Research Article

Serological Assay for SARS-CoV-2 in Domestic and Wild Animals in Middle and Southeast Regions in Brazil

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Abstract

The new coronavirus pandemic has become a major public health crisis and, from a One Health perspective, the need to assess transmissibility to animal species and its consequences has become essential to assess the possible risks of maintaining this agent infectious in other hosts in the nature. Therefore, the present study consisted of a serological survey of SARS-CoV-2 in 950 species of domestic and wild animals, from the states of Mato Grosso do Sul, Minas Gerais and São Paulo, Central and Southeastern regions in Brazil. The serological study used the ELISA ID Screen® "SARS-CoV-2 Double Antigen Multi Species" (ID-Vet.®) test, according to the recommendations in the manufacturer's manual and capable to detect antibodies in different species. The frequency found was 1.37% (13/950) of animals carrying anti-SARS-CoV-2 antibodies, being identified in dogs (2.63%), horses (1.46%), cattle (1. 09%) and bats (2.70%). The tapir was the only seronegative species for Covid-19. The horses belonging to the Military Police of the State of São Paulo were able to be monitored and observed that some animals showed a long period of antibody persistance, as occurs in humans. This serological survey contributed to evaluating possible hosts of SARS-CoV-2 in different animal species from the perspective of One Health, in Brazil

Keywords: Covid-19, Pandemic, Animal hosts, Epidemiology, serodiagnosis, One health

Introduction

The emergence of a new coronavirus in 2019, which emerged in the city of Wuhan/China, which affected the population at a global level, was declared a pandemic on March 11, 2020 by the World Health Organization (WHO). The virus called SARS-CoV-2 caused a clinical picture of acute respiratory syndrome, evidenced by cough, fever and fatigue in infected individuals, most of the time, fatal and whose main route of transmission was respiratory [1].

The Covid-19 pandemic quickly spread to all continents, affected different hosts, humans and animals and, because it has affected the world economy, it constitutes a problem of only one Health. The most affected countries were the emerging countries [2-6].

SARS-CoV-2 is a single-stranded RNA virus belonging to the Coronaviridae family, making it the seventh virus in the family capable of infecting humans [2-3]. Regarding its origin, it is assumed that the virus has a zoonotic origin, given its similarity with other bat SARS-CoV and MERS-CoV viruses, and was subsequently transmitted to humans through

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a pathogenic spillover event followed by adaptation, or via a possible mammalian intermediate host such as pangolins, cats, ferrets or minks [1, 5, 7]. The pathway of entry of the virus into the human cell occurs through the interaction between the virus receptor-binding domain (RBD) and the angiotensin convertase 2 (ACE2) receptor, which has orthologous genes in other mammals [1, 8-9]. Given that the S1 subunit and RBD are the major sources of genetic variability of the virus, their genetic plasticities may allow SARS-CoV-2 to initially bind to and subsequently adapt to the ACE2 receptor of several animal species, increasing the likelihood of the virus crossing the species barrier [10-11].

In addition to humans, several cases of animal infection by SARS-CoV-2 have been reported in several countries since the beginning of the pandemic in 2020, affecting domestic, wild, zoo, and farm animals [12-18], where at least 32 animal species in 32 countries have been infected by the virus [11, 19]. The first confirmed cases occurred in domestic animals that had had contact with humans infected with SARS-CoV-2, which initiated debates about the transmissibility of the virus from animals [4]. According to studies by Shi et al., 2020, dogs were asymptomatic for the respiratory symptoms of the disease, indicating inefficient replication of the virus in their bodies, and therefore, presenting low susceptibility to infection; Cats affected by the disease exhibited respiratory symptoms and the ability to transmit the virus through their airways, suggesting high replication, transmission, and seroconversion [3, 12]. Regarding farm animals, studies indicate that cattle, chickens, and ducks have little or no susceptibility to the SARS-CoV-2 virus [4, 8, 20-22].

In the last three decades, the emergence of diseases of zoonotic origin has become an increasing risk with the outbreaks of Ebola, avian influenza and the SARS and MERS coronavirus syndromes. Its prevention strategies are difficult to implement, since they involve several factors such as genetic evolution of the virus, demographic changes, interaction between wild, domestic and human animals, as well as environmental and climatic conditions of the ecosystem [23]. According to the Editorial "Emerging Zoonoses: A One Health Challenge", the One Health Approach is necessary to prevent the emergence of new zoonotic diseases by promoting the interdependence of human, animal and environmental health, thus involving multidisciplinary strategies such as the implementation of programs, monitoring policies and research that can communicate with each other at the academic and public health level [24].

With the scarcity of research and articles on the transmission and effects of SARS-CoV-2 infection in animals, it is essential that new studies be carried out in order to understand their susceptibility to contamination, given that some species can act as natural reservoirs of the virus, promoting its constant replication and mutation and, consequently, may interfere with transmissibility, pathogenicity and adaptation of the virus, in addition to having the potential to impair the effect of vaccines already applied against the new coronavirus in humans [6].

The general objective of this project was to carry out a serological survey in different animal species, whether domestic or wild, in order to detect if there is the presence of antibodies to SARS-CoV-2, and thus, evidence the risk of transmission to animals hosts.

Materials and Methods

This study was approved by the Ethics Committee on the Use of Animals (CEUA-IB), under protocol number 181/22, highlighting that this study was in accordance with the Ethical Principles in Animal Experimentation adopted by the Brazilian Society of Science in Laboratory Animals (SBCAL/COBEA), by the National Council for the Control of Animal Experimentation (CONCEA) and the Brazilian Guideline for the Care and Use of Animals for Scientific and Didactic Purposes (DBCA).

Sampling

For laboratory analysis, 950 samples of animal serum were selected, 886 of which were from domestic animals (with 367 cattle sera, 38 canine sera and 481 equine sera) and 64 samples from wild animals (27 serums belonging to tapirs and 37 from bats).The bovine serum samples were randomly selected from the Biobank of the Bovine Virus Laboratory of the Biological Institute of São Paulo, from 2020 to 2022, without distinction between sex and age of the animals, from the States of São Paulo and Minas Gerais.

Samples of canine and equine serums were collected from 2022 to 2023, respectively, from the kennel and cavalry of the Military Police of São Paulo. Based on the serological results obtained, serological monitoring of horses belonging to the Military Police was carried out to assess the persistence of anti-SARS-CoV-2 antibodies. Thus, the number of final analyses was greater than the number of animals that had their sera collected. Samples of bat serum (*Desmodus rotundus*) were collected in São Paulo in July 2022, while samples of tapirs (*Tapirus terrestris*) were collected in August 2022 in the state of Mato Grosso do Sul.

All serum samples were frozen and transported to the Bovine Virus Laboratory of the Biological Institute of São Paulo for further laboratory analysis.

ELISA antibody test

To carry out this project, it was necessary to request the approval of the Ministry of Agriculture, Livestock and Supply for the import of the indirect ELISA kit for SARS-CoV-2 from the manufacturer IDVet. Laboratory analysis was conducted using IDVet®'s "ID Screen® SARS-CoV-2

Double Antigen Multi-Species" ELISA kit, which is capable of detecting the presence of antibodies in serum or whole blood samples. This kit had a specificity of 99.1% and high sensitivity, according to previous studies [21]. The laboratory procedure followed the manufacturer's recommendations. In general, N antigens (SARS-CoV-2 nucleocapsid) were adsorped to a 96-cavity plate and samples were subsequently added to screen for antibodies [25] and negative and positive controls. After completing the incubation time of 45 minutes, in an oven at 37ºC, the plate was subjected to five washes followed by drying and addition of the anti-nucleoprotein conjugate labeled with the peroxidase enzyme (HRP). After completing the incubation time of 30 minutes at room temperature, the plate was subjected to five washes followed by drying and addition of the substrate. The plate was incubated for 20 minutes at room temperature and protected from light followed by the addition of the blocking solution. The positive control, as well as the serum samples containing antibodies, revealed a yellow color. The plate was read in a spectrophotometer at 450nm, to measure by spectrophotometry, the optical density and estimate the concentration of anti-SARS-CoV-2 antibodies in the sample.

Criteria for test validation:

- Positive control: average value > 0.35

- Positive control/Negative control: mean value > 3

Criteria for test interpretation:

- Amostra reagente: S/P% ≥60

- Non-reactive sample: S/P%<50

- Inconclusive display: 50<S/P%<60

At the end of the laboratory analysis, the results were transferred to an Excel spreadsheet for evaluation in the different domestic and wild animal species for statistical analysis. The animals seropositives in the ELISA test, were repeated in triplicate, in order to confirm the first observed result.

Results

The different animal species were analyzed from 2020 to 2023, i.e., at the beginning and end of the Covid-19 pandemic and beloging to three Brazilian states: São Paulo, Mato Grosso do Sul, and Minas Gerais. Of the population of animals studied, it was found that the majority belonged to the State of São Paulo (825), followed by Minas Gerais (98) and Mato Grosso do Sul (27), as described in Table 1.Of the 950 serum samples analyzed, 13 (1.3%) animals were seropositives for SARS-CoV-2, which 1 canine, 8 horses, 4 cattle and 1 bat. From the serum samples of tapirs, all were non-reactive. Figure 1 shows the frequency of SARS-CoV-2 detected by species and locality (Figure 1).

Table 1: Brazilian States evaluated for SARS-CoV-2, according year.

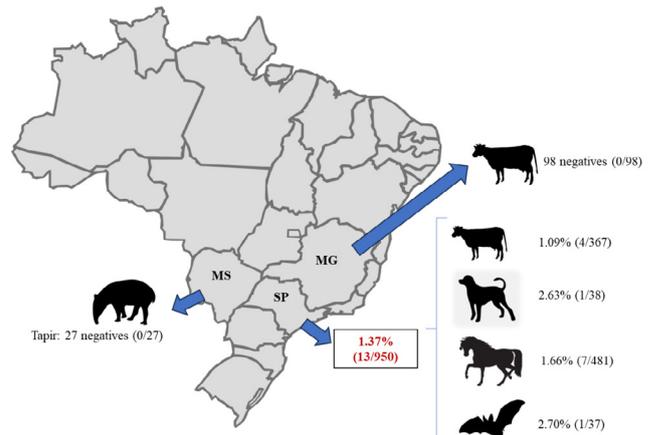| Brazilian States	Year			Total analyzed
	2020	2021	2022	
São Paulo	215	40	570	825
Minas Gerais	60	38	0	98
Mato Grosso do Sul	0	0	27	27
Total analyzed	275	78	597	950

Figure 1: Frequency of SARS-CoV-2 according to animal species and Brazilian States.

MG = Minas Gerais, MS = Mato Grosso do Sul, SP = São Paulo

When assessing the frequency of SARS-CoV-2 according to sex, it was observed that 672 males, 268 females, and 11 uninformed were analyzed, as shown in Table 3.

Bovine Seroprevalence

The study in cattle was carried out in the period of 2020 and 2021, that is, during the Covid-19 pandemic. Of the 367 cattle analyzed, 1.09% (4/367) of reactive animals were detected, and only seropositive cattle from the State of São Paulo were identified. Table 1 stratifies them into two groups: Group 1 – presence of respiratory symptoms, with 61 cattle being analyzed and 2 seropositive animals identified, and Group 2 – absence of respiratory symptoms, 306 cattle being analyzed and 2 seropositive were identified. The 61 cattle belonging to Group 1 were tested for bovine respiratory syncytial virus (BRSV) or bovine parainfluenza type 3 (bPI3), and nine animals were identified for BRSV, six for bPI3 and five for antibodies to the two viral species (BRSV and bPI3), with antibody titers ranging from Log 0.3 to 3. There were no reports that these animals were vaccinated for BRSV and bPI3. The two cattle tested positive for covid-19, one was female and seronegative for BRSV and bPI3 and the other did not have the sex informed and presented co-infection for both bovine respiratory viruses. The two cattle belonging to group 2 reagents for covid-19 were male and had no history of vaccination for BRSV and bPI3.

Table 3: Frequency of antibodies anti-SARS-CoV-2 according to species and gender.

Gender	Equine		Bovine		Canine		Bat		Tapir		Total analyzed
	Reactor	Non reactor	Reactor	Non reactor	Reactor	Non reactor	Reactor	Non reactor	Reactor	Non reactor	
Male	6	316	2	300	0	25	0	9	0	15	673
Female	1	157	1	53	1	13	1	27	0	12	266
Not informed	n/a	n/a	1	10	n/a	n/a	n/a	n/a	n/a	n/a	11
Total	7	473	4	363	1	38	1	36	0	27	950

n/a not applicable

All seropositive samples were retested in triplicate to confirm the initial diagnosis.

Equine Seroprevalence

The horses were analyzed in 2022 to 2023. Of the 481 equine serum samples, 321 were males and 160 females, which seven animals seropositive were identified (six male and one female) and six of them were monitored for the persistence of anti-SARS-CoV-2 antibodies over time (Figure 2). The horses 1 and 2 only presented antibodies in the first analysis on May 2022. The horses 3 and 4 showed a persistence of antibodies until January 2023. The horse 6 was analyzed on June 2022 and presented antibodies anti-SARS-CoV-2, however on September 2022 was considered seronegative in ELISA test. Considering that horses may moved up to another Military Police unit, the same animal was not tested on January 2023.

Canine Seroprevalence

Of the 38 dog samples analyzed, only one animal was seropositive, and its analysis was repeated in triplicate to confirm the laboratory diagnosis. A new serum was collected from this animal one month after the first analysis. Both serum samples were tested in triplicate, in the same reaction, with a reactive result of 172.52% (1st analysis) and inconclusive (2nd analysis), as it presented 58.69% (inconclusive 50-60%), as shown in Figure 3. When comparing the two results, it was found that the level of antibodies decreased between the first and second analyses.

Table 2: Frequency of cattle reactive for SARS-CoV-2, classified according to respiratory symptoms and gender.

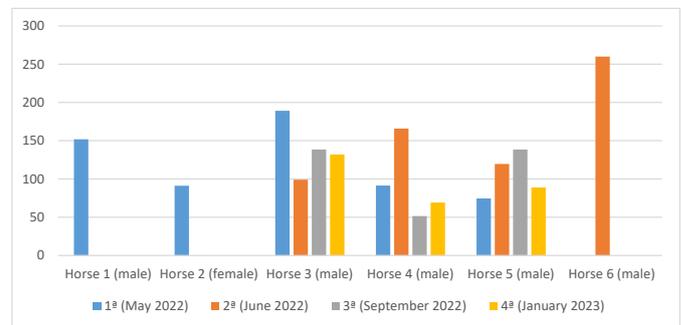| Respiratory symptoms	Total number of samples analysed	Number of animals reactive to Covid-19	Gender	Frequency (%)
Presence	61	1	Female	3.28
		1	not informed	
Absence	306	2	Male	0.65

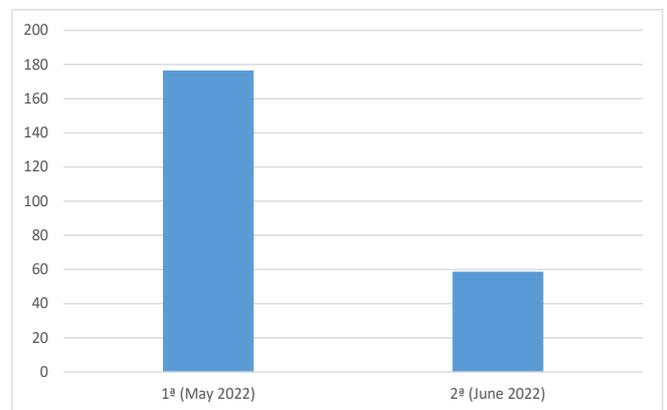**Figure 2:** Evaluation of persistence of antibodies anti-SARS-CoV-2 in horses.

Figure 3: Evaluation of persistence of antibodies anti-SARS-CoV-2 in only one dog seropositive.

Tapir (Tapirus terrestris)

Of the 27 tapir serum samples, all were seronegative for COVID-19.

Bat (Desmodus rotundus)

A total of 37 bats of the hematophagous species, *Desmodus rotundus*, were analyzed, and one male animal was identified with antibodies against SARS CoV-2 (S/P% 64.1).

Discussion

The COVID-19 pandemic has brought reflections on the one-health approach, the importance of integrated action to prevent emerging and re-emerging diseases [40]. The World Organization for Animal Health quickly positioned itself for institutions working in animal health to propose to conduct studies on covid-19 in animals [41]. Brazil has reported more than 37 million cases of COVID-19, according to WHO data. With the high prevalence of cases among humans, human-animal transmission has become proportionally increased [26-29]. The present study in domestic and wild animals comprised the pandemic period from 2020 to 2022 and post-pandemic, represented by the oscillation of SARS CoV-2 waves in humans in the State of São Paulo and its different variants detected over time.

This randomized study identified the ability of SARS CoV-2 to infect different animal species, whether domestic or wild, with or without clinical symptoms compatible with respiratory diseases. The frequency of anti-SARS-CoV-2 antibodies was higher in domestic animals, although the sample number was lower for wild animals. In the literature consulted, the main way SARS-CoV-2 is transmitted to animals is through close contact with humans. Thus, it was evidenced that horses and dogs belonging to the Military Police of São Paulo, because they carry out policing rounds and have intense contact with other humans and with the police officers themselves, explain a higher prevalence of anti-SARS-CoV-2 antibodies in relation to other animal species, such as cattle, tapir and bats.

Few cases of SARS-CoV-2 infection in horses are reported, however, studies indicate that the equine ACE2 receptor (eqACE2) has an affinity for the RBD of the virus, similar to what occurs in humans [32-33]. The exposure of horses to SARS-CoV-2 occurs, however, it is not always detectable by RT-PCR and, for this reason, studies that show the presence of anti-SARS-CoV-2 antibodies in these animals indicate that there was a previous infection [34]. In the present study, when monitoring the horses, it was found that in two animals, the presence of antibodies was detected for up to eight months, approximately, which corroborates findings in the literature, which detected horses for at least 21 days [33]. In humans, it was evidenced that neutralizing antibodies to SARS-CoV-2 lasted up to 10 months [35].

In the bovine species, the frequency of animals carrying anti-SARS-CoV-2 antibodies, with or without respiratory symptoms, was similar to the findings of the literature. Wernike et. al, detected a seroprevalence of 1.1% (11/1000) in cattle from different farms [31]. The absence of severe symptoms related to covid-19 in cattle can be explained by immunological cross-protection, possibly between SARS-CoV-2 and the bovine coronavirus, due to the high homology observed between the epitopes of the spike protein of these two viruses, given that the latter already affects cattle [30]. In Brazil, there are cases of bovine coronavirus associated with diarrhea and associated with respiratory problems, called winter dysentery [38-39], and a vaccine is adopted to control it. In the present study, we did not obtain information on whether the animals received vaccine for bovine coronavirus and whether there would be cross-reactivity in the ELISA test for SARS CoV-2.

Similar to what happens with cattle, the high genetic similarity between SARS-CoV-2 and the canine respiratory coronavirus (CRCoV) can promote some immune protection, preventing them from contracting the severe form of covid-19 [30]. In the present study, only one animal seropositive for Covid-19 was detected, however, the persistence time is unknown, since only two samples were evaluated, and the last one was considered inconclusive, which denotes a gradual drop in antibodies. Decaro *et al.*, 2022 detected antibodies in the serum of dogs around 10 months after contact with the SARS-CoV-2 virus, where the animals were monitored soon after their exposure and infection by the virus, confirmed by RT-PCR [36]. The fact that the Military Police dogs received a vaccine against coronavirus disease may have promoted cross-protection and, consequently, a more efficient response to SARS CoV-2 infection, since the animal did not present, during the study period, any respiratory or enteric condition. Further studies should be conducted to evaluate these issues.

The serological results of covid-19 in wild animals corroborate the need for human contact to promote a higher rate of infection in these species. In the literature, cases of wild animals with anti-SARS-CoV-2 antibodies have been described, but it is believed that these animals became infected by foraging in regions close to human contact [37]. In the present study, the fact that the seropositive bat came into contact with humans, although remote, is possible since this animal belonged to a region that carried out anthropogenic activity. Another possibility is a cross-reactivity with other bat coronaviruses. The antigen used in the ELISA kit has high similarity with other coronaviruses such as SARS and MERS natural to bats.

Conclusions

This research showed the susceptibility of domestic and wild animals to the SARS-CoV-2 virus, through the identification of animals with anti-SARS-CoV-2 antibodies. The persistence of antibodies in animals was observed in horses, similar to what occurs in humans. This study contributed to the understanding of SARS-CoV-2 in domestic and wild animals, in line with what was proposed by the World Organization for Animal Health to promote one health.

Author Contributions

All the authors contribute equally to develop this research

Funding

Institutional Review Board Statement

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of the Biological Institute of São Paulo (protocol code 181/22, 05/10/2022).

Acknowledgments

Conflicts of Interest

The authors declare no conflict of interest.

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