DOI: 10.7589/JWD-D-24-00028

Journal of Wildlife Diseases, 61(2), 2025, pp. 396–407
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Wildlife Sentinel: Development of Multispecies Protein A-ELISA for Detection of SARS-CoV-2 Antibodies in Zoo Animals as a Proof of Concept for Wildlife Surveillance

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ABSTRACT: Coronavirus disease 2019 (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 highlighted infections of wild animals by SARS-CoV-2. Nevertheless, 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 underused diagnostic parameter for health assessments in wild animals, primarily due to the absence of effective diagnostic tools. A protein A–based indirect ELISA can serve as an efficient method to identify IgG antibodies against different pathogens in wildlife surveillance programs. To develop a multispecies protein A-ELISA for IgG detection against SARS-CoV-2, we used 44 animal species serum samples to ascertain the protein A binding affinity, and 88 serum samples, chosen for the strong binding affinity to protein A, were used to identify IgG antibodies against SARS-CoV-2. The serum samples were obtained from animals housed in Safari Park Dvůr Králové, Czech Republic. The zoo animals are in proximity to humans, facilitating the exploration of potential reverse transmission events of SARS-CoV-2 from humans to animals. Also, they undergo routine veterinary examinations, providing convenient access to blood samples. Therefore, they can be easily used for the 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 (*Ceratotherium simum simum*) and one Persian leopard (*Panthera pardus tulliana*).

Key words: COVID-19, indirect ELISA, proof of concept, SARS-CoV-2, serologic surveillance, virus neutralization test, wildlife surveillance, zoo animals.

INTRODUCTION

Since the onset of the coronavirus disease 2019 (COVID-19) pandemic, many animal species exhibit susceptibility to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Various surveillance modalities have been used to ascertain the vulnerability of wild animals to SARS-CoV-2 infections, including pathogen identification, serologic determination, clinical investigation, and parameter monitoring (Clayton et al. 2022; Italiya, Bhavsar, et al. 2023). 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 may have experienced infection over an extended duration (Decaro et al. 2022). During the pandemic, several studies serologic surveillance carried out on animals, such as wild white-tailed deer (*Odocoileus virginiana*) from US states, pet animals, and stray cats (*Felis catus*) in Spain, revealed the presence of neutralizing antibodies (Chandler

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et al. 2021; Barroso-Arévalo et al. 2022; Villanueva-Saz et al. 2022). It is known that SARS-CoV-2 can cause dangerous life-threating infections in some animals, especially large felids (Giraldo-Ramirez et al. 2021).

Conducting epidemiologic research in wildlife is essential for understanding the biology of SARS-CoV-2 in different animal species. Development of new serologic assays directly in wildlife is a formidable undertaking due to the complexities associated with procuring blood samples from a wide variety of wild species. Conversely, zoos or captive animals present as advantageous opportunities for serologic investigations, providing representative samples that span various species. The accessibility of these samples is facilitated by regular veterinary interventions within zoo settings. Also, 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 (*Panthera tigris*) and lions (*Panthera leo*) were found positive for SARS-CoV-2 infection at the Bronx Zoo, New York, US (McAloose et al. 2020), where epidemiologic and genomic data indicated human-to-tiger transmission, although the specific combination was unique. Similarly, a coatimundi (*Nasua nasua*) and a fishing cat (*Prionailurus viverrinus*) were found positive in an Illinois zoo (Brookfield, US; Allender et al. 2022), and other animal species such as spotted hyenas (*Crocuta crocuta*, Sparrer et al. 2023), Canadian lynx (*Lynx canadensis*, Tewari et al. 2023), Eurasian river otter (*Lutra lutra,* Padilla-Blanco et al. 2022), and gorillas (*Gorilla gorilla,* Nagy et al. 2022) have tested positive in various zoos around the world. Genomic data from infected zookeepers suggest human-to-animal transmission in these cases. Many zoo-kept SARS-CoV-2 permissive animals are endangered and suffer from other serious threats in the wild.

The ELISA is the most commonly used serologic assay that plays a pivotal role in advancing epidemiologic investigations, offering a cost-effective, sensitive, and specific tool for detecting antibodies in diverse populations (Shah and Maghsoudlou 2016). The ELISA is one of the commonly used serologic techniques for detecting antibodies against SARS-CoV-2. Conventional ELISA necessitates the use of a particular conjugate against immunoglobulin G (IgG) and requires optimization for each species of animal. Use of protein A, isolated from the cell wall of *Staphylococcus aureus*, streamlines this process by demonstrating a specific affinity for the fragment crystallizable (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).

The advancement of novel serologic assays, particularly ELISA, is crucial for accurate detection of pathogen-specific antibodies in wildlife and zoo populations, facilitating improved disease surveillance and epidemiologic studies. Our aim was to use a protein A–based indirect ELISA method to generate baseline data on protein A interactions with antibodies from various wild animal species and to establish proof of concept for using protein A–based ELISA in wildlife surveillance, using SARS-CoV-2 as an example and a viral neutralization test for result confirmation.

MATERIALS AND METHODS

Serum sample collection

Blood samples were obtained from 88 animals representing 37 species, 10 families, and four orders from Dvůr Králové (Dvůr Králové nad Labem, Czechia, European Union) in the period from May 2020 to January 2022 (Table 1). Animals were not sampled primarily for SARS-CoV-2 serosurveillance but for many different routine veterinary interventions. Only sera remaining after the primary analyses were used in this study; thus, no ethical oversight was required. The positive reference human serum was acquired in May 2021 from a human patient who recovered from COVID-19.

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TABLE 1. Determination of binding efficiency of protein A with antibodies of different species from Safari Park Dvůr Králové (Czechia, EU) in the period from May 2020 to January 2022 using an ELISA performed in triplicate on each sample. The mean and SE of the optical density (OD) are provided for each sample.

Family	Common name	Taxonomic name	Sex[a]	OD (mean±SE)
Hominidae	Human (positive control)	*Homo sapiens*	M	0.90±0.12
Equidae	Somali wild ass	*Equus africanus somaliensis*	F	1.5554±0.09
Suidae	Common warthog	*Phacochoerus africanus*	F	1.502±0.08
Hominidae	Orangutan	*Pongo pygmaeus*	ND[b]	1.4708±0.24
Bovidae	Sitatunga	*Tragelaphus spekii*	F	1.4162±0.06
Canidae	Jackal	*Canis aureus*	M	1.3655±0.24
Equidae	Maneless zebra	*Equus quagga borensis*	F	1.2954±0.17
Bovidae	Zebu	*Bos taurus indicus*	F	1.1906±0.10
Felidae	Cheetah	*Acinonyx jubatus*	M	1.1577±0.10
Eupleridae	Fossa	*Cryptoprocta ferox*	F	1.01±0.08
Rhinocerotidae	Eastern black rhinoceros	*Diceros bicornis michaeli*	F	0.98±0.43
Canidae	Bat-eared fox	*Otocyon megalotis*	F	0.95±0.14
	African wild dog	*Lycaon pictus*	F	0.93±0.14
Bovidae	Bongo	*Tragelaphus eurycerus*	F	0.92±0.15
	Common eland	*Taurotragus oryx*	F	0.92±0.05
Equidae	Chapmann's zebra	*Equus quagga chapmani*	ND	0.9172±0.15
	Grévy's zebra	*Equus grevyi*	F	0.91±0.11
Bovidae	Dahomey dwarf cattle	*Bos taurus africanus*	F	0.90±0.09
	Impala	*Aepyceros melampus*	F	0.88±0.04
	Lesser kudu	*Tragelaphus imberbis*	M	0.87±0.15
	Greater kudu	*Tragelaphus strepsiceros*	F	0.87±0.14
Equidae	Grant's zebra	*Equus quagga boehmi*	F	0.84±0.02
Felidae	Serval	*Leptailurus serval*	F	0.83±0.06
Camelidae	Dromedary	*Camelus dromedarius*	M	0.81±0.04
Felidae	Lion	*Panthera leo*	F	0.7731±0.36
Suidae	Red river hog	*Potamochoerus porcus*	F	0.77±0.04
Felidae	Persian leopard	*Panthera pardus tulliana*	F	0.76±0.08
Hyaenidae	Striped hyena	*Hyaena hyaena*	F	0.74±0.05
Bovidae	African forest buffalo	*Syncerus caffer nanus*	F	0.73±0.04
	Thomson's gazelle	*Eudorcas thomsonii*	M	0.71±0.06
	Sable antelope	*Hippotragus niger*	F	0.70±0.10
	Nyala	*Tragelaphus angasii*	M	0.70±0.04
Rhinocerotidae	White rhinoceros	*Ceratotherium simum*	F	0.68±0.03
Equidae	Plains zebra	*Equus quagga*	F	0.67±0.08
Bovidae	Roan antelope	*Hippotragus equinus*	M	0.65±0.05
	Black wildebeest	*Connochaetes gnou*	M	0.62±0.18
Bovidae	White-bearded wildebeest	*Connochaetes taurinus albojubatus*	F	0.5151±0.00
	Mountain reedbuck	*Redunca fulvorufula*	F	0.4842±0.05
	Scimitar-horned oryx	*Oryx dammah*	F	0.4760±0.09
	Lechwe	*Kobus leche*	F	0.377043±0.04
	Nile lechwe	*Kobus megaceros*	F	0.4709±0.09
	Cameroon dwarf goat	*Capra aegagrus hircus*	F	0.3473±0.02
	Daha gazelle	*Nanger dama*	F	0.3366±0.02
	Somalian sheep	*Ovis aries*	F	0.3048±0.01
	Blue wildebeest	*Connochaetes taurinus*	F	0.2496±0.01
Negative control	NA[c]	NA	NA	0.3986±0.01

[a] M = male; F = female.
[b] ND = data not available.
[c] NA = not applicable.

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Preparation of antigen

We prepared an antigen using a heat-inactivated sample of SARS-CoV-2 (strain: human/Czech Republic/951/2020), provided by Dr. Jan Weber from the Institute of Organic Chemistry and Biochemistry in Prague, Czech Republic. For virus propagation, Vero cells (ATCC CCL-81; American Type Culture Collection, Manassas, Virginia, USA) were cultured in Dulbecco modified Eagle's medium (DMEM enriched with 10% newborn calf serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 1% glutamine (all reagents sourced from Sigma-Aldrich, Prague, Czech Republic). This virus was passaged through Vero cells a maximum of five times before use.

Determination of protein A and HRP horseradish peroxidase binding efficiency to antibodies of different animal species

The capability of protein A to bind to IgG of a given species was determined by an ELISA. For each species, one to five individuals (depending on availability) were tested. Tested sera were diluted in phosphate buffered saline (PBS), pH 7.4 (Carl Roth, Karlsruhe, Germany) in a 1:10 ratio, and 100 µL of diluted serum was added to each well of flat-bottomed microplates (SPL Immunoplate, Downey, California, USA) for coating. 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 rinsed three times with washing buffer (1× PBS with 0.05% Tween 20). The wells were then blocked by incubating with 100 µL of 2% bovine serum albumin (BSA) in PBS and washed again. Next, 100 µL of a protein A-horseradish peroxidase (protein A-HRP) conjugate (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at a dilution of 1:1,000 in PBS was added to each well. Plates were incubated for 1 h at 4 C and washed, as stated previously. The wells were then incubated with 50 µL of chromogenic substrate (SureBlue TMB PLUS2, Kementec, Taastrup, Denmark). After 15 min at room temperature (in a dark area), the reaction was halted by the addition of 50 µL of 2 M sulfuric acid. A photometer (Infinite 200 PRO, Tecan, Männedorf, Switzerland) 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 semiquantitatively determined by interpreting optical density (OD) 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. Each sample was tested in triplicate.

Nonspecies-dependent ELISA

Initially, we used checkerboard titration to identify the ideal concentrations of all chemicals, as well as the optimal volumes and reaction conditions. Also, sera were only chosen for the analysis if the IgG of that species had shown a medium or high binding affinity with protein A-HRP. The final optimal conditions for ELISA (SPL Immunoplate) using the protein A-HRP instead the secondary antibody were as follows: Wells were loaded with 100 µL of inactivated SARS-CoV-2 (virus titer 10^6 plaque-forming units [PFU]/mL), diluted in PBS, pH 7.4, in a 1:2,000 ratio, and incubated overnight at 4 C in a humid environment, and then rinsed three times with washing buffer (1× PBS with 0.05% Tween 20). The wells were then blocked by incubating with 100 µL of 2% BSA in PBS for 2 h at room temperature and washed. Next, the wells were incubated overnight at 4 C in a humid environment with animal sera diluted in a 1:800 ratio in PBS and then washed three times. Protein A-HRP (Thermo Fisher Scientific) diluted in a 1:200 ratio was then used instead of the secondary antibody. After that, the wells were washed a final time, and the final incubation with 50 µL of SureBlue TMB HRP substrate (Seracare, Manasaas, Virginia, USA) was added. The reaction was developed for 15 min and inhibited by 50 µL of 2 M H_2SO_4. The photometric measurements were taken at a wavelength of 450 nm, as described earlier. Each sample was tested in triplicate. As the positive control usually had an absorption value of about 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).

Virus neutralization test

A virus neutralization test (VNT) was performed in a Biosafety Level 3 laboratory setting. The samples for VNT were chosen based on the

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high absorption rate in the ELISA experiment. Inactivated animal sera were diluted (1:4 in complete DMEM (supplemented with 10% fetal bovine serum, 1% antibiotics, and 1% glutamine; Biosera, Boussens, France). Next, twofold serial dilutions of sera (50 µL) were incubated for 90 min at 37 C with 10^3 PFU/mL of SARS-CoV-2 (50 µL per well) in a 96-well plate. Vero cells (ATCC CCL-81) were added (5×10^4 cells in 100 µL per well), and after 4 days of incubation (37 C and 5% CO_2), the cytopathic effect was investigated. The highest serum dilution that inhibited the cytopathic effect of the SARS-CoV-2 virus was regarded as the end point titer (Brzuska et al. 2023).

RESULTS

Determination of binding affinity of protein A

The ability of the protein A-HRP conjugate to bind to the sera of different animal species varied, depending on the species. As shown in Table 1, 24 of 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 eight had no or low binding affinity (absorption 0.2–0.6). Only serum samples from species with a high or medium affinity for protein A-HRP were used in the subsequent studies.

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. Overall, 16 zoo animal serum samples tested positive in the 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 (one tested positive twice repeatedly), one striped hyena (*Hyaena hyaena*), two white rhinoceros (*Ceratotherium simum*, one tested positive twice), and one red river hog (*Potamochoerus porcus*). These high-absorbance samples were then tested with the VNT. In VNT, three serum samples demonstrated the ability to inhibit growth of SARS-CoV-2 but only at 1:10, the lowest dilution tested (Table 3). The positively tested animals in VNT were one Persian leopard and two white rhinoceros, one of which was tested twice nearly a year apart but was only positive in the VNT, with serum taken at the first time point.

Health status of seropositive animals with SARS-CoV-2 antibodies

None of the seropositive (ELISA or VNT) animals showed any respiratory-related signs 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 no individuals were detected as being infected with the SARS-CoV-2.

DISCUSSION

The ELISA is a widely used 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 microorganism was able to replicate within such an animal host. It is possible that the animal came into contact with the microorganism and that the pathogen 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 serologic surveillance of various pathogens in diverse wildlife populations. The critical point in ELISA is usually the use of secondary antibodies against the Fc region of the species' immunoglobulin (Liyanage et al. 2023). Secondary antibodies are often unavailable for many wild animal species; consequently, less specific antibodies designed for the domesticated counterparts must be used (Smit 2017). In other species, there is no appropriate domesticated counterpart. Therefore, a non–species-specific

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TABLE 2. Results of the non–species-specific indirect ELISA (performed in triplicate for each sample) for antibodies against severe acute respiratory syndrome coronavirus 2 in species from Safari Park Dvůr Králové (Czechia, EU) in the period from May 2020 to January 2022. The samples highlighted in bold were used for additional virus neutralization testing.

No.	Family	Common name	Taxonomic name	Sex[a]	Date of sample collection	Optical density (mean+SE)
1	Hominidae	Human (positive control)	*Homo sapiens*	M	7 May 2021	0.89±0.07
2	Bovidae	Greater kudu	*Tragelaphus strepsiceros*	F	22 July 2020	0.26±0
3		Lesser kudu	*Tragelaphus imberbis*	M	23 June 2020	0.3±0.02
4		Thomson's gazelle	*Eudorcas thomsonii*	M	23 July 2020	0.21±0.01
5		Impala	*Aepyceros melampus*	F	30 April 2020	0.19±0.01
6		African forest buffalo	*Syncerus caffer nanus*	F	16 July 2020	0.18±0.01
7		Roan antelope	*Hippotragus equinus*	M	6 August 2020	0.19±0.03
8		Dahomey dwarf cattle	*Bos taurus africanus*	F	1 October 2020	0.26±0.03
9		Bongo	*Boocercus euryceros isaaci*	F	20 August 2020	0.69±0.04
10		Dahomey dwarf cattle	*Bos taurus africanus*	F	9 June 2020	0.22±0.01
11		Dahomey dwarf cattle	*Bos taurus africanus*	M	9 June 2020	0.28±0.04
12		Sable antelope	*Hippotragus niger*	F	24 June 2020	0.18±0.01
13		Sable antelope	*Hippotragus niger*	M	23 June 2020	0.19±0.01
14		Common eland	*Taurotragus oryx*	F	5 November 2020	0.25±0.06
15		Nyala	*Tragelaphus angasii*	M	21 October 2020	0.19±0
16		Common eland	*Taurotragus oryx*	F	5 November 2020	0.2±0.01
17		Greater kudu	*Tragelaphus strepsiceros*	M	7 May 2020	0.55±0.07
18		Black wildebeest	*Connochaetes gnou*	M	1 June 2020	0.2±0.01
19		Sable antelope	*Hippotragus niger*	F	4 June 2020	0.23±0.01
20		Nyala	*Tragelaphus angasii*	F	2 June 2020	0.22±0.01
21		Lesser kudu	*Tragelaphus imberbis*	F	23 June 2020	0.45±0.1
22		Lesser kudu	*Tragelaphus imberbis*	F	21 July 2020	0.42±0.12
23		Thomson's gazelle	*Eudorcas thomsonii*	F	4 September 2020	0.26±0.05
24		Greater kudu	*Tragelaphus strepsiceros*	F	18 September 2020	0.47±0.14
25		Greater kudu	*Tragelaphus strepsiceros*	F	23 July 2020	0.65±0.38
26		Mountain reedbuck[b]	*Redunca fulvorufula*	F	15 April 2021	0.38±0.06
27		Mountain reedbuck[b]	*Redunca fulvorufula*	F	25 October 2021	0.31±0.00
28		Lechwe	*Kobus leche*	F	5 February 2021	0.36±0.01
29		Lesser kudu	*Tragelaphus imberbis*	F	19 March 2021	0.35±0.05
30		Sable antelope	*Hippotragus niger*	F	25 June 2021	0.34±0.07
31		Lesser kudu	*Tragelaphus imberbis*	F	19 March 2021	0.34±0.01
32		Lesser kudu	*Tragelaphus imberbis*	M	17 March 2021	0.33±0.03
33		White-bearded wildebeest	*Connochaetes taurinus albojubatus*	F	15 October 2021	0.32±0.01
34		Lesser kudu	*Tragelaphus imberbis*	M	19 March 2021	0.32±0.01
35		Mountain reedbuck	*Redunca fulvorufula*	F	16 April 2021	0.32±0.01
36		Zebu	*Bos taurus indicus*	F	22 January 2021	0.31±0.01
37		Mountain reedbuck	*Redunca fulvorufula*	F	6 January 2021	0.31±0.02
38		Thomson's gazelle	*Eudorcas thomsonii*	ND[c]	16 February 2021	0.30±0.02
39		Impala	*Aepyceros melampus*	M	17 March 2021	0.31±0.02
40		Thomson's gazelle	*Eudorcas thomsonii*	M	14 April 2021	0.29±0.00
41		Sitatunga	*Tragelaphus spekii*	F	12 November 2021	0.36±0.01
42		Somali sheep	*Ovis aries*	F	20 January 2021	0.32±0.01
43	Camelidae	Dromedary	*Camelus dromedarius*	M	28 October 2020	0.38±0.06

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TABLE 2. Continued.

No.	Family	Common name	Taxonomic name	Sex[a]	Date of sample collection	Optical density (mean+SE)
44	Canidae	African wild dog	*Lycaon pictus*	F	5 August 2020	0.5±0.14
45		**Bat-eared fox**	***Otocyon megalotis***	**F**	**31 August 2020**	**0.92±0.06**
46		**African wild dog**	***Lycaon pictus***	**F**	**5 August 2020**	**0.91±0.18**
47		African wild dog	*Lycaon pictus*	F	5 August 2020	0.64±0.13
48		Jackal	*Canis aureus*	M	ND	0.59±0.02
49		Jackal	*Canis aureus*	F	ND	0.57±0.05
50	Equidae	**Grévy's zebra**	***Equus grevyi***	**F**	**29 April 2020**	**1.17±0.22**
51		Plains zebra	*Equus quagga*	F	28 May 2020	0.19±0.03
52		Plains zebra[b]	*Equus quagga*	F	28 April 2020	0.19±0
53		Plains zebra[b]	*Equus quagga*	F	16 April 2021	0.34±0.01
54		Plains zebra	*Equus quagga*	F	28 May 2020	0.25±0.02
55		Plains zebra	*Equus quagga*	F	26 June 2020	0.2±0
56		Plains zebra	*Equus quagga*	F	28 May 2020	0.24±0
57		**Grévy's zebra**	***Equus grevyi***	**F**	**30 April 2020**	**0.94±0.31**
58		Grévy's zebra	*Equus grevyi*	F	1 June 2020	0.45±0.04
59		Grant's zebra	*Equus quagga boehmi*	F	14 April 2021	0.57±0.07
60		Maneless zebra	*Equus quagga borensis*	M	23 December 2021	0.38±0.03
61		Grant's zebra	*Equus quagga boehmi*	F	24 June 2021	0.37±0.03
62		Somali wild ass	*Equus africanus somaliensis*	F	7 January 2021	0.36±0.05
63		Maneless zebra	*Equus quagga borensis*	F	14 April 2021	0.36±0.02
64		Maneless zebra	*Equus quagga borensis*	ND	4 July 2021	0.34±0.03
65		Chapmann's zebra	*Equus quagga chapmani*	ND	4 January 2022	0.34±0.00
66		Grant's zebra	*Equus quagga boehmi*	F	15 April 2021	0.33±0.01
67		Chapmann's zebra	*Equus quagga chapmani*	ND	2 April 2021	0.33±0.00
68		Maneless zebra	*Equus quagga borensis*	F	31 March 2021	0.32±0.00
69		Maneless zebra	*Equus quagga borensis*	F	3 August 2021	0.32±0.01
70	Eupleridae	**Fossa**	***Cryptoprocta ferox***	**F**	**12 November 2020**	**0.86±0.01**
71	Felidae	**Serval**	***Leptailurus serval***	**F**	**5 August 2020**	**1.12±0.1**
72		**Persian leopard**	***Panthera pardus saxicolor***	**M**	**19 September 2020**	**0.93±0.12**
73		**Serval**	***Leptailurus serval***	**M**	**5 August 2020**	**0.86±0.1**
74		**Persian leopard**	***Panthera pardus saxicolor***	**F**	**18 August 2020**	**1.01±0.14**
75		**Lion**	***Panthera leo***	**F**	**22 January 2021**	**0.81±0.10**
76		**Lion**[b]	***Panthera leo***	**M**	**15 April 2021**	**0.76±0.04**
77		**Lion**[b]	***Panthera leo***	**M**	**23 November 2021**	**0.72±0.09**
78		Lion	*Panthera leo*	M	28 November 2021	0.60±0.01
79		Lion	*Panthera leo*	ND	25 November 2021	0.37±0.01
80		Cheetah	*Acinonyx jubatus*	M	25 July 2021	0.56±0.01
81		Cheetah	*Acinonyx jubatus*	F	25 July 2021	0.49±0.03
82		Cheetah	*Acinonyx jubatus*	ND	ND	0.36±0.01
83	Hominidae	Orangutan	*Pongo pygmaeus*	ND	26 October 2021	0.59±0.01
84	Hyaenidae	**Striped hyena**	***Hyaena***	**F**	**2 October 2020**	**1.48±0.54**
85	Rhinocerotidae	**White rhinoceros**[b]	***Ceratotherium simum simum***	**F**	**21 July 2020**	**0.84±0.08**
86		White rhinoceros[b]	*Ceratotherium simum simum*	F	21 July 2021	0.67±0.05

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TABLE 2. Continued.

No.	Family	Common name	Taxonomic name	Sex[a]	Date of sample collection	Optical density (mean+SE)
87		**White rhinoceros**	***Ceratotherium simum simum***	**F**	**23 June 2020**	**1.06±0.31**
88	Suidae	**Red river hog**	***Potamochoerus porcus***	**F**	**4 August 2020**	**0.88±0.03**
89		Common warthog	*Phacochoerus africanus*	F	10 November 2021	0.43±0.03
90	Negative control	NA[d]	NA	NA	NA	0.21±0.01

[a] M = male; F = female.
[b] The same individual sampled and tested twice with distinct time periods.
[c] ND = data not available.
[d] NA = not applicable.

protein A-ELISA is useful tool. Staphylococcal protein A (spA) is a prominent IgG-binding receptor derived from *S. aureus* and characterized by its five unique IgG-binding domains. It plays a critical role in various immunologic applications, particularly in ELISA (Eliasson et al. 1989). Using molecular dynamics simulations to compare the binding strengths of spA and streptococcal protein G (spG) with human IgG, spA has been found to have significantly stronger binding affinity than spG (Lee et al. 2021). However, similar studies are lacking for wild animal species; thus, investigating the binding affinity of protein A with IgG from wildlife species is necessary for developing novel ELISAs usable in multiple species. A study has suggested a lack of discernible correlation between the phylogenetic similarity of families and the 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 (Stöbel et al. 2002). The affinity of protein A for various animal species has been demonstrated previously in zoo animals but not for all species (Stöbel et al. 2002). Therefore, we rescreened protein A-HRP binding ability to sera of all the animal species that we tested in our experiment. In combination with the previously published data (Stöbel et al. 2002), 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 thus may be used for

TABLE 3. Results of the indirect ELISA and virus neutralization test (VNT) for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in samples from the three animals from Safari Park Dvůr Králové (Czechia, EU) that tested positive in both tests and from the human positive control.

No.[a]	Sample ID	Indirect 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	7 May 2021
74	Persian leopard (*Panthera pardus saxicolor*)	1.01	Inhibition of SARS-CoV-2 in dilution up to 1:10, then negative	18 August 2020
85	White rhinoceros (*Ceratotherium simum simum*)	0.84	Inhibition of SARS-CoV-2 in dilution up to 1:10, then negative	21 July 2020
87	White rhinoceros (*Ceratotherium simum simum*)	1.06	Inhibition of SARS-CoV-2 in dilution up to 1:10, then negative	23 June 2020

[a] No. = the sample number and is the same as in Table 2. Only positive samples are shown.

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development of indirect ELISA for any particular microorganism. Of the 18 species that we tested that had also been tested by Stöbel et al. (2002), we found that sera from 12 species exhibited binding efficiencies consistent with those previously reported, while sera from six species: Roan antelope (*Hippotragus equinus*); black wildebeest (*Connochaetes gnou*); mountain reedbuck (*Redunca fulvorufula*); scimitar-horned oryx (*Oryx dammah*); Nile lechwe (*Kobus megaceros*); and Somalian sheep (*Ovis aries*) showed no detectable binding. However, antibodies from these wild bovine species have shown a stronger binding affinity with protein G (Kramsky et al. 2003). Potential reasons for this might be the existence of a lower amount of Ig or total protein in the serum (Liyanage et al. 2023). 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). Given the diverse range of species tested, our findings underscore the importance of characterizing the binding properties of protein A across wildlife species, which could enhance the reliability and applicability of immunoassays in various ecologic contexts. Multispecies Protein A-ELISAs have been developed previously and effectively used in wildlife to detect antibodies against numerous pathogens such as *Brucella*, *Mycobacterium bovis*, *paratuberculosis*, foot-and-mouth disease, tick-borne encephalitis virus, parapoxvirus, and others (Inoshima et al. 1999; Nymo et al. 2013; Pruvot et al. 2013; Hosamani et al. 2015; Inagaki et al. 2016; Bernitz et al. 2021). Our study has added to this list a multispecies Protein A-ELISA capable of detecting antibodies against SARS-CoV-2. Our evaluation in a range of zoo-kept animals demonstrates the potential applicability of multispecies Protein A-ELISA in wildlife serologic surveillance.

Typically, COVID-19 is transmitted from human to human by aerosolized particles, with documented bidirectional transmission between people and animals (Saw et al. 2021; Clayton et al. 2022). Animal infection cases pique interest in viral 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. Further, from a conservation standpoint, it is important to recognize the hazards of SARS-CoV-2 infection to threatened wild animals. Unlike direct virus cultivation or viral nucleic acid detection methods, which offer high specificity and precise virus identification, serologic test results can be challenging to interpret. This difficulty arises partly from potential cross-reactivity with antibodies directed against closely related viral species (Meyer et al, 2014). The specificity of detection can be confirmed through VNT, which serves as a complementary assay to verify ELISA results (Lu et al. 2021).

Although 16 of our samples showed a high absorbance (>0.7) in the ELISA, only three sera, demonstrated the ability to neutralize SARS-CoV-2, albeit only at low dilutions. The positive VNT for the Persian leopard was not surprising, as big cats are known to be sensitive to SARS-CoV-2 infection and has been reported in many felids, including lions, tigers, snow leopards (*Uncia uncia*) and others in zoos (Bartlett et al. 2021; Mitchell et al. 2021; Wang et al. 2022). In addition, a free-living Indian leopard (*Panthera pardus*) was infected with SARS-CoV-2 (Mahajan et al. 2022). The other sera testing positive by VNT (at lower titration, 1:10) were from two southern white rhinoceros, providing the first records of the presence of antibodies against SARS-CoV-2 in white rhinoceros. Notably, SARS-CoV-2 RNA had been found in a fecal sample of white rhinoceros during small-scale coronavirus surveillance in Bandia Reserve, Senegal (Italiya, Vacek, Matějů, et al. 2023). Despite SARS-CoV-2 infection not being reported in any other member of Perissodactyla, bioinformatic, functional, and genetic analyses of SARS-CoV-2 receptor angiotensin-converting enzyme 2 orthologs support

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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, no state of emergency was declared (see Supplementary Material Fig. S1), thus, the zoos and other places for leisure activities in Czechia were open to the public, counterepidemic measures were minimal, and new waves were not expected by the general public. No zookeepers from exhibits of the rhinoceros and carnivores had shown any COVID-19-related symptoms or tested positive before the collection dates of the positive samples.

Our study was subject to some limitations, one of which pertains to the validation of the species nonspecific ELISA developed in-house. The ELISA we used has not undergone validation and was thus used only for preliminary screening purposes. Also, the current ELISA did not test for additional SARS-CoV-2 variants of concern. Our use of whole-virus antigen may provide reduced specificity as a result of an increased probability of nonspecific binding of copurified cellular proteins and nontarget viral proteins, such as nucleocapsid, membrane, and envelope proteins in ELISA. Therefore, only samples positively tested by VNT, which is understood as the gold standard of serologic methods but is very labor intensive and time consuming for a large number of samples, can be considered as confirmed positive for antibodies against SARS-CoV-2.

Our proof-of-concept study confirms that protein A–based multispecies ELISA can serve as a valuable tool for the development of various serologic assays to monitor disease status in wildlife. Nevertheless, it is crucial to conduct validation and assessment of tests on zoo animals prior to implementation in surveillance programs. Also, zoo-kept animals can be understood as sentinels in surveillance for animal species susceptible to SARS-CoV-2 infection (Italiya et al. 2024). As many of the SARS-CoV-2-permissive animals (including those detected in this study) are threatened, this information is crucial for further protection and should be considered in the preparation of conservation strategies of these animals in the wild.

ACKNOWLEDGMENTS

We thank staff in the Dvůr Králové Zoo for animal management, sample collection, and processing. We thank colleagues from the Czech University of Life Sciences Prague and the Veterinary Research Institute, Czechia, for contributions to this study. This study was supported by the Ministry of Health of the Czech Republic (grant NU21-05-00143) and the Czech University of Life Sciences Prague (grant IGA 2024305).

SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/JWD-D-24-00028.

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Submitted for publication 20 February 2024.
Accepted 4 December 2024.

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