47]

2.7 Sampling strategies

During targeted surveillance or pathogen-specific surveillance, studies are conducted in which statistical inferences about the population of interest are very limited [50]. This is usually caused by many factors, for example, limited numbers of sampled individuals since most of the sampling is opportunistic and large sampling campaigns can be too expensive to perform. Then, sampling can be very complicated or impossible due to either laws and regulations or practical issues, as these animals can be too difficult to trap and handle. During targeted surveillance studies, a cohort of the population of interest is targeted based on a high risk for exposure and susceptibility rates [11]. These studies may focus on populations of animals that seem to be in good health conditions [50]. Regarding SARS-CoV-2 virus detection in wild animals, target species populations can be divided into three groups based on previous known natural infection events, experimental studies, and in silico studies: high-risk susceptibility (or first target group), medium-risk (or second target group), and low-risk (or third target group).

The viral spike proteins (S) are the primary determinant of the host cell [51]. They play a key role in the attachment process to the host cell-surface receptor, ACE2 protein, during host cell entry [52]. There are several mammalian species that conserve these protein sequences. Based on the presence of ACE2 receptors, it is possible to predict the permissive animal species for natural infection with SARS-CoV-2. The transmembrane serine protease-2 also plays a key role in the attachment of the virus to the host cell [53]. However, in silico studies are limited to host

cell entrance, and replication may also depend on numerous other variables, such as proteases CTSL (Cathepsin L) and ADAM-17 (a disintegrin and metalloprotease domain) [54]. The expression of ACE2 proteins in different species not only indicates the possibilities of natural infection but also shows host entry and the involvement of different tissue types, as well as the clinical expression of the disease, which were revealed by studies with COVID-19 human patients [55]. Based on these bioinformatic studies, Alexander *et al.* [56] identified five animal species that are highly susceptible to SARS-CoV-2 infections, including the Rhesus macaque, house cat, tiger, lion, and golden Syrian hamster.

Since the beginning of the pandemic, several animal species have been found to be susceptible to infection, which supports the in silico findings. For instance, the exposure of SARS-CoV-2 in white-tailed deer was demonstrated by serosurveillance [57], which supported the in silico modeling data [58]. Therefore, based on high-risk susceptibility of these animals, as shown through in silico findings, experimental infection results, and some natural infection cases, animals such as while-tailed deer could be the first target animal population for pathogen-specific surveillance or serosurveillance. On the other hand, animal species that are identified as high-risk regarding susceptibility based on in silico findings, but no natural infection events or experimental infection cases are recorded yet, fall under the second target animal population.

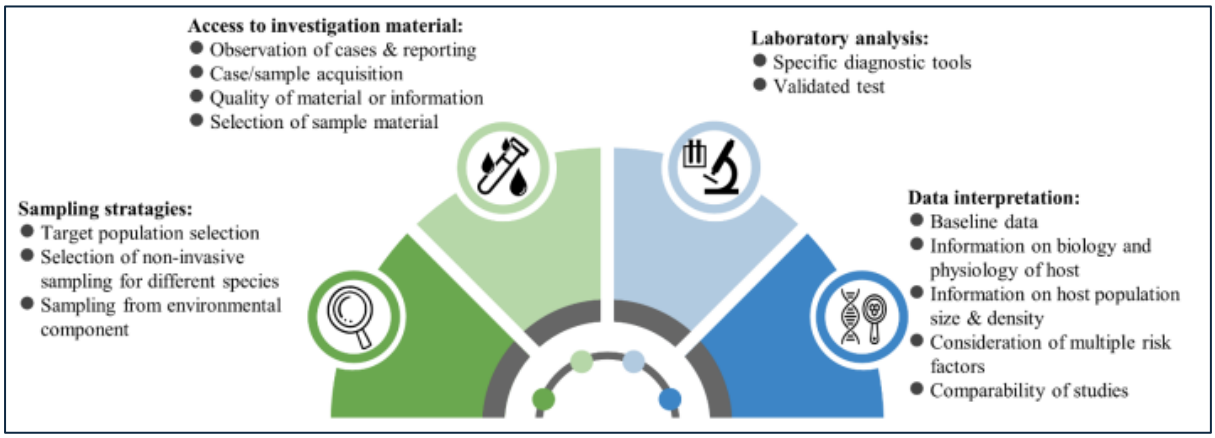


Figure 2.1: Fundamental challenges of severe acute respiratory syndrome coronavirus-2 screening in wildlife.

2.8 Access to investigation material

Sampling methods are primarily selected based on the chosen surveillance modalities. It also includes a stratified random sampling of the population of interest. During stratified random sampling, a subunit of the population is sampled based on known risk factors [59]. A sample

can be collected opportunistically during routine operations, or animals can be handled and captured for sampling purposes. Among the invasive and non-invasive methods of sampling, non-invasive sampling methods are always preferred in wildlife surveillance [60]

Sample selection for surveillance also depends on the chosen analysis strategy and targeted virus tissue tropism in different animal species. Depending on the expression of ACE2 receptors in different tissues of different animals, the susceptibility of infection and its clinical manifestation vary [58]. Based on that, the clinical outcome of the disease and sampling strategies can be determined. For example, SARS-CoV-2 was detected in rectal swabs from infected ferrets and dogs [61]. Thus, non-invasive samples were also selected as investigatory materials based on tissue tropism and experimental studies. In Figure 2.2 [21], the expression of the ACE2 gene in different tissues of different species has been demonstrated. Aguiló-Gisbert et al., 2021 [19] detected SARS-CoV-2 in 2 of 13 feral dark brown American minks (Neovison vison) trapped in the Valencian community (Eastern Spain) during an invasive species trapping campaign. The virus was found in mesenteric lymph nodes of animals. Sampling dead animals could also be an option; however, scavengers can remove them rapidly, as mentioned.

In terms of environmental sampling, it is critical to collect samples from common water sources for wildlife as well as from human waste in the local ecosystem because it has been discovered that infected human waste can contaminate the local ecosystem and serve as a source of infection [20].

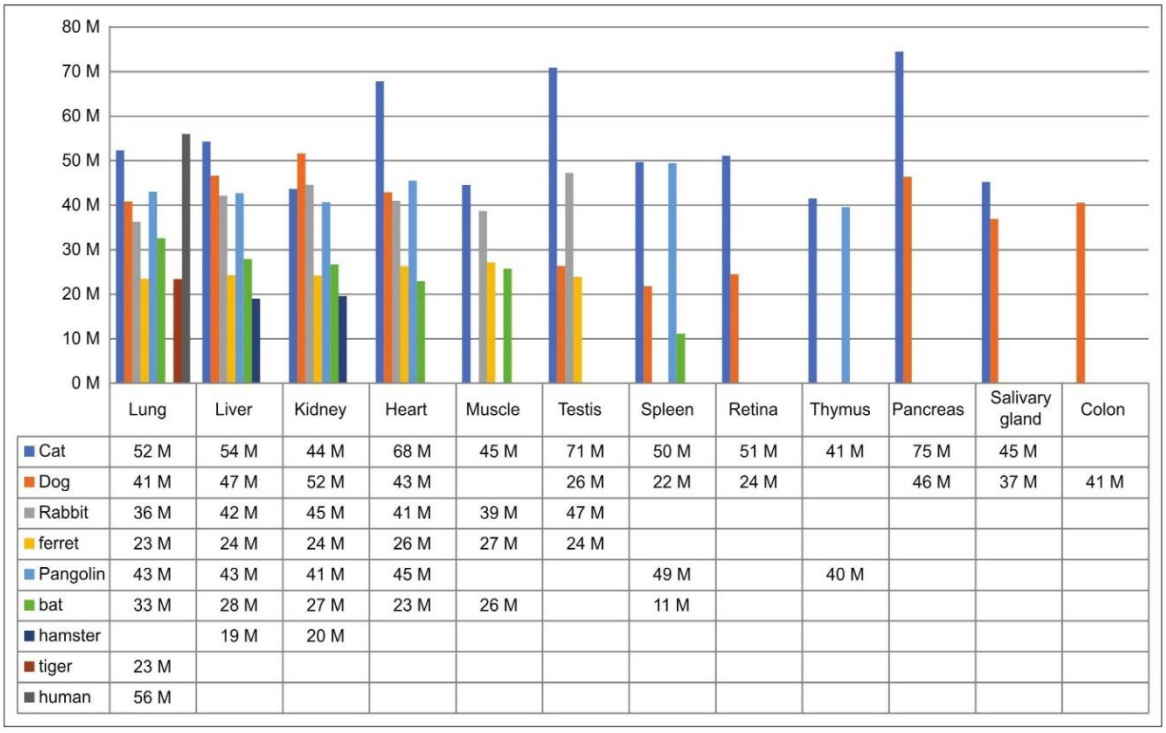
2.9 Laboratory analysis and data interpretation

Since the beginning of the pandemic, several diagnostic tests have been developed. The diagnostic assay includes virus culture, nucleic acid testing assays, and immunological assays. Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is one of the best methods for detecting SARS-CoV-2 RNA [62]. However, loop-mediated isothermal amplification could serve as an alternative method to RT-qPCR to detect SARS-CoV-2 RNA. This method can be used without the need of specialized equipment and trained analysts [63].

There has also been an immunological assay enzyme-linked immunosorbent assay (ELISA) methodology developed to diagnose the presence of antibodies against SARS-CoV-2 in animals. For example, Wernike et al. 2021 developed an indirect multispecies ELISA based on

the receptor-binding domain for ferrets, raccoon dogs, hamsters, rabbits, chickens, cattle, and cats [64]. Serological surveillance (using commercial ELISA kit) revealed the presence of antibodies against SARS-CoV-2 in sheep and goats, confirmed by a virus neutralization test [65].

Together with the development and validation of an assay, data interpretation also plays a crucial role. For serological assays, cross-immunity against similar virus antigens is the major drawback. Following the detection of virus nucleic acid, it is critical to perform sequencing to identify novel changes or mutations in the virus genome to overcome its future consequences. Further, actions should be taken based on the achieved results, For example, several mass culling of minks were carried out after the identification of infection spillover and mutation [66].



	Lung	Liver	Kidney	Heart	Muscle	Testis	Spleen	Retina	Thymus	Pancreas	Salivary gland	Colon
Cat	52 M	54 M	44 M	68 M	45 M	71 M	50 M	51 M	41 M	75 M	45 M	
Dog	41 M	47 M	52 M	43 M		26 M	22 M	24 M		46 M	37 M	41 M
Rabbit	36 M	42 M	45 M	41 M	39 M	47 M						
ferret	23 M	24 M	24 M	26 M	27 M	24 M						
Pangolin	43 M	43 M	41 M	45 M			49 M		40 M			
bat	33 M	28 M	27 M	23 M	26 M		11 M					
hamster		19 M	20 M									
tiger	23 M											
human	56 M											

Figure 2.2: Expression of the angiotensin-converting enzyme 2 gene in different tissues of different species (original transcripts per kilobase of exon model per million mapped reads). The bar graph was prepared based on data from [21].

2.10 Conclusion

To develop strategies and identify challenges for SARS-CoV-2 screening, the current knowledge of SARS-CoV-2 infection in animals plays a significant role. Continued assessment of the risk of SARS-CoV-2 infection in animals aids in breaking the link between virus exposure and wild-living animals. Natural infection cases reported in different zoos worldwide provide baseline data on the severity of infections and virus biology in wild animals. Collective data from various sources, such as in silico studies, experimental infection case studies, and natural infection, aid in the development of mass wildlife screening strategies and the resolution of challenges.

In the future, continued upgrading of knowledge and identification of new animal hosts susceptible to SARS-CoV-2 infection during the current pandemic situation will help to modify disease surveillance strategies in wildlife.

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Competing Interests

The authors declare that they have no competing interests.

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CHAPTER 3

First Detection of SARS-CoV-2 in White Rhinoceros during a Small-Scale Coronavirus Surveillance in the Bandia Reserve, Senegal

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First Detection of SARS-CoV-2 in White Rhinoceros during a Small-Scale Coronavirus Surveillance in the Bandia Reserve, Senegal

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Simple Summary

This study focuses on the molecular surveillance of coronaviruses in wildlife in Senegal. Fecal samples were collected from various species of wild animals, both in the Bandia Reserve and Ngaparou. The results revealed the absence of coronaviruses in hedgehogs, non-human primates, and a giraffe. However, a positive sample obtained from a white rhinoceros yielded SARS-CoV-2 through sequencing of the RdRp gene. This finding represents the first documented case of molecular detection of SARS-CoV-2 in white rhinoceros, expanding our understanding of potential hosts of the virus. This finding expands our understanding of potential hosts of SARS-CoV-2 and highlights the importance of using wildlife monitoring to improve coronavirus surveillance.

3.1 Abstract

The SARS-CoV-2 pandemic has heightened interest in the monitoring and surveillance of coronaviruses in wildlife. Testing for the virus in animals can provide valuable insights into viral reservoirs, transmission, and pathogenesis. In this study, we present the results of the molecular surveillance project focused on coronaviruses in Senegalese wildlife. During the project, we screened fecal samples of the wild animals living in the Bandia Reserve (ten non-human primates, one giraffe, and two white rhinoceros) and the free-living urban population of African four-toed hedgehogs in Ngaparou. The results showed the absence of coronaviruses in hedgehogs, non-human primates, and a giraffe. A single positive sample was obtained from a white rhinoceros. The sequencing results of amplified RdRp gene confirmed that the detected virus was SARS-CoV-2. This study represents the first documented instance of molecular detection of SARS-CoV-2 in white rhinoceros and, therefore, extends our knowledge of possible SARS-CoV-2 hosts.

Keywords: *SARS CoV-2; coronaviruses; wildlife surveillance; molecular detection*

3.2 Introduction:

Coronaviruses (CoVs) infect a wide range of animal species, having a particular affinity for their respiratory and intestinal systems. The severity of infections caused by these viruses can vary greatly, ranging from asymptomatic cases to fatal outcomes [1]. Coronaviruses (*Coronaviridae* family, *Orthocoronavirinae* subfamily) have a single-stranded positive-sense RNA genome (+ssRNA), which is the largest among all known viruses, spanning a length of 25 to 33 kilobases [2]. The genomic RNA of CoVs is capable of func-tioning as an mRNA and is considered infectious in its purified form [1]. The subfamily *Orthocoronavirinae* is divided into four genera (*Alphacoronavirus, Betacoronavirus, Gammacoronavirus,* and *Deltacoronavirus*), and SARS-CoV-2 belong to the genus *betacoronavirus*. While bats (*Chiroptera*) have long been recognized as the primary reservoir of CoVs, various other mammalian species also harbor specific CoVs. Hedgehogs, in particular, exhibit a high susceptibility to CoVs, as evidenced by documented occurrences of MERS-CoV-related CoVs

in several European countries, including France, Germany, Italy, the United Kingdom, and Poland [3-5]. Furthermore, research has identified the possibility of coronavirus infections in non-human primates. For instance, wild chimpanzees in Côte d'Ivoire [6] and native hamadryas baboons (Papio hamadryas hamadryas) in Saudi Arabia have been found to be susceptible to these viruses [7].

In Africa, surveillance efforts to detect coronavirus nucleic acid in non-bat wildlife, livestock, and domestic animals have been limited. Several surveillance studies have been conducted, including the investigation of human coronavirus OC43 (HCoV-OC43) transmission between humans and chimpanzees in Côte d'Ivoire, MERS-CoV-specific monitoring in livestock animals in Ghana, and general surveillance among wild animals in Gabon [8]. The overall proportion of positive coronavirus RNA detected in these studies was less than 1%. The findings include positive cases identified in non-human primate (14 chimpanzees), ungulate (1 bush duiker), carnivore (1 African palm civet), and rodent species (13 individuals) [8].

Over the past two decades, the Coronaviridae family has been associated with three significant epidemic and pandemic outbreaks, which were primarily attributed to the betacoronavirus genus. The most recent and notable of these outbreaks was the COVID-19 in humans caused by SARS-CoV-2, which has shed light on the potential for reversezoonosis, wherein viruses can transmit from humans to animals. Over the past three years, several instances of symptomatic and asymptomatic natural SARS-CoV-2 infection have been reported in various animal species, further emphasizing the potential for interspecies transmission [9, 10]. Molecular and serological diagnostic methodologies are frequently used to detect SARS-CoV-2 infections in animal and human populations. Various laboratory techniques are commonly employed to characterize strains implicated in outbreaks, such as RT-PCR, RT-LAMP, virus isolation, and sequencing, including next-generation sequencing [11]. Several cases of active SARS-CoV-2 infections have been identified in zoos, free-ranging wild animals, and domestic animals through pathogen-specific surveillance studies [9]. There have been documented instances of lions, tigers, cougars, leopards, lynx, otters, coati, giant anteater, binturong, and gorillas being confirmed to be positive forSARS-CoV-2 using PCR and genetic-sequencing techniques [9, 12-16]. The virus neutralization test (VNT), the surrogate virus neutralization test (sVNT), and the enzyme linked immune sorbent assay (ELISA) have been used to detect previous exposure to SARS-CoV-2by evaluating antibody immune responses. In order to detect antibodies against nucleoprotein (N), a commercial double-antigen poly specific ELISA has

been used for all susceptible animal species, demonstrating high sensitivity [17]. For instance, in July 2021, a study revealed that 40% of free-ranging white-tailed deer (Odocoileus virginianus) tested positive for antibodies against SARS-CoV-2 in four US states. This finding identified white-tailed deer as a wildlife host of the disease, providing evidence of their susceptibility to SARS-CoV-2 infection [18]. Additionally, a case of natural SARS-CoV-2 infection was recorded in a free-range black-tailed marmoset (Mico melanurus) studied in an urban area in the Central-West region of Brazil, highlighting the occurrence of the virus in non-human primate populations[19]. The study conducted in the Campania region of Italy revealed the existence of serological evidence indicating SARS-CoV-2 infection in lactating cows. However, the investigation did not detect the existence of neutralizing antibodies against bovine coronavirus (BCoV) [20].

These examples highlight the significance of understanding the potential role of various animal species in the transmission and maintenance of CoVs, including SARS-CoV-2. Monitoring and surveillance efforts across diverse wildlife populations are vital to the identification of potential reservoirs, the assessment of the risk of zoonotic transmission, and the implementation of appropriate preventive measures to mitigate future outbreaks.

This study aimed to investigate the potential presence of CoVs in two distinct populations: the fauna of the Bandia Reserve and the free-living four-toed hedgehogs (*Atelerix albiventris*) in Ngaparou town. The rationale behind this investigation stems from our university's ongoing long-term monitoring project, which focuses on studying the wildlife inhabiting these specific areas.

3.3 Materials and Methods

3.3.1. Sample Collection and Study Region

This study focused on two specific sites in western Senegal: The Bandia Reserve and the coastal town of Ngaparou (Figure 1).

The Bandia Reserve, which spans an area of 3500 hectares and is located 65 km from Dakar, was established in 1990 with the aim of conserving wildlife. The reserve boasts a diverse ecosystem, harboring more than 120 bird species and 18 large animal species, both native and

non-native to Senegal. Ngaparou, on the other hand, is a coastal town situated 75 km south of Dakar and located 33 km away from the Bandia Reserve [21].

In May 2022, fresh animal fecal samples were collected from both the Bandia Reserve and Ngaparou. Twenty hedgehogs were captured in Ngaparou in order to obtain fecal samples. Subsequently, the captured animals were released back into their original habitat. In the Bandia Reserve, ten samples were collected randomly from non-human primates inhabiting the area. The reserve is home to two distinct species of non-human primates: patas monkeys (*Erythrocebus patas*) and green vervet monkeys (*Chlorocebus pygerythrus*), which coexist within the same habitat. Due to the similarities in the dimensions and morphology of their fecal matter, it was challenging to differentiate the source species or individual based on the collected samples. Additionally, observations indicated that these two species often reside in the same social groups, further complicating the identification process.

Furthermore, three fresh fecal specimens were obtained from the Bandia Reserve, consisting of two samples from a white rhinoceros (*Ceratotherium simum*) and one sample from a giraffe (*Giraffa camelopardalis*). To ensure sample integrity, all collected samples were promptly stored on ice and processed on the day of collection.

3.3.2. Nucleic Acid Extraction and RT-PCR

The field-based RNA extraction process was conducted using the Quick-DNA/RNA Viral MagBead kit (Zymo Research, Irvine, CA, USA). This kit utilizes magnetic bead-based techniques that do not require centrifugation, enabling the extraction of RNA from freshly collected fecal samples in the field. A total of 33 samples (20 samples from four-toed hedgehogs, 10 samples from non-human primates, 2 samples from white rhinoceros, and 1 sample from a giraffe) were subjected to RNA analysis using RT-PCR. The one-step RT-PCR kit (QIAGEN, Germantown, MD, USA) and the portable miniPCR® mini8 thermal cycler (miniPCR, Cambridge, MA, USA) were employed for this purpose.

The RT-PCR system consisted of a 25-microliter reaction volume containing the following components: 5 µL of 5× QIAGEN OneStep RT-PCR buffer, 1 µL of dNTP (resulting in a final concentration of 400 µM for each dNTP), 1 µL each of upstream and downstream primers (at a concentration of 25 µmol/L), 0.25 µL of RNAsin (at a concentration of 40 µ/µL), 1 µL of enzyme mix, and 2 µL of RNA template; the remaining volume was filled with RNase-free

water to reach a total volume of 25 µL. Two distinct sets of primers were used to selectively amplify specific regions within the RNA-dependent RNA polymerase (RdRP) gene, which is a highly conserved gene among coronaviruses. The first primer set consisted of forward (5'-AARTTYTAYGGHGGYTGG-3') and reverse (5'-GARCARAATTCATGHGGDCC-3') primers targeting a 668-base pair fragment of the polymerase gene. The experiment commenced via an initial reverse transcription process at a temperature of 50 ◦C for a period of 30 min. This step was followed by PCR activation at 95 ◦C for 15 min. The amplification phase consisted of 35 cycles, each involving 40 s at 94 ◦C, 40 s at 52 ◦C, and 1 min at 72 ◦C. Finally, a final extension step was carried out at 72 ◦C for 10 min, as described by Hu et al. [22]. Similarly, the second primer set consisted of forward (5'-GGGDTGGGAYTAYCCHAARTGYGA-3') and reverse (5'-TARCAVACAACISYRTCRTCA-3') primers targeting a 452-base pair fragment of the polymerase gene. The experiment commenced via an initial reverse transcription process at a temperature of 50 ◦C for a period of 30 min. This step was followed by PCR activation at 95 ◦C for 15 min. The amplification phase consisted of 35 cycles, each involving 40 s at 94 ◦C, 40 s at 50 ◦C, and 1 min at 72 ◦C. Finally, a final extension step was carried out at 72 ◦C for 10 min, as described by Hasoksuz M et al. [23].

The RT-PCR products were visualized using a portable electrophoresis system BlueGel™ (miniPCR, USA). To ensure the accuracy of the results, the RT-PCR screening conducted in the field did not incorporate a positive control to mitigate the risk of false positives resulting from cross-contamination. However, the effectiveness of the RT-PCR reactions in producing positive results using positive control samples was separately evaluated in a laboratory setting at the standard university laboratory. This evaluation was carried out before the commencement of the in-field experiment, ensuring the reliability of the in-field RT-PCR screening process.

3.3.3. Cloning and Sequencing of RT-PCR Products

The amplified products of RT-PCR positive samples were sent to the Center for Infectious Animal Diseases (FTZ) in Prague for sequencing in order to circumvent any potential legal complications associated with the transfer of biological specimens. Prior to sequencing, these transported amplified products underwent a cloning process in the pJET vector to enhance the quality of the resulting sequences.

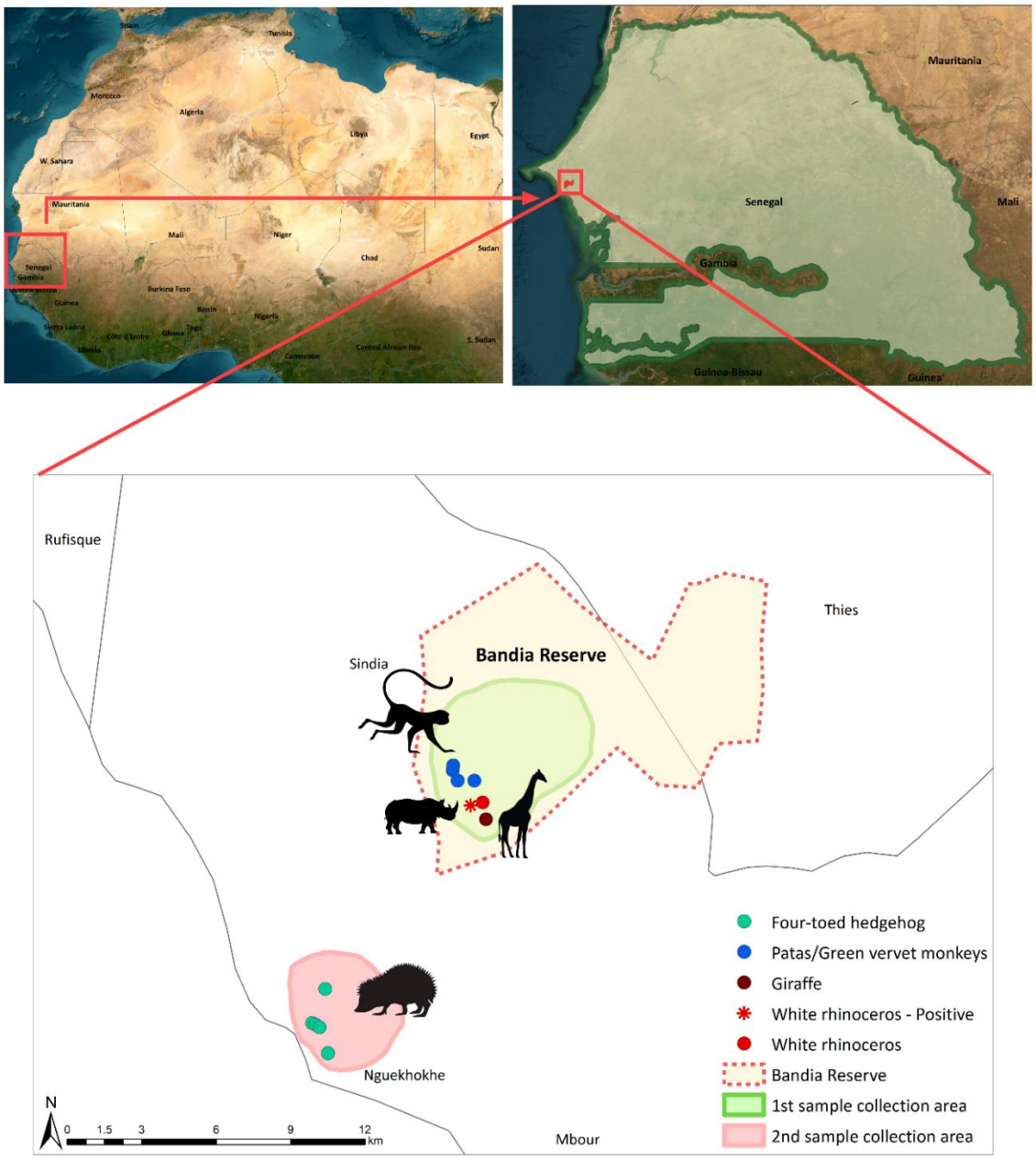

Figure 3.1. Map of the location and sampling points of the study area. The sampling locations of the four-toed hedgehog, patas and green vervet monkeys, giraffe, and rhinoceros are depicted using green, blue, dark red, and red circles, respectively. The red asterisk highlights the positive sample derived from a white rhinoceros. The green background depicts the sampling site inside of the Bandia Reserve, where fresh fecal samples of patas and green vervet monkeys, giraffe, and white rhinoceros were collected. The light red area shows the locations in Ngaparou at which fresh fecal samples of the four-toed hedgehog were collected. The boundaries of the Bandia Reserve are represented using a red dotted line. The figure was generated using ArcMap 10.8.2.

The amplification product was treated to create blunt ends and then ligated into the pJET1.2/blunt vector. Subsequently, Sanger sequencing was performed on the resulting plasmid using two plasmid-specific primers provided by the pJET2.1 vector: the forward sequencing primer (5'-d(CGACTCACTATAGGGAGAGCGGC)-3') and the reverse sequencing primer (5'-d(AAGAACATCGATTTTCCATGGCAG)-3'). The obtained sequence data were analyzed using the Geneious software (Version 2022.2) and compared to existing sequences in the GenBankTM dataset via basic local alignment tool (BLAST) analysis [24].

3.4. Results and Discussion

SARS-CoV-2, which is a coronavirus initially identified in Wuhan, China, in late 2019, has rapidly spread worldwide, leading to the COVID-19 pandemic [25]. Since the emergence of COVID-19 pandemic, numerous cases of SARS-CoV-2 infection in animals have been reported [26]. Observational and experimental studies on a range of non-human mammalian species, including free-living, captive, domestic, and farmed animals, have identified at least 54 species susceptible to the virus [27].

In order to monitor the potential presence of SARS-CoV-2 in animals, extensive surveillance programs have been implemented. For instance, a study conducted from January to March 2021 focused on monitoring free-ranging white-tailed deer in Northeast Ohio, revealing their vulnerability to COVID-19 through real-time RT-PCR testing [14]. Furthermore, in India, SARS-CoV-2 was detected in a free-ranging leopard (*Panthera pardus fusca*) and cases of natural infection in captive wild animals in zoos are well documented [28, 29]. These instances emphasize the need for comprehensive risk analysis to evaluate the potential transmission of the virus from animals to humans. Additionally, continuous surveillance is crucial to gain a deeper understanding of the role that animals play in the spread of the virus.

In this study, we identified a positive case of coronavirus infection in a white rhinoceros using the RT-PCR assay. Subsequent sequencing of a short fragment of the RdRp gene confirmed the presence of SARS-CoV-2 in the rhinoceros' sample. Comparisons between the rhinoceros host cell entry receptor ACE2 and its human counterpart ACE2 revealed homology, suggesting the potential for SARS-CoV-2 infection in rhinoceros [30]. However, it is important to note that in silico studies solely focusing on host cell entry may have limitations, as successful viral replication could also rely on various other factors, such as tissue proteases TMPRSS2, CTSL, or ADAM-17 [31]. Further investigations are required to fully understand the susceptibility and

implications of SARS-CoV-2 infection in rhinoceros and its potential role in the transmission dynamics of the virus.

The results identified in the Bandia Reserve, which is a semi-enclosed wildlife sanctuary known for tourism, raise concerns about the potential transmission of infections to animals. This concern is primarily due to the possibility of direct or indirect human interaction with wild animals through activities such as providing feed, which is a common practice, or engaging in wildlife safari tours. It is important to consider that the fresh fecal sample collected from the white rhinoceros may also be influenced by environmental contaminants.

Currently, our understanding of SARS-CoV-2 biology in rhinoceroses is limited, underscoring the need to continue surveillance studies in rhinoceros populations to identify any similar occurrences and potential spillover events. In our investigation, we found no evidence of coronaviruses in the four-toed hedgehog from Ngaparou, as well as in nonhuman primates and giraffes from the Bandia Reserve. It is worth noting that throughout the period of sample collection in May 2022, the prevalence of SARS-CoV-2 in the human population was minimal, with most instances ranging from zero to a maximum of twelve cases [32].

This study, to our best knowledge, represents the first documented instance of molecular detection of SARS-CoV-2 in white rhinoceros, but it does have a few limitations. Firstly, it is important to note that the investigation was carried out on a limited number of samples, thereby limiting the generalizability of the findings to the entire populations of four-toed hedgehogs, patas and green vervet monkeys, and giraffes. Consequently, it is not possible to definitively conclude that these animal populations were entirely free of the CoVs. The primary objective of the study was to assess the prevalence of the CoVs in animals; therefore, the use of specific primers targeting SARS-CoV-2 during the fieldwork was not prioritized. Instead, the focus was on detecting the presence of coronaviruses or bovine-like coronaviruses in general. In such cases, sequencing of the RT-PCR amplicon alone would have been sufficient to identify and differentiate the viral presence. Furthermore, the field conditions presented logistical challenges, as we lacked deep freezers for long-term storage of virus RNA. Consequently, we opted to transport the more stable DNA amplicon for sequencing, as opposed to the relatively unstable virus RNA. Subsequently, to circumvent any potential legal complications associated with transporting biological samples, only the PCR products were transported to the Czech Republic for sequencing. This decision resulted in our inability to perform amplification of the spike (S) or receptor-binding domain (RBD) genes for the purpose of identifying variants.

Another limitation of this study is that we cannot definitively rule out the possibility of the passive transit of the virus through the digestive system. Our sampling methodology aimed to minimize the likelihood of detecting virus remnants from passive transit. By directly collecting fresh fecal samples from the animals in their natural habitats, we aimed to capture active shedding of the virus, which would indicate an active infection rather than passive transit. Future research should address these limitations to gain a comprehensive understanding of the status of coronaviruses in the studied animal populations.

3.5. Conclusions

In conclusion, this study sheds light on the presence of coronaviruses in wildlife populations in Senegal, specifically in the Bandia Reserve and Ngaparou. While no coronaviruses were detected in four-toed hedgehogs, non-human primates, and a giraffe, the molecular surveillance revealed the presence of SARS-CoV-2 in a white rhinoceros. This finding expands our understanding of potential hosts of SARS-CoV-2 and highlights the importance of wildlife monitoring for coronavirus surveillance. To obtain a comprehensive understanding of the prevalence, transmission, and impact of coronaviruses in wildlife populations, as well as to elucidate the dynamics of viral spillover events, further research and enhanced surveillance measures are warranted.

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CHAPTER 4

Serological Screening of SARS-CoV-2 Infection in Several Mammalian Species in Wilhelma Zoo, Stuttgart, Germany

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Serological Screening of SARS-CoV-2 Infection in Several Mammalian Species in Wilhelma Zoo, Stuttgart, Germany

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4.1. Abstract

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) affects both humans and a wide range of mammalian species globally. Between July 2022 and January 2023, fifteen blood samples were collected from twelve different animal species during veterinary examinations, as well as for health control at Wilhelma Zoo, Germany. These samples were later analyzed for the presence of SARS-CoV-2 antibodies. The serum analysis from two gorillas indicated the presence of antibodies specific to the nucleocapsid protein of SARS-CoV-2, suggesting previous infection. These gorillas were sampled in August and September 2022, during which time they exhibited symptoms such as apathy, anorexia, vomiting, and moderate diarrhea—symptoms not typically associated with COVID-19. Given that several periods of other unusual signs have been observed in the gorillas kept in Wilhelma Zoo since the onset of the COVID-19 pandemic, it remains uncertain whether these symptoms were directly related to SARS-CoV-2 infection or if these gorillas underwent clinically inapparent infection before. Nonetheless, this study underscores the importance of ongoing animal screening in zoos to better understand the spread of SARS-CoV-2 among different animal species.

Keywords: *COVID-19; zoo animals; western lowland gorillas; serological surveillance*

4.2. Introduction

The COVID-19 pandemic, caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), is believed to have originated from bats and subsequently transmitted to humans through an intermediary animal host [1]. Globally, both symptomatic and asymptomatic cases of SARS-CoV-2 infection in animals have been reported in various zoos and among free-living wildlife [2, 3]. Further, reverse zoonotic spillovers from SARS-CoV-2-infected animals to humans have also been reported [4].

Notably, wild felines have shown high susceptibility to life-threatening infections of SARS-CoV-2 [5, 6, 7]. The in-silico analysis has identified Old World primates as highly susceptible to SARS-CoV-2 infection due to the similarity between their ACE2 receptor and that of humans [8]. Despite this expected susceptibility, natural infections have so far been observed only in gorillas, and their prevalence is relatively low compared to those in felids [3]. In captivity, SARS-CoV-2-infected animals have exhibited a range of clinical signs, such as cough, nasal discharge, and behavior changes like reduced appetite and lethargy. Captive western lowland gorillas are reported to display diverse clinical signs, including fever, coughing, and lethargy.

Zoos play a very important role in public health by enrolling standardized epidemiological surveillance of their zoological collections [9]. Investigating the SARS-CoV-2transmission among various zoo species helps to identify potential virus reservoirs within wildlife populations. This study specifically examines the potential SARS-CoV-2 infection in symptomatic western lowland gorillas (*Gorilla gorilla gorilla*) and other asymptomatic mammals across different species by sampling and screening their sera.

4.3. Methods

A total of fifteen blood samples (5 mL, clotted) were collected from twelve distinct species across seven families between July 2022 and January 2023 by zoo veterinarians. Given that blood collection in zoo animals is known to be a stressful procedure, these samples were obtained during routine veterinary examinations rather than for the explicit purpose of testing for SARS-CoV-2 antibodies.

Serum was extracted from all fifteen samples. Samples were collected in VACUETTE® TUBE 5 ml CAT Serum Separator Clot Activator (Greiner Bio-One GmbH, Frickenhausen, Germany).

To obtain serum, blood was allowed to clot at room temperature for at least 40 minutes before centrifugation at 3400 RPM for ten minutes. The detection of SARS-CoV-2 antibodies in the serum specimens was performed using the ID Screen® SARS-CoV-2 Double Antigen Multi-species ELISA kit (ID VET, Montpellier, France). This double antigen enzyme-linked immunosorbent assay (ELISA) protocol aimed to identify immunoglobulin G (IgG) antibodies specific to the nucleocapsid protein of SARS-CoV-2 in the serum samples. The ELISA assays were conducted in accordance with the instructions provided by the manufacturer. The optical densities (ODs) were measured at a wavelength of 450 nm. The optical density (OD) of each sample was determined by calculating the proportion of signal to background (S/P%). According to the manufacturer's instructions, serum samples with S/P% values exceeding 60% were categorized as positive.

4.4. Results and Discussion

Except for two samples derived from gorillas, all the other samples tested negative in the ELISA assay, as detailed in Table 1. These gorillas had not been vaccinated against SARS-CoV-2.

Table 4.1. List of animal species used in this study: highlighted samples denote positives in ELISA assay (S/P% > 60); name of the individual animal (if given) is in brackets.

Animal Family	**Animal Species**	**Scientific Name**	**Date of sample collection**	**S/P%**
Equidae	Shetland Pony	*Equus caballus caballus*	26 July 2022	19.22679
Suidae	Kunekune Pig	*Sus scrofa domesticus*	1 August 2022	-7.27843
Bovidae	Alpine Ibex	*Capra ibex*	13 August 2022	-8.11235

Hominidae	**Western lowland gorilla (Tuana)**	***Gorilla gorilla gorilla***	**16 August 2022**	**719.3502**
Bovidae	Border Leicester	*Ovis aries*	23 August 2022	20.60776
Bovidae	Scimitar Oryx	*Oryx dammah*	24 August 2022	-8.57934
Hominidae	**Western lowland gorilla (Undi)**	***Gorilla gorilla gorilla***	**22 September 2022**	**84.59922**
Felidae	Asiatic Lion	*Panthera leo persica*	23 September 2022	-5.07022
Equidae	Shetland Pony	*Equus caballus caballus*	27 September 2022	6.911505
Hominidae	Bonobo	*Pan paniscus*	19 November 2022	-21.2615
Felidae	Snow Leopard	*Panthera uncia*	22 November 2022	-10.6208
Bovidae	Domestic Yak	*Bos grunniens*	23 November 2022	-17.152
Bovidae	Domestic Yak (Sonam)	*Bos grunniens*	30 November 2022	1.441009
Cervidae	Milu	*Elaphurus davidianus*	5 January 2023	8.08566
Equidae	Somali Wild Ass	*Equus africanus somaliensis*	17 January 2023	-10.1271

The western lowland gorilla population at Wilhelma Zoo consists of eleven individuals: six females and five males. The examined blood samples were collected between August and September 2022, when two female gorillas exhibited apathy and multiple gastrointestinal

symptoms. Undi, a 51-year-old female, displayed clinical symptoms including anorexia, signs of fever, lameness, and stiffness in her movements. Tuana, a 17-year-old, experienced milder symptoms. Due to the severity of these clinical manifestations, the zoo veterinarians sedated the animals to perform examinations, provide medical care, and collect blood samples. It took Undi approximately six weeks and multiple treatments to fully recover. Tuana recovered within a few days.

Since the onset of the COVID-19 pandemic, gorillas at Wilhelma Zoo have displayed symptoms of SARS-CoV-2 infection on several occasions. In February 2022, Kibo (31-yearold silverback) and Milele (10-year-old female) presented with dry cough; however, the subsequent SARS-CoV-2 nasal and fecal tests yielded negative results. In April 2022, Pelu, a 4-year-old male, showed mild coughing symptoms but was not tested for SARS-CoV-2 and did not receive any medical intervention, while the other members of the group remained asymptomatic. Furthermore, alongside the two previously mentioned females, Mutasi, a 28-year-old female, was anorexic after displaying vomiting and mild diarrhea in August 2022. She recovered within a few days without treatment. Later, in September 2022, two older adult gorillas, Kibo and Kolo, a 36-year-old female, showed lameness and stiff walking but no other symptoms. Due to the mild nature of these symptoms, sedation for the diagnosis was not deemed necessary.

Only two published cases of SARS-CoV-2 infections in gorillas are described in the literature, and it is also worthwhile to report asymptomatic infections. For example, in November 2021, multiple western lowland gorillas and Asiatic lions at Rotterdam Zoo in the Netherlands exhibited fever, coughing, and lethargy, and an outbreak of COVID-19 was confirmed in both species through positive SARS-CoV-2 RT-qPCR tests. The contact tracing identified two zookeepers who tested positive for SARS-CoV-2 [10]. Nagy et al., 2022 described a COVID-19 case in the gorillas in a zoo in the Czech Republic. Clinical signs reminiscent of COVID-19 disease, such as tiredness, fatigue, dry cough, and loss of appetite, were observed. The fecal specimens showed weak positivity by RT-qPCR [11]. Unlike the literature, our study did not observe the typical respiratory signs of SARS-CoV2 in the positive animals. Therefore, COVID-19 was not considered the primary problem, the serological testing was conducted almost half a year after the onset of the symptoms, and the contact tracing was not carried out during the sample collection period. Nevertheless, the zookeepers were advised to get vaccinated and were required to wear personal protective equipment, especially FFP2 face masks. None of the zookeepers showed any signs of SARS-CoV-2 infection.

This study has several limitations. First, further confirmatory tests were not conducted. Additionally, the presence of antibodies in gorillas does not confirm an active SARS-CoV-2 infection at the time of sampling as it may result from a past asymptomatic infection.

In conclusion, our study underscores the importance of continued surveillance for SARS-CoV-2 in zoo species, particularly given the documented instances and potential of asymptomatic transmission patterns. Further investigation is essential to fully describe the possible symptoms associated with SARS-CoV-2 infection in different species and effectively address the concerns related to the zoonotic or reverse-zoonotic transmission of SARS-CoV-2.

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CHAPTER 5

Wildlife Sentinel: Development of Multi-Species Protein A-ELISA Assay for Detection of SARS-CoV-2 Antibodies in Zoo Animals as a Proof of Concept for Wildlife Surveillance

Adapted from: Italiya, J., Petra Straková, Lukáš Pavlačík, Jiří Váhala, Jaroslav Haimy Hyjánek, Jiří Salát, Daniel Růžek, Dominika Komárková, and Jiří Černý
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Wildlife Sentinel: Development of Multi-Species Protein A-ELISA Assay for Detection of SARS-CoV-2 Antibodies in Zoo Animals as a Proof of Concept for Wildlife Surveillance

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5.1. Abstract:

COVID-19, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), first emerged in Wuhan in 2019 and rapidly spread worldwide. During the course of the COVID-19 pandemic, numerous reports have highlighted infections of wild animals by SARS-CoV-2. However, further research is required to understand the virus potential to infect various animal species, which is crucial for evaluating its future evolution and the potential reemergence of SARS-CoV-2.

The total concentration of immunoglobulin G (IgG) represents a valuable yet underutilized diagnostic parameter for health assessments in wild animals, primarily attributed to the absence of effective diagnostic tools. The utilization of Protein A-based indirect ELISA can serve as an efficient method to identify IgG antibodies against different pathogens in wildlife surveillance programs. For the development of Multi-Species Protein A-ELISA Assay for IgG detection against SARS-CoV-2, we utilized 44 animal species serum samples in order to ascertain their Protein A binding affinity. A total of 88 serum samples were used to identify IgG antibodies against SARS-CoV-2. The samples were chosen based on their strong binding affinity to protein-A. The serum samples were obtained from animals housed in Safari Park Dvůr Králové, Czech Republic. The zoo animals maintain close proximity to humans, facilitating the exploration of potential reverse transmission events of SARS-CoV-2 from humans to animals. Additionally, they undergo routine veterinary examinations, providing convenient access to blood samples. Therefore, they can be easily used for development of Protein A based Indirect ELISA for wildlife disease surveillance programs.

Based on the ELISA results, antibodies to SARS-CoV-2 were detected in the sera of 16 animals. To further confirm these findings, the ELISA-positive samples were subjected to virus neutralization assays. This additional testing revealed the presence of SARS-CoV-2 neutralizing antibodies in the serum of two white rhinoceros and one Persian leopard. It contributes to our understanding of the virus's potential host range and its interactions with various animal species.

Keywords: *COVID-19, SARS-CoV-2, serological surveillance, indirect ELISA, virus neutralization test (VNT), wildlife surveillance, zoo animals, proof of concept*

5.2. Introduction:

Since the onset of the COVID-19 pandemic, it has been shown that many animal species exhibit susceptibility to SARS-CoV-2 infection. Various surveillance modalities have been used to ascertain the vulnerability of wild animals to SARS-CoV-2 infections. These modalities include pathogen identification, serological determination, clinical investigation, and parameter monitoring (Clayton et al., 2022, Italiya et al., 2023a). Due to the prolonged presence of antibodies in the host organism after infection, the implementation of serosurveillance is a useful approach for the detection of animals that have experienced infection over an extended duration (Decaro et al., 2022). During pandemic, several studies were carried out on animal serological surveillance, e.g., wild white-tailed deer from US states, pet animals, and stray cats in Spain revealed presence of neutralizing antibodies (Barroso-Arévalo et al., 2022, Villanueva-Saz et al., 2022, Chandler et al., 2021). SARS-CoV-2 can cause dangerous life-threating infections in some animals, especially large felids (Giraldo-Ramirez et al., 2021).

Conducting epidemiological research in wildlife is essential for understanding the biology of SARS-CoV-2 in different animal species. Development of new serological assays directly in wildlife is a formidable undertaking due to the complexities associated with procuring blood samples from a wide variety of wild species. On the other hand, zoos or captive animals present themselves as advantageous subjects for serological investigations, providing representative samples that span various species. The accessibility of these samples is facilitated by regular veterinary interventions within zoo settings. Additionally, the close and direct interaction between humans and zoo animals provides a unique opportunity to observe the transmission dynamics of SARS-CoV-2 from humans to animals (Dusseldorp et al., 2023). For example, tigers and lions were found positive for SARS-CoV-2 infection in Bronx Zoo, USA (McAloose et al., 2020), a coatimundi and a fishing cat were found positive in Illinois Zoo, USA (Allender et al., 2022), and other animal species like hyenas (Sparrer et al., 2023), Canadian lynx (Tewari et al., 2023), Eurasian river otter (Padilla-Blanco et al., 2022), and gorillas (Nagy et al., 2022) also found positive in other zoos all around the world. Many zoo-kept SARS-CoV-2 permissive animals are endangered and suffer from other serious threats in the wild.

The Enzyme-linked immunosorbent assay (ELISA) is the most commonly used serological assay. The ELISA plays a pivotal role in advancing epidemiological investigations, offering a cost-effective, sensitive, and specific tool for detecting antibodies in diverse populations (Shah and Maghsoudlou, 2016). The Indirect ELISA is a one of the commonly used serological

techniques for detecting antibodies against SARS-CoV-2. Conventional ELISA necessitates the use of a particular anti-IgG conjugate and requires different optimization for each species of animal. However, the introduction of Protein A, isolated from the cell wall of Staphylococcus aureus, streamlines this process by demonstrating a specific affinity for the Fc segment of IgG of multiple mammalian species (Surolia et al., 1982). The use of protein A conjugate in ELISA has proven to be efficient in detecting infection caused by several infectious agents in both wild and domestic animals (Al-Adhami and Gajadhar, 2014). Hence, there is a need for novel diagnostic techniques that enhance diagnosis of many different animal species and epidemiological monitoring in wildlife and zoo settings by serological surveillance. In this study, we proposed a Protein A-based indirect ELISA method to address this gap, aiming to improve the detection of anti-F1 antibodies from various SARS-CoV-2 animal hosts within a single, comprehensive protocol. The obtained results were then validated by a viral neutralization test.

5.3. Materials and Methods:

5.3.1. Serum sample collection:

Blood samples were obtained from 88 animals, which represent 37 species, 10 families, and 4 orders from Safari Park Dvůr Králové (Czechia, EU) in the period from May 2020 to January 2022 (Table 1). Animals were not sampled primary for SARS-CoV-2 serosurveillance but due to many different routine veterinary interventions. Only sera remaining after the primary analyses were further used in this study. The positive reference human serum was acquired in May 2021 from a human patient who had undergone COVID-19 recovery.

5.3.2. Preparation of antigen:

Antigen was prepared by using heat-inactivated sample of SARS-CoV-2 (strain: human/Czech Republic/951/2020, provided by Dr. Jan Weber, Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic).

5.3.3. Determination of protein A-HRP binding efficiency to antibodies of different animal species:

The capability of protein A to bind to immunoglobulin G of a certain species was determined by an ELISA. Overall, 1-5 individuals (depending on availability) per species were tested. Tested sera were diluted in PBS, pH 7.4 (Carl Roth, Germany) in a 1:10 ratio. 100 ml of diluted serum was added to each well of the flat-bottomed microplates (SPL Immunoplate, USA) in order to coat it. Three wells without any serum and three wells with human serum were loaded on each plate as negative (blank) and positive controls, respectively. Plates were incubated overnight at 4°C in a humid environment and then rinsed three times with PBS. Further the wells were blocked by incubating with 100 ml of 2% BSA in PBS and washed again. Following that, 100 ml of protein A-HRP (ThermoFihser Scientific, USA) at a dilution of 1:1,000 in PBS were added to each well. Plates were incubated for one hour at 4°C and washed as stated previously. The wells were then submerged in a 50 ml solution of the enzyme substrate TMB PLUS2® (Kementec, Denmark). After 15 minutes at room temperature (in a dark area), the reaction was halted by the addition of 50 ml of 2M H2SO4. The photometer Infinite® 200 PRO (Dynatech, Germany) was used to determine the absorption at 450 nm. The measurements were conducted using the negative control as a baseline. The binding affinities of proteins to antibodies from a particular species were semi quantitatively determined by interpreting absorption as none (mean absorption below 0.3), low (mean absorption 0.3-0.6), medium (mean absorption 0.6-0.8), or high (mean absorption above 0.8) binding potential at a 1:10 dilution.

5.3.4. Non-species dependent ELISA:

Initially, checkerboard titration was used to identify the ideal concentrations of all chemicals, as well as the optimal volumes and reaction conditions. Additionally, only sera from those animal species were chosen for the analysis, which immunoglobulin G had a medium or high binding affinity with protein A-HRP. The final optimal conditions for ELISA using the protein A/HRP instead the secondary antibody were as follows: The wells were loaded with 100 ml of inactivated SARS-CoV-2 (virus titre 106 PFU/ml) diluted in PBS, pH 7.4, in a 1:2000 ratio, and incubated overnight at 4°C in a humid environment and then rinsed three times with PBS. Further, the wells were blocked by incubating with 100 ml of 2% BSA in PBS for 2 hours at

room temperature and washed. Then the wells were incubated overnight at 4°C in a humid environment with animal sera diluted in a 1:800 ratio in PBS and washed three times again. Protein A-HRP (ThermoFihser Scientific, USA) diluted in a 1:200 ratio was then used instead of the secondary antibody. After that, the wells were washed last time, and the final incubation with 50 ml of SureBlue HRP substrate (Seracare, USA) was added. The reaction was developed for 15 minutes, and then inhibited by 50 ml of 2M H2SO4. The photometric measurements were taken at a wavelength of 450 nm as described earlier. As the positive control had usually an absorption value of ~0.9 the results were classified into three categories based on the OD value: no absorption (less than 0.3), low absorption (0.3-0.6), medium absorption (0.6-0.7), and high absorption (more than 0.7).

5.3.5. A virus neutralization test (VNT):

The samples for VNT were chosen based on their high absorption rate in the ELISA experiment. Inactivated animal sera were diluted (1:4) in complete DMEM medium (supplemented with 10% foetal bovine serum, 1% antibiotics, and 1% glutamine, Biosera, France). In the next step, 2-fold serial dilutions of sera (50 µl) were incubated for 90 minutes at 37 °C with 103 PFU/ml of SARS-CoV-2 (50 µl per well) in a 96-well plate. Then, Vero cells (ATCC CCL-81) were added (5x104 cells in 100 µl per well) and after 4 days of incubation (37 °C and 5% CO2), the cytopathic effect was investigated. The highest serum dilution that inhibited the cytopathic effect of the SARS-CoV-2 virus was regarded as the endpoint titer (Brzuska et al., 2023).

5.4. Results:

5.4.1. Determination of binding affinity of Protein A:

The ability of Protein A-HRP conjugate to bind to the sera of different animal species varied depending on the species. Twenty-four of the 45 species (including Human) samples had a high binding affinity (absorption above 0.8) and 12 had a medium binding affinity (absorption 0.6-0.8) while 8 had a no binding affinity/low binding affinity (absorption 0.3-0.6). None of the tested samples had absorption lower than 0.3 (Table 1). Only serum samples from species with a high or medium affinity for Protein A-HRP were used in subsequent studies.

5.4.2. Detection of antibodies against SARS-CoV-2 by indirect ELISA and VNT:

Based on the results of the Protein A binding affinity assay, samples from 88 individuals from 36 species were selected for investigation of antibodies against SARS-CoV-2 by non-species dependent indirect ELISA. Sixteen zoo animal serum samples were tested positive in ELISA (Table 2). The samples with a high absorbance (higher than 0.7) in ELISA included one bat-eared fox (*Otocyon megalotis*), one African wild dog (*Lycaon pictus*), two Grévy's zebras (*Equus grevyi*), one fossa (*Cryptoprocta ferox*), two servals (*Leptailurus serval*), two Persian leopards (*Panthera pardus saxicolor*), three lions (*Panthera leo*), one tested positive twice repeatedly, one striped hyena (*Hyaena hyaena*), two white rhinoceros (*Ceratotherium simum simum*), one tested positive twice repeatedly, and one red river hog (*Potamochoerus porcus*). The samples with a high absorbance (higher than 0.7) were chosen for further VNT. In VNT, three serum samples demonstrated the ability to inhibit growth of SARS-CoV-2 but only in the lowest dilution tested (1:10) (Table 3). The positively tested animals in VNT were one Persian leopard (*Panthera pardus saxicolor*), and two white rhinoceros (*Ceratotherium simum simum*), one tested twice, but only once positively.

5.4.3. Health status of SARS-CoV-2 antibodies seropositive animals:

The animals have not shown any respiratory-related signs or symptoms associated with COVID-19. The zookeepers and other personnel who had direct or indirect contact with animals that tested positive for SARS-CoV-2 underwent several self-tests but were unable to detect any persons infected with the SARS-CoV-2.

Table 5.1. Determination of binding efficiency of protein A with different species antibodies

No.	Family	English name	Latin name	Sex	Mean O.D. (Mean ± SE)
1	*Hominidae*	Human (Positive control)	*Homo sapiens*	M	0.90 ± 0.12
2	*Equidae*	Somali wild ass	*Equus africanus somaliensis*	F	1.5554 ± 0.09
3	*Suidae*	Savanna pig	*Phacochoerus africanus*	F	1.502 ± 0.08
4	*Hominidae*	Orangutan	*Pongo pygmaeus*		1.4708 ± 0.24
5	*Bovidae*	Sitatunga	*Tragelaphus spekii*	F	1.4162 ± 0.06
6	*Canidae*	Jackal	*Canis aureus*	M	1.3655 ± 0.24
7	*Equidae*	Maneless zebra	*Equus quagga borensis*	F	1.2954 ± 0.17
8	*Bovidae*	Zebu	*Bos taurus indicus*	F	1.1906 ± 0.10
9	*Felidae*	Cheetah	*Acinonyx jubatus*	M	1.1577 ± 0.10
10	*Eupleridae*	The fossa	*Cryptoprocta ferox*	F	1.01 ± 0.08
11	*Rhinocerotidae*	Eastern black rhinoceros	*Diceros bicornis michaeli*	F	0.98 ± 0.43
12	*Canidae*	The bat-eared fox	*Otocyon megalotis*	F	0.95 ± 0.14
13		The African wild dog	*Lycaon pictus*	F	0.93 ± 0.14
14	*Bovidae*	The bongo	*Tragelaphus eurycerus*	F	0.92 ± 0.15

15		Common eland	*Taurotragus oryx*	F	0.92 ± 0.05
16	*Equidae*	Chapmann's Zebra	*Equus quagga chapmani*		0.9172 ± 0.15
17		The Grévy's zebra / The imperial zebra	*Equus grevyi*	F	0.91 ± 0.11
18	*Bovidae*	The Dwarf Dahomey cattle	*Bos taurus africanus*	F	0.90 ± 0.09
19		The impala	*Aepyceros melampus*	F	0.88 ± 0.04
20		Lesser kudu	*Tragelaphus imberbis*	M	0.87 ± 0.15
21		The greater kudu	*Tragelaphus strepsiceros*	F	0.87 ± 0.14
22	*Equidae*	The Grant's zebra	*Equus quagga boehmi*	F	0.84 ± 0.02
23	*Felidae*	The serval	*Leptailurus serval*	F	0.83 ± 0.06
24	*Camelidae*	The dromedary / The Arabian camel	*Camelus dromedarius*	M	0.81 ± 0.04
25	*Felidae*	Lion	*Panthera leo*	F	0.7731 ± 0.36
26	*Suidae*	The red river hog	*Potamochoerus porcus*	F	0.77 ± 0.04

27	*Felidae*	The Persian leopard	*Panthera pardus tulliana*	F	0.76 ± 0.08
28	*Hyaenidae*	The striped hyena	*Hyaena hyaena*	F	0.74 ± 0.05
29	*Bovidae*	The African forest buffalo	*Syncerus caffer nanus*	F	0.73 ± 0.04
30		Thomson's gazelle	*Eudorcas thomsonii*	M	0.71 ± 0.06
31		Sable antelope	*Hippotragus niger*	F	0.70 ± 0.10
32		Nyala	*Tragelaphus angasii*	M	0.70 ± 0.04
33	*Rhinocerotidae*	White rhinoceros	*Ceratotherium simum*	F	0.68 ± 0.03
34	*Equidae*	The plains zebra	*Equus quagga*	F	0.67 ± 0.08
35	*Bovidae*	Roan Antelope	*Hippotragus equinus*	M	0.65 ± 0.05
36		Black wildebeest	*Connochaetes gnou*	M	0.62 ± 0.18
37	*Bovidae*	White-bearded wildebeest	*Connochaetes taurinus albojubatus*	F	0.5151 ± 0.00
38		Mountain reedbuck	*Redunca fulvorufula*	F	0.4842 ± 0.05
39		The scimitar oryx	*Oryx dammah*	F	0.4760 ± 0.09

40		Lechwe	*Kobus leche*	F	0.377043 ± 0.04
41		The Nile lechwe o	*Kobus megaceros*	F	0.4709 ± 0.09
42		Cameroon dwarf goat	*Capra aegagrus hircus*	F	0.3473 ± 0.02
43		Daha gazelle	*Nanger dama*	F	0.3366 ± 0.02
44		Somalian sheep	*Ovis aries*	F	0.3048 ± 0.01
45		Blue wildebeest	*Connochaetes taurinus*	F	0.2496 ± 0.01
46	Negative control	Blank			0.3986 ± 0.01

Table 5.2. Non-species specific indirect-ELISA result (The samples highlighted in bold were used for additional VNT testing). The symbol "*" denotes the same individual sampled and screed twice with distinct time periods.

NO.	Family	English name	Latin name	Sex	Date of sample collection	Mean O.D.
1	*Hominidae*	Human (Positive control)	*Homo sapiens*	M	07-05-2021	0.89 ± 0.07
2	*Bovidae*	Greater kudu	*Tragelaphus strepsiceros*	F	22-07-2020	0.26 ± 0
3		Lesser kudu	*Tragelaphus imberbis*	M	23-06-2020	0.3 ± 0.02
4		Thomson's gazelle	*Eudorcas thomsonii*	M	23-07-2020	0.21 ± 0.01
5		Impala	*Aepyceros melampus*	F	30-04-2020	0.19 ± 0.01
6		African forest buffalo	*Syncerus caffer nanus*	F	16-07-2020	0.18 ± 0.01
7		Roan Antelope	*Hippotragus equinus*	M	06-08-2020	0.19 ± 0.03
8		Dwarf dahomey cattle	*Bos taurus africanus*	F	01-10-2020	0.26 ± 0.03
9		Bongo	*Boocercus euryceros isaaci*	F	20-08-2020	0.69 ± 0.04
10		Dwarf dahomey cattle	*Bos taurus africanus*	F	09-06-2020	0.22 ± 0.01
11		Dwarf dahomey cattle	*Bos taurus africanus*	M	09-06-2020	0.28 ± 0.04
12		Sable antelope	*Hippotragus niger*	F	24-06-2020	0.18 ± 0.01
13		Sable antelope	*Hippotragus niger*	M	23-06-2020	0.19 ± 0.01
14		Common eland	*Taurotragus oryx*	F	05-11-2020	0.25 ± 0.06
15		Nyala	*Tragelaphus angasii*	M	21-10-2020	0.19 ± 0

16	*Bovidae*	Common eland	*Taurotragus oryx*	F	05-11-2020	0.2 ± 0.01
17		Greater kudu	*Tragelaphus strepsiceros*	M	07-05-2020	0.55 ± 0.07
18		Black wildebeest	*Connochaetes gnou*	M	01-06-2020	0.2 ± 0.01
19		Sable antelope	*Hippotragus niger*	F	04-06-2020	0.23 ± 0.01
20		Nyala	*Tragelaphus angasii*	F	02-06-2020	0.22 ± 0.01
21		Lesser kudu	*Tragelaphus imberbis*	F	23-06-2020	0.45 ± 0.1
22		Lesser kudu	*Tragelaphus imberbis*	F	21-07-2020	0.42 ± 0.12
23		Thomson's gazelle	*Eudorcas thomsonii*	F	04-09-2020	0.26 ± 0.05
24		Greater kudu	*Tragelaphus strepsiceros*	F	18-09-2020	0.47 ± 0.14
25		Greater kudu	*Tragelaphus strepsiceros*	F	23-07-2020	0.65 ± 0.38
26		Mountain reedbuck *	*Redunca fulvorufula*	F	15-04-2021	0.38 ± 0.06
27		Mountain reedbuck *	*Redunca fulvorufula*	F	25-10-2021	0.31 ± 0.00
28		Lechwe	*Kobus leche*	F	05-02-2021	0.36 ± 0.01
29		Lesser kudu	*Tragelaphus imberbis*	F	19-03-2021	0.35 ± 0.05
30		Sable antelope	*Hippotragus niger*	F	25-06-2021	0.34 ± 0.07
31		Lesser kudu	*Tragelaphus imberbis*	F	19-03-2021	0.34 ± 0.01
32		Lesser kudu	*Tragelaphus imberbis*	M	17-03-2021	0.33 ± 0.03
33		White-bearded wildebeest	*Connochaetes taurinus albojubatus*	F	15-10-2021	0.32 ± 0.01
34		Lesser kudu	*Tragelaphus imberbis*	M	19-03-2021	0.32 ± 0.01

35		Mountain reedbuck	*Redunca fulvorufula*	F	16-04-2021	0.32 ± 0.01
36		Zebu	*Bos taurus indicus*	F	22-01-2021	0.31 ± 0.01
37		Mountain reedbuck	*Redunca fulvorufula*	F	06-01-2021	0.31 ± 0.02
38		Thomson's gazelle	*Eudorcas thomsonii*		16-02-2021	0.30 ± 0.02
39		Impala	*Aepyceros melampus*	M	17-03-2021	0.31 ± 0.02
40		Thomson's gazelle	*Eudorcas thomsonii*	M	14-04-2021	0.29 ± 0.00
41		Sitatunga	*Tragelaphus spekii*	F	12-11-2021	0.36 ± 0.01
42		Somali sheep	*Ovis aries*	F	20-01-2021	0.32 ± 0.01
43	*Camelidae*	Dromedary / The Arabian camel	*Camelus dromedarius*	M	28-10-2020	0.38 ± 0.06
44	*Canidae*	African wild dog	*Lycaon pictus*	F	05-08-2020	0.5 ± 0.14
45		**Bat-eared fox**	***Otocyon megalotis***	**F**	**31-08-2020**	**0.92 ± 0.06**
46		**African wild dog**	***Lycaon pictus***	**F**	**05-08-2020**	**0.91 ± 0.18**
47		African wild dog	*Lycaon pictus*	F	05-08-2020	0.64 ± 0.13
48		Jackal	*Canis aureus*	M		0.59 ± 0.02
49		Jackal	*Canis aureus*	F		0.57 ± 0.05
50	*Equidae*	**Grévy's zebra**	***Equus grevyi***	**F**	**29-04-2020**	**1.17 ± 0.22**
51		Plains zebra	*Equus quagga*	F	28-05-2020	0.19 ± 0.03
52		Plains zebra *	*Equus quagga*	F	28-04-2020	0.19 ± 0
53		Plains zebra *	*Equus quagga*	F	16-04-2021	0.34 ± 0.01
54		Plains zebra	*Equus quagga*	F	28-05-2020	0.25 ± 0.02

55		Plains zebra	*Equus quagga*	F	26-06-2020	0.2 ± 0
56		Plains zebra	*Equus quagga*	F	28-05-2020	0.24 ± 0
57		**Grévy's zebra**	***Equus grevyi***	**F**	**30-04-2020**	**0.94 ± 0.31**
58		Grévy's zebra	*Equus grevyi*	F	01-06-2020	0.45 ± 0.04
59		Grant's zebra	*Equus quagga boehmi*	F	14-04-2021	0.57 ± 0.07
60		Maneless zebra	*Equus quagga borensis*	M	23-12-2021	0.38 ± 0.03
61		Grant's zebra	*Equus quagga boehmi*	F	24-06-2021	0.37 ± 0.03
62		Somali wild ass	*Equus africanus somaliensis*	F	07-01-2021	0.36 ± 0.05
63		Maneless zebra	*Equus quagga borensis*	F	14-04-2021	0.36 ± 0.02
64		Maneless zebra	*Equus quagga borensis*		04-07-2021	0.34 ± 0.03
65		Chapmann's Zebra	*Equus quagga chapmani*		04-01-2022	0.34 ± 0.00
66		Grant's zebra	*Equus quagga boehmi*	F	15-04-2021	0.33 ± 0.01
67		Chapmann's Zebra	*Equus quagga chapmani*		02-04-2021	0.33 ± 0.00
68		Maneless zebra	*Equus quagga borensis*	F	31-03-2021	0.32 ± 0.00
69		Maneless zebra	*Equus quagga borensis*	F	03-08-2021	0.32 ± 0.01
70	*Eupleridae*	**Fossa**	***Cryptoprocta ferox***	**F**	**12-11-2020**	**0.86 ± 0.01**
71	*Felidae*	**Serval**	***Leptailurus serval***	**F**	**05-08-2020**	**1.12 ± 0.1**
72		**Persian leopard**	***Panthera pardus saxicolor***	**M**	**19-09-2020**	**0.93 ± 0.12**
73		**Serval**	***Leptailurus serval***	**M**	**05-08-2020**	**0.86 ± 0.1**
74		**Persian leopard**	***Panthera pardus saxicolor***	**F**	**18-08-2020**	**1.01 ± 0.14**

75		**Lion**	***Panthera leo***	**F**	**22-01-2021**	**0.81 ± 0.10**
76		**Lion ***	***Panthera leo***	**M**	**15-04-2021**	**0.76 ± 0.04**
77		**Lion ***	***Panthera leo***	**M**	**23-11-2021**	**0.72 ± 0.09**
78		Lion	*Panthera leo*	M	28-11-2021	0.60 ± 0.01
79		Lion	*Panthera leo*		25-11-2021	0.37 ± 0.01
80		Cheetah	*Acinonyx jubatus*	M	25-07-2021	0.56 ± 0.01
81		Cheetah	*Acinonyx jubatus*	F	25-07-2021	0.49 ± 0.03
82		Cheetah	*Acinonyx jubatus*			0.36 ± 0.01
83	*Hominidae*	Orangutan	*Pongo pygmaeus*		26-10-2021	0.59 ± 0.01
84	*Hyaenidae*	**Striped hyena**	***Hyaena hyaena***	**F**	**02-10-2020**	**1.48 ± 0.54**
85	*Rhinocerotidae*	**White rhinoceros ***	***Ceratotherium simum simum***	**F**	**21-07-2020**	**0.84 ± 0.08**
86		White rhinoceros *	*Ceratotherium simum simum*	F	21-07-2021	0.67 ± 0.05
87		**White rhinoceros**	***Ceratotherium simum simum***	**F**	**23-06-2020**	**1.06 ± 0.31**
88	*Suidae*	**The red river hog**	***Potamochoerus porcus***	**F**	**04-08-2020**	**0.88 ± 0.03**
89		Savanna pig	*Phacochoerus africanus*	F	10-11-2021	0.43 ± 0.03
90	Negative control	Blank				0.21 ± 0.01

Table 5.3. The results of the indirect ELISA and VNT assays.

No.	Sample ID	In-Direct ELISA	VNT Result	Date of collection
1	Positive Control (human)	0.89	Inhibition of SARS-CoV-2 in dilutions up to 1:40, then negative	07-05-2021
74	Persian leopard	1.01	Inhibition of SARS-CoV-2 in dilution up to 1:10, then negative	18-08-2020
85	Southern white rhinoceros	0.84	Inhibition of SARS-CoV-2 in dilution up to 1:10, then negative	21-07-2020
87	Southern white rhinoceros	1.06	Inhibition of SARS-CoV-2 in dilution up to 1:10, then negative	23-06-2020

NO denotes the sample number and is the same as in the Table 2. Only positive samples are shown

5.5. Discussion:

ELISA is a critical method for detecting specific antibodies against a particular microorganism in animal sera. Nevertheless, the presence of antibodies in the serum of an animal does not mean that the virus is able to replicate within such an animal host. It could be possible that the animal only came into contact with the virus which was not able to start a productive infection. Considering the complex dynamics of viral interactions in wildlife, the establishment of a non-species-specific ELISA holds significance for the comprehensive serological surveillance of various pathogens in diverse wildlife populations. The critical point in ELISA is usually to employ secondary antibodies against the Fc region of the species' immunoglobulin (Liyanage et al., 2023). Such secondary antibodies are usually not available for most of the wild-living animals and therefore less specific antibodies targeted against their domesticated relatives have to be used. In certain species, there is no other option available, therefore developing a non-

species-specific Protein A-ELISA a useful tool in such circumstances. A study suggests that there is no discernible correlation between the phylogenetic similarity of families and their capacity to interact with protein A. Consequently, it is necessary to evaluate the binding affinity of serum IgG from different wild species (families that were not previously studied) to protein A before the non-species-specific Protein A-ELISA would be applied. In the past, the affinity of Protein A for various animal species has been demonstrated in zoo animals. It is not, though, described for all species (Stöbel et al., 2002b). Therefore, we rescreened Protein A-HRP binding ability to sera of all animal species we tested in our experiment. In correspondence with the previously published data (Stöbel et al., 2002a), our results demonstrated that Protein A-HRP can be used for the detection of specific antibodies in the sera of many animal species (Table 1) and can be used for development of indirect-ELISA for any particular microorganisms. The sera of twelve animal species had similar outcomes to those described by Stobel et al., while six species, including Roan Antelope, Black wildebeest, mountain reedbuck, scimitar oryx, Nile lechwe, and Somalian sheep, demonstrated opposite results. The potential reason might be attributed to the existence of a lower amount of Ig or total protein in the serum (Liyanage et al., 2023). The result of the ability of protein-A to bind to IgG in a species-independent manner has been extensively used for antibody purification and the development of species-independent indirect ELISAs for different wildlife species (Al-Adhami and Gajadhar, 2014, Zarrineh et al., 2020). Several Multi-Species Protein A-ELISA Assays have been developed and effectively used in wildlife to detect antibodies against numerous pathogens such as Brucella, paratuberculosis, foot-and-mouth disease virus, tick-borne encephalitis virus, parapoxvirus and others (Pruvot et al., 2013, Hosamani et al., 2015, Inagaki et al., 2016, Nymo et al., 2013, Inoshima et al., 1999). In this study, we developed Multi-Species Protein A-ELISA assays capable of detecting antibodies against SARS-CoV-2. The assays were specifically evaluated in zoo animals to demonstrate their applicability in wildlife serological surveillance, establishing a proof of concept for their versatile use.

COVID-19, a disease caused by SARS-CoV-2, is typically transmitted from human to human by aerosolized particles with documented bi-directional transmission between people and animals (Saw et al., 2021, Clayton et al., 2022). Animal infection cases pique interest in virus pathogenesis in animals and possible subsequent transmission between animals and from animals to humans, as well as virus mutation. Due to the close spatial proximity of zoo animals to humans, there is a considerable risk of infection spreading from humans to these animals and vice versa. This can help us to understand SARS-CoV-2 ecology on a wide scale and

identify its new potential animal hosts. Further, from a conservation standpoint, it is important to recognize the hazards of SARS-CoV-2 infection to threatened wild animals. Moreover, contrary to other methods of direct virus cultivation or viral nucleic acid detection, which are very specific and allow exact identification of the virus, the results of serological tests are sometimes hard to interpret, for example, due to possible cross-reactivity with antibodies targeted on closely related virus species. The specificity of the detection can be improved by VNT (Lu et al., 2021). The presence of cross-reactivity between anti-SARS-CoV-2 antibodies and antibodies against other viruses was observed by the use of EUROIMMUN IgA and IgG ELISAs in serum samples obtained before the onset of the COVID-19 pandemic. Nevertheless, none of the individuals exhibited measurable levels of neutralizing antibodies against the live Wuhan strain of SARS-CoV-2 (Hunsawong et al., 2022). Further, monoclonal antibodies generated against the structural proteins of SARS-CoV, including the nucleocapsid, spike, envelope, and membrane proteins exhibited significant cross-reactivity towards SARS-CoV-2 proteins, while spike antibodies of SARS-CoV demonstrate minimal cross-neutralization of the SARS-CoV-2 (Bates et al., 2021). Currently, there is a lack of empirical evidence on the cross-reactivity between animal coronaviruses and SARS-CoV-2. Nevertheless, the comparative computational research conducted on the epitopes of the nucleocapsid protein of SARS-CoV-2 in coronaviruses that are taxonomically related revealed significant structural resemblances with SARS-CoV and Bat CoV but exhibited lower levels of similarity with Dromedarius CoV and Pangolin CoV (Tilocca et al., 2020).

Further, we screened the sera of 92 animals from 43 animal species for presence of SARS-CoV-2 antibodies. We found total 16 samples with a high absorbance (higher than 0.7) in ELISA and were further tested by VNT. In VNT sera from three animals (two white rhinoceros, and one Persian leopard) were shown to be able to neutralize SARS-CoV-2 despite only in small dilutions. Big cats are known to be sensitive to SARS-CoV-2 infection. SARS-CoV-2 infection of many felids including lions, tigers, snow leopards and others have been reported from zoos all over the world (Bartlett et al., 2021, Wang et al., 2022, Mitchell et al., 2021). Nevertheless, also infections of wild living felids have been observed when a free-living Indian leopard has been found to be infected with SARS-CoV-2 (Mahajan et al., 2022). The other two animals tested positive by VNT (at lower titration 1:10) are two southern white rhinoceros. According to our knowledge, this is the first evidence of the presence of antibodies against SARS-CoV-2 in white rhinoceros. The SARS-CoV-2 RNA was found in a fecal sample of white rhinoceros in our previous studies during small-scale coronavirus surveillance in Bandia

Reserve, Senegal (Italiya et al., 2023b). Despite SARS-CoV-2 infection not being reported in any other member of Perissodactyla, bioinformatic, functional, and genetic analyses of SARS-CoV-2 receptor ACE2 orthologs support the idea that SARS-CoV-2 can infect Rhinocerotidae (Liu et al., 2021).

All positive samples identified in this study were collected between the first and second waves of COVID-19 in Czechia in the summer and fall of 2020. During this time, daily reported COVID-19 cases in the human population were very low in the whole of Czechia (including the Trutnov district where the zoo is located). Therefore, during this time period, the state of emergency was not declared (Supplementary Figure 1), and thus, the zoos and other places for leisure activities in Czechia were open to the public but the counter-epidemic measures were minimal and new waves were not expected by the general public. The health statuses of zookeepers from the rhinoceros and carnivores' departments before the positive sample collection dates, none of the employees had any COVID-19 related symptoms or was tested positive.

This study is subject to some limitations, one of which pertains to the validation of the species nonspecific ELISA assay developed in-house. The ELISA used in this experiment has not undergone validation and was thus employed only for preliminary screening purposes. In addition, the current ELISA did not test for additional SARS-CoV-2 variants of concerns. The present investigation used the whole virus antigen, which may demonstrate reduced specificity as a result of an increased probability of non-specific binding of co-purified cellular proteins and non-target viral proteins such as nucleocapsid, membrane, and envelope proteins in ELISA assay. Therefore, only samples positively tested by VNT, which is understood as a gold standard of serological methods, but which is very laborious and time consuming to be performed on a large number of samples, were considered as positive.

Protein A-based multispecies ELISA can serve as a valuable tool for the development of various serological assays to monitor disease status in wildlife. Nevertheless, it is crucial to conduct validation and assessment of tests on zoo animals prior to their implementation in surveillance programs. The zoo kept animals can be understood as sentinels in surveillance for animal species susceptible to SARS-CoV-2 infection. As many of the SARS-CoV-2 permissive animals (including those detected in this study) are threatened, this information is crucial for their further protection and should be considered in the preparation of conservation strategies of these animals in the wild.

Author contributions:

JI, JC, JS: research conceptualization and design. JI, JC, PS, LP, JV, JH, DK, DR: data collection. JI, JC, JS, PS, LK, JV, JH: analysis and interpretation of results. JI: manuscript draft preparation. JI, JC: statistical analysis. JC, JS: supervision. All authors contributed to the article and approved the submitted version.

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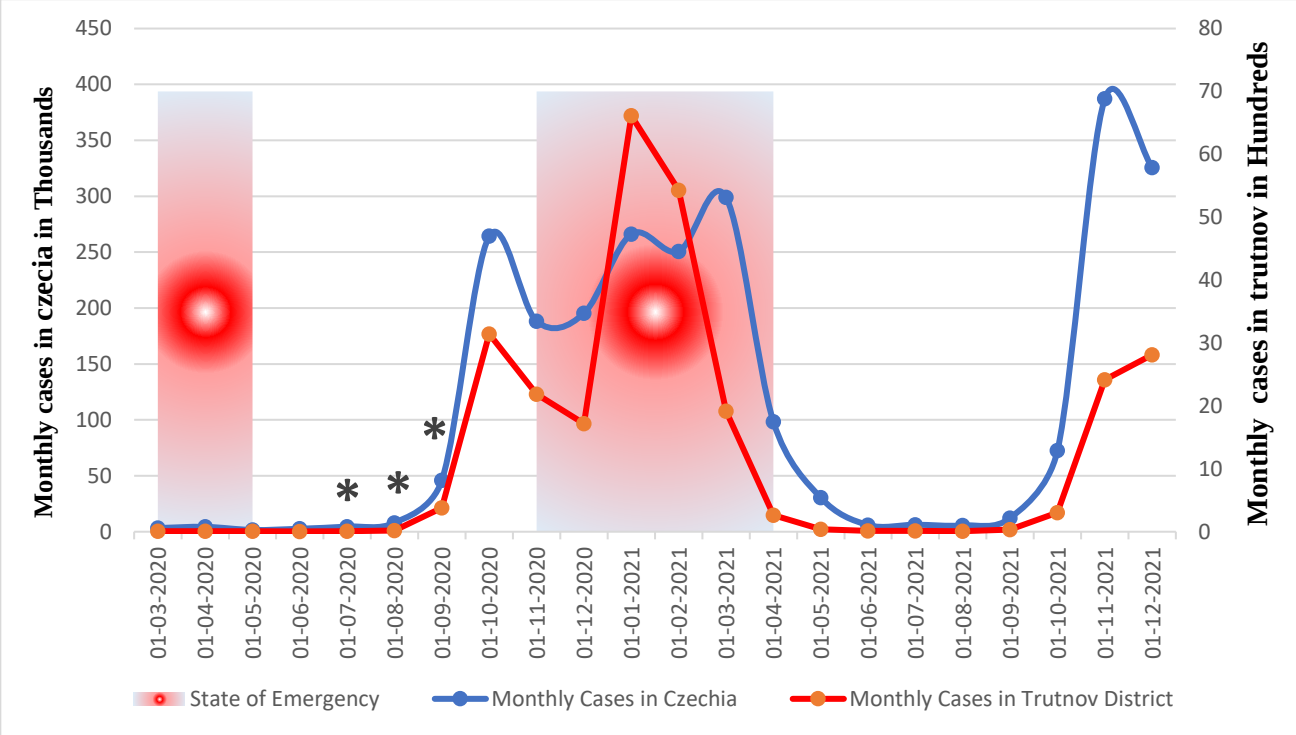

Supplementary Figure 5.1. Date of sample collection (*), which were determined to be positive for VNT, together with the daily number of cases in the Czech Republic and Trutnov district during and after the declaration of a state of emergency.

CHAPTER 6

General discussion

The dissertation sought to explore the possible exposure and spillover events of the newly emerged SARS-CoV-2 in zoo settings and wild living animals. Assessing the impacts of the newly emerged virus on wildlife, along with its potential to persist and mutate in animal reservoirs, is essential for evaluating future pandemic risks. In order to conduct intensive surveillance, particularly in wildlife, it is necessary to assess and devise a strategy for SARS-CoV-2 screening in wildlife. SARS-CoV-2 is a generalist pathogen with the ability to infect at least one nonhuman animal species from almost every group of mammals. The continuous and prolonged spread of SARS-CoV-2 among humans is anticipated to increase the likelihood of secondary animal reservoirs emerging. There is evidence that characteristics related to the virus, host, and environment have a role in the transmission of infections across other species. However, the factors that cause these advances from one host to another remain largely unknown (Kuchipudi et al., 2023).

The rise of infectious diseases in recent decades, exacerbated by causes such as climate change and human activities, highlights the need to comprehend zoonotic diseases (Esposito et al., 2023). The recent outbreak of COVID-19, caused by SARS-CoV-2 is one example and highlighted the need for surveillance efforts, particularly in wildlife populations. Implementing surveillance strategies in wild animal populations has distinct obstacles, such as restricted animal accessibility, limitations in disease detection, and financial implications (Perez et al., 2011). To overcome these challenges, it is necessary to use innovative sample approaches, such as non-invasive techniques, and to strategically plan to focus on animal populations that are at higher risk of virus exposure. Conducting a risk assessment to evaluate the potential for SARS-CoV-2 exposure in animals is the first step that has to be taken prior to implementing surveillance strategies. Risk assessment entails a thorough examination of the source of the virus, the extent of viral exposure in wildlife, and the potential impacts of exposure on animals (Logeot et al., 2022). Before implementing wildlife surveillance for SARS-CoV-2 screening, it is essential to evaluate various surveillance modalities. These modalities encompass pathogen determination, serological surveillance, clinical investigation, and parameter monitoring, among others (WHO, 2022). Pathogen determination in the context of SARS-CoV-2 wildlife surveillance can be readily accomplished through non-invasive sampling methods,

owing to the virus's presence in fecal samples from infected animals (Sanyal et al., 2022). On the other hand, serological surveillance can offer insights into previous events of virus infection through the detection of antibodies, the collection of samples remains a significant obstacle for this method of surveillance (Ryser-Degiorgis & Pierre, 2013).

Several animal species were reported to be sensitive to SARS-CoV-2 but did not exhibit clinical disease development, thus clinical investigation and parameter monitoring are only applicable to species that exhibit disease symptoms (Rutherford et al., 2022). A number of fundamental obstacles may materialize during SARS-CoV-2 wildlife surveillance, including sampling strategy, access to investigation materials, laboratory analysis, and data interpretation. Therefore, it is critical to develop effective strategies in order to effectively resolve these challenges. To carry out surveillance studies for SARS-CoV-2 in zoo settings is comparatively easier than wildlife due to its controlled environment where animals are housed in confined spaces, making it easier to monitor their health and behavior. Secondly, animals in zoos are usually accustomed to human presence and handling, which facilitates sample collection and medical examinations (Joffrin et al., 2023).

Among surveillance methodologies in free-ranging wildlife, pathogen determination stands out as paramount and feasible way. In the context of SARS-CoV-2, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) emerges as the prevalent technique for detection (Li et al., 2020). However, implementing this method presents challenges stemming from constraints in sample preservation and transportation to advanced molecular biology facilities. Performing on-site genomic RNA extraction and PCR amplification can overcome challenges related to nucleic acid degradation and yield rapid outcomes. To address these hurdles, we utilized a mobile molecular biology laboratory in the field, facilitating the detection of coronaviruses in animal samples. Target genes for SARS-CoV-2 detection by RT-PCR assay typically include the E gene, ORF 1ab, and N gene (Corman et al., 2020). SARS-CoV-2 variant tracking is conducted by analyzing the whole S-gene by long-range RT-PCR and Sanger sequencing (Matsubara et al., 2022). In our study, we employed widely used Betacoronavirus primers to target the highly conserved region of the RdRP (RNA-dependent RNA polymerase) gene, a part of ORF1a. For the small-scale coronavirus surveillance, we selected the Bandia Reserve in Senegal as our study site, targeting both free-ranging wildlife species within the reserve and the nearby population of free-living hedgehogs. Employing non-invasive methods, fecal samples were collected from these animals for pathogen detection, followed by on-site genomic extraction and RT-PCR analysis.

Betacoronaviruses have been documented in several mammalian species, encompassing humans, bats, rodents, carnivores, and ungulates (Ghai et al., 2021). In our investigation, we did not detect the presence of any coronaviruses in fecal samples from hedgehogs, primates, and giraffes. The limited sample size used in this study does not negate the presence of coronavirus in these species. Our findings revealed only one positive case in a rhinoceros, with all other samples testing negative. Subsequent sequencing of the RdRP gene amplicon from the positive case unveiled a sequence of SARS-CoV-2. The identification of the SARS-CoV-2 virus in the fecal sample of a free-living rhinoceros is the first reported instance since the pandemic began. The Bandia reserve is a partially enclosed wildlife sanctuary that is renowned for its tourism activities. However, there are concerns regarding the possible transmission of viruses to the animals from humans in the reserve. This apprehension stems primarily from the potential for direct or indirect human contact with wild animals via activities such as wildlife safari excursions or the provision of feed, which is a prevalent practice.

Serological surveillance is another widely utilized method for understanding the spread of pathogens within populations and their ecological contexts. Serological assays offer a reliable means of evaluating exposure to SARS-CoV-2 and are valuable for comprehending the spread of the virus and the evolution of the pandemic (Tanne & Hopkins, 2020). Different serological assay can be used in order to identify SARS-CoV-2 antibodies in animals includes ELISA (Enzyme Linked Immunosorbent Assay), Western blot, LFIA (Lateral Flow Immunoassay), sVNT (Surrogate Virus Neutralization Test), and VNT (Virus Neutralization Test) (Diezma-Díaz et al., 2023). During the SARS-CoV-2 pandemic, serological surveillance has been extensively employed to investigate the transmission of the virus from humans to animals. This is particularly crucial because wild animals have the ability to acquire illnesses without exhibiting any signs (Meekins et al., 2021). Antibodies in the bloodstream of these individuals enable the detection of these infections. However, only a limited number of serological tests have been developed to identify specific IgG antibodies against SARS-CoV-2 in animals. These tests have been used in epidemiological studies, but they have not undergone a thorough validation process, most likely because there is no comprehensive set of well-characterized reference sera available (Mohit et al., 2021).

Humans are the dominant SARS-CoV-2 host species (Lytras et al., 2021). During the SARS-CoV-2 pandemic, many wild animal species housed in zoos worldwide were found to be infected due to close contact with COVID-19 asymptomatic humans. Therefore, zoos serve as crucial venues for studying the susceptibility of different animal species to SARS-CoV-2

infection. Investigating SARS-CoV-2 transmission among various zoo species helps identify potential virus reservoirs within wildlife populations. To conduct serological surveillance of SARS-CoV-2 in wild animals, it is necessary to develop and validate ELISA tests for different species. This is a challenging task due to the unavailability of reference sera from various species. In such cases, different diagnostic techniques, such as protein A-based indirect ELISA and double antigen sandwich ELISA, can be used. However, confirmatory tests such as surrogate virus neutralization tests (sVNT) and virus neutralization tests (VNT) are also required.

In our study, we developed a multispecies protein A-ELISA assay for detecting SARS-CoV-2 antibodies in zoo animals. For the assay development and implementation for surveillance, a total of 88 samples were obtained from different animal species, representing 45 species, 10 families, and 4 orders, from Safari Park Dvůr Králové (Czechia) between May 2020 and January 2022. The binding efficiency of protein A-HRP with antibodies from different animal species revealed that 24 of the 45 species (including humans) had a high binding affinity (absorption above 0.8), 12 had a medium binding affinity (absorption 0.6-0.8), and 8 had low or no binding affinity (absorption 0.3-0.6). Based on these results, samples with high and medium binding affinity were selected for further ELISA assay. Sixteen zoo animal serum samples tested positive in the multispecies protein A-ELISA. However, our in-house developed ELISA assay was not validated, highlighting the need for further confirmatory tests. These 16 samples were subsequently tested by virus neutralization tests (VNT). In the VNT, three serum samples demonstrated the ability to inhibit the growth of SARS-CoV-2, but only at the lowest dilution tested (1:10), which cannot be considered positive.

The second serological screening for SARS-CoV-2 infection was conducted in several mammalian species at Wilhelma Zoo in Stuttgart, Germany, using the commercially available ID Screen® SARS-CoV-2 Double Antigen Multi-species ELISA kit from ID vet. This kit is designed to detect antibodies against the nucleocapsid protein of SARS-CoV-2 in serum or plasma samples from various animal species. The serological surveillance at Wilhelma Zoo revealed that, except for two Western lowland gorilla samples, all other samples tested negative for SARS-CoV-2 antibodies. In our study, a total of fifteen blood samples were collected from twelve distinct species across seven families by zoo veterinarians between July 2022 and January 2023. These samples were not initially collected solely for SARS-CoV-2 serological screening. Blood samples were collected between August and September 2022 from two female gorillas exhibiting apathy and multiple gastrointestinal symptoms. Undi, a 51-year-old

female, presented with severe clinical signs including anorexia, fever, lameness, and stiffness in her movements. Tuana, a 17-year-old female, experienced milder symptoms. Due to the severity of these clinical manifestations, zoo veterinarians sedated the animals to perform examinations, administer medical care, and collect blood samples. Undi required approximately six weeks and multiple treatments to achieve full recovery, whereas Tuana recovered within a few days. In our study, the animals did not show the typical respiratory signs of SARS-CoV-2 observed in some previous studies (Dusseldorp et al., 2023; Nagy et al., 2022). However, it is important to note that SARS-CoV-2 in gorillas has been reported in only a few zoos worldwide. Therefore, it is crucial to carry out passive surveillance for SARS-CoV-2 to understand the potential for asymptomatic infections and the possibility of zoo animals serving as reservoir hosts.

CHAPTER 7

General conclusion

The current dissertation investigates SARS-CoV-2 in zoo-kept and wild-living animals using various surveillance methodologies. The following overall conclusions can be drawn from the three research outputs of this study:

a. Understanding the impacts of the newly emerged SARS-CoV-2 virus on wildlife, and its potential to persist and mutate in animal reservoirs, is essential for evaluating future pandemic risks. Developing screening strategies is necessary. Our studies explored different surveillance methods for wildlife surveillance based on available information of SARS-CoV-2 cases in animals.

b. Small-scale coronavirus surveillance at Bandia Reserve, utilizing mobile molecular biology laboratories, revealed the presence of the SARS-CoV-2 genome in noninvasive fecal samples from white rhinoceroses, marking the first detection of the virus in this species by our group.

c. Serological surveillance conducted at Wilhelma Zoo in Stuttgart, Germany, detected antibodies specific to the nucleocapsid protein of SARS-CoV-2 in two gorillas, indicating previous infection.

d. We developed a multi-species protein A-ELISA assay for detecting SARS-CoV-2 antibodies in zoo animals, implemented it on animals at Dvůr Králové Zoo, and found SARS-CoV-2 antibodies in several animals. However, subsequent confirmatory tests (VNT assay) detected neutralizing antibodies at very low levels (1:10) in two rhinoceroses and a Persian leopard.

CHAPTER 8

General references

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CHAPTER 9

CURRICULUM VITAE

EDUCATION

PhD STUDIES 2020 – Present

Tropical Agrobiology and Bioresource Management. Czech University of Life Sciences Prague.

Thesis title: SARS-CoV-2 in zoo-kept and wild-living animals

07/2017 – 30/08/2019

MASTER OF VETERINARY SCIENCE (M.V.Sc.)

Subject: Animal Biotechnology and veterinary microbiology

Thesis Transcriptome Profiling to evaluate effect of herbal plant extract on bull spermatozoa

Anand agricultural university, Anand, India.

07/2012 – 07/2017

DOCTOR OF VETERINARY MEDICINE (B.V.Sc.&A.H.)

Junagadh Agricultural University, Junagadh, India

INTERNSHIP

01/05/2022 03/07/2022

Université Cheikh Anta Diop de Dakar (SN) - Erasmus+ internship for training

WORKING EXPERIENCE

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JUNIOR RESEARCHER, CZECH UNIVERSITY OF LIFE SCIENCES.

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VETERINARY TECHNICIAN, CZECH UNIVERSITY OF LIFE SCIENCES, PRAGUE, CZECHIA

12/12/2021 – 30/09/2022

JUNIOR RESEARCHER, CZECH UNIVERSITY OF LIFE SCIENCES, PRAGUE, CZECHIA

25/09/2020 – 10/12/2020

VETERINARY SPECIALIST (SENIOR EXECUTIVE), GVK EMRI (EMERGENCY MANAGEMENT AND RESEARCH INSTITUTE), SURAT, INDIA

07/2019 – 05/2020

VETERINARY OFFICER, GOVERNMENT OF INDIA, SURAT, INDIA

SCIENTIFIC PUBLICATIONS

1. **Italiya, J.**; Knauf-Witzens, T.; Weigold, A.; Černý, J. Serological Screening of SARS-CoV-2 Infection in Several Mammalian Species in Wilhelma Zoo, Stuttgart, Germany. Pathogens 2024, 13, 612. https://doi.org/10.3390/pathogens13080612

2. **Italiya, J.**, Panchal, K. J., Jakhesara, S. J., Joshi, C. G., & Koringa, P. G. (2024). In vitro impact of ethanolic extract of Bryonia laciniosa seed on Gir bull spermatozoa: A comprehensive evaluation through transcriptome profiling. Frontiers in Veterinary Science, 11, 1419573. https://doi.org/10.3389/fvets.2024.1419573

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4. **Italiya J**, Vacek V, Matějů P, Dering C, Celina SS, Ndiaye A, Černý J. First Detection of SARS-CoV-2 in White Rhinoceros during a Small-Scale Coronavirus Surveillance in the Bandia Reserve, Senegal. Animals. 2023; 13(16):2593. https://doi.org/10.3390/ani13162593

5. **Italiya, J.**, Bhavsar, T., & Černý, J. (2023). Assessment and strategy development for SARS-CoV-2 screening in wildlife: A review. Veterinary World, 16(6), 1193. 10.14202/vetworld.2023.1193-1200

6. **Italiya, J. M.**, Patel, M. R., Golaviya, A. V., Patel, S. S., Thakkar, B. K., Jakhesara, S. J., ... & Koringa, P. G. (2023). RNA-sequencing attest increased sperm motility in bovine spermatozoa treated with ethanolic extract of Putranjiva roxburghii. *3 Biotech*, *13*(1), 33. https://doi.org/10.1007/s13205-022-03452-4

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8. Patel, S., Shah, T., Sabara, P., Bhatia, D., Panchal, K., **Italiya, J.**, ... & Rank, D. N. (2020). Understanding functional implication of β-casein gene variants in four cattle breeds characterized using AmpliSeq approach. 3 Biotech, 10, 1-8. https://doi.org/10.1007/s13205-020-02410-2