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VETERINARY MICROBIOLOGY - SHORT COMMUNICATION

Lack of molecular evidence of fecal-borne viruses in capybaras from São Paulo state, Brazil, 2018–2020: a minor public health issue

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Abstract

Capybara (*Hydrochoerus hydrochaeris*) is the world's largest rodent species distributed throughout South America. These animals are incredibly tolerant to anthropogenic environments and are occupying large urban centers. Capybaras are known to carry potentially zoonotic agents, including *R. rickettsia*, *Leishmania* spp., *Leptospira* spp., *Trypanosoma* spp., *Salmonella* spp., *Toxoplasma gondii*, and rabies virus. Focusing on the importance of monitoring potential sources of emerging zoonotic viruses and new viral reservoirs, the aim of the present study was to assess the presence of fecal-borne viruses in the feces of capybaras living in urban parks in São Paulo state, Brazil. A total of 337 fecal samples were collected between 2018 and 2020 and screened for the following: (i) Rotavirus group A (RVA) by ELISA; (ii) non-RVA species and Picobirnavirus (PBV) using PAGE; (iii) Human Bocaparvovirus (HBoV), Bufavirus (BuV), Tusavirus (TuV), and Cutavirus (CuV) qPCR; (iv) Human Enterovirus (EV), Norovirus GII (NoV), and Hantavirus by in houses RT-qPCR; (v) SARS-CoV-2 via commercial RT-qPCR kit assay; and (vi) Astrovirus (AstV) and Adenovirus (AdV) using conventional nested (RT)-PCRs. All fecal samples tested were negative for fecal-borne viruses. This study adds further evidence that the fecal-borne viruses is a minor public health issue in Brazilian capybaras, at least during the surveillance period and surveyed areas. Continuous monitoring of sylvatic animals is essential to prevent and control the emergence or re-emergence of newly discovered virus as well as viruses with known zoonotic potential.

Keywords Capybaras · Zoonosis · Molecular surveillance · Fecal-borne viruses · Virus reservoir

Zoonosis is an infectious disease that is transmitted between animals and humans and can be caused by bacteria, viruses, parasites, and even prions [1]. In recent years, there has been an emergence of zoonotic infections, especially viral-borne diseases, including the recently Zika virus outbreak and SARS-CoV-2 pandemic [2, 3]. Wild animal species in close contact with humans are often involved in viral-borne infections [1] and should be carefully considered when assessing zoonotic disease emergence. Continuing wildlife surveillance is crucial for mitigating the consequences of emerging zoonotic viruses.

Rodents are the most species-rich order of mammals and well known recognized as reservoirs associated with new virus emergence [1]. Capybara (*Hydrochoerus hydrochaeris*) is the world's largest rodent species, displaying semi-aquatic habit and distributed throughout South America. Their high reproductive capacity, generalist feeding habits, and minimal habitat quality requirements have contributed to overpopulation in numerous regions of Brazil. In addition, capybaras are regularly found in close proximity to human population in urban parks across the country [4–6]. *R. rickettsii* is transmitted to humans mainly by the tick *Amblyomma sculptum*,

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which uses the capybara as its main host [7]. Capybaras are also known to carry other potentially zoonotic agents, including *Leishmania* spp., *Leptospira* spp., *Trypanosoma* spp., *Salmonella* spp., *Toxoplasma gondii*, and rabies virus [8, 9]. Therefore, monitoring these animals in areas cohabited by humans is vital to help outline measures in order to decrease the risk of a zoonotic emergence. The role of these rodents as asymptomatic hosts for potential zoonotic fecal-borne viruses has been poorly investigated in the country [4–6].

Enteric viruses can be transmitted by the fecal–oral route directly or by indirect contact via contaminated fluids, including surface water, food, and carriers such as fomites. In addition, enteric viruses are usually very stable in the environment and can survive under different conditions once these pathogens must tolerate the hostile conditions of the gastrointestinal tract [10]. Recently, there have been a growing number of reports describing the interspecies transmission of enteric viruses between animal and humans [1, 11]. Rotavirus, Picobirnavirus, Norovirus, Astrovirus, Bocaparvovirus, Enterovirus, and Adenovirus infect a wide range of vertebrates including human, domestic and wild mammals, especially rodents [1, 11, 12]. Moreover, rodents are also a source of Coronaviruses and Hantaviruses as well as newly identified viruses [12]. It is important to highlight that virus fecal shedding do not necessarily require replication in the intestine, like hepatitis A and E viruses that is shed in the feces despite their liver tropism [13]. In the same way, not all viruses that are shed in feces infect new hosts via the fecal–oral route, standing out the respiratory viruses [2], but in both cases, viral DNA/RNA can be detected in stool samples.

Focusing on the importance of monitoring potential sources of emerging zoonotic viruses and new viral reservoirs, the aim of the present study was to assess the presence of fecal-borne viruses in the feces of capybaras living in urban parks in São Paulo state, Brazil.

A total of 337 fecal samples were collected between 2018 and 2020 from 4 urban parks located in 2 municipalities of São Paulo state, Brazil. The (i) Yacht Club Santo Amaro (YCSA) is placed on the shores of the Guarapiranga Reservoir (Socorro District); the (ii) Novo Rio Pinheiros bike lane is placed on the banks of the Pinheiros River, both of them located at south region of São Paulo City (Santo Amaro District); the (iii) Tietê Ecological Park (PET) is placed on the east region of São Paulo City (Cangaíba District); and the (iv) Municipal Dam Park is placed on the east region of São José do Rio Preto City (Fig. 1). The 4 parks inhabited by capybaras present low water quality conditions, and the pollutants are associated with anthropogenic environments, mostly emerging contaminants resultant from incomplete degradation in sewage treatment plants (https://cetesb.sp.gov.br/infoaguas/). Stool samples were obtained *in natura* and immediately stored at − 20 °C. All frozen specimens were taken to the Enteric Disease Laboratory, Virology Center, Adolfo Lutz Institute, São Paulo, Brazil. All animal procedures in this study were approved by the Ethics Committee on Animal Use (CEUA) guidelines of the Adolfo Lutz Institute, IAL, São Paulo, SP, Brazil (nº 04/2018) and by Chico Mendes Institute for Biodiversity Conservation (ICMBio), Ministry of the Environment (No 77659–1/2021).

Controls for (RT-) PCR inhibitors or for DNA/RNA extraction efficiency are not available for animal origin specimens. In order to improve the reliability of negative PCR test result eventually obtained (i.e., assure that a negative PCR test result represent a true negative in the sample), we proposed to verify the viability of the capybaras stools screened in the present study using MALDI-TOF MS (matrix-assisted laser desorption ionization–time of flight mass spectrometry) and seeded experimental specimens with rotavirus and adenovirus reference strains (representing enteric RNA and DNA viruses, respectively). MALDI-TOF MS is a rapid and simple technique for microbial identification [14]. The detection of bacteria and fungi in capybara feces ensures an adequate logistics applied in the collection, packaging, and transport of feces samples from the field to the laboratory for viral testing. In addition, the capacity of recovering viruses artificially seeded in stools specimens offer the opportunity to evaluate the presence of potential PCR inhibitory factors. Therefore, based on collection site (representative sampling), a total of 35 stool samples were selected for MALDI-TOF MS investigation and 20 fecal specimens for seeded experiments.

One positive Rotavirus group A (RVA) G2P[4] specimen (GenBank accession No. JQ693560) and one positive Human Adenovirus F-40 (HAdV-F40) (GenBank accession No. MH201117) stool sample representing enteric RNA viruses and DNA viruses, respectively, were used to spike experimental specimens. The reference RVA and HAdV-F40 samples used here belong to the Enteric Diseases Laboratory collection, a regional reference center for viral gastroenteritis surveillance. The titers of positive RVA and HAdV-F samples used for the spiking experiments were not established; hence, detection limits were not evaluated. The aim of the seeded experiments was exclusively tested the sample matrices (stool) for containing potential PCR inhibitory factors. A total of 20 capybaras stool samples was spiked with the reference RVA G2P[4] and HAdV-F40 specimens. Briefly, 1 g of experimental stool and 1 g of each reference sample were added to 1 mL of TRIS/HCL in 1.5-mL microtubes. The suspensions were vigorously vortexed and centrifuged at $5000 \times g$ for 30 min. The clarified supernatants were transferred to 2-mL cryotubes and stored at − 20 °C for subsequent direct DNA/RNA extraction using *Bio Gene DNA/RNA Viral* (Quibasa – Química Básica Ltda, Belo Horizonte, BH, Brazil) according to the

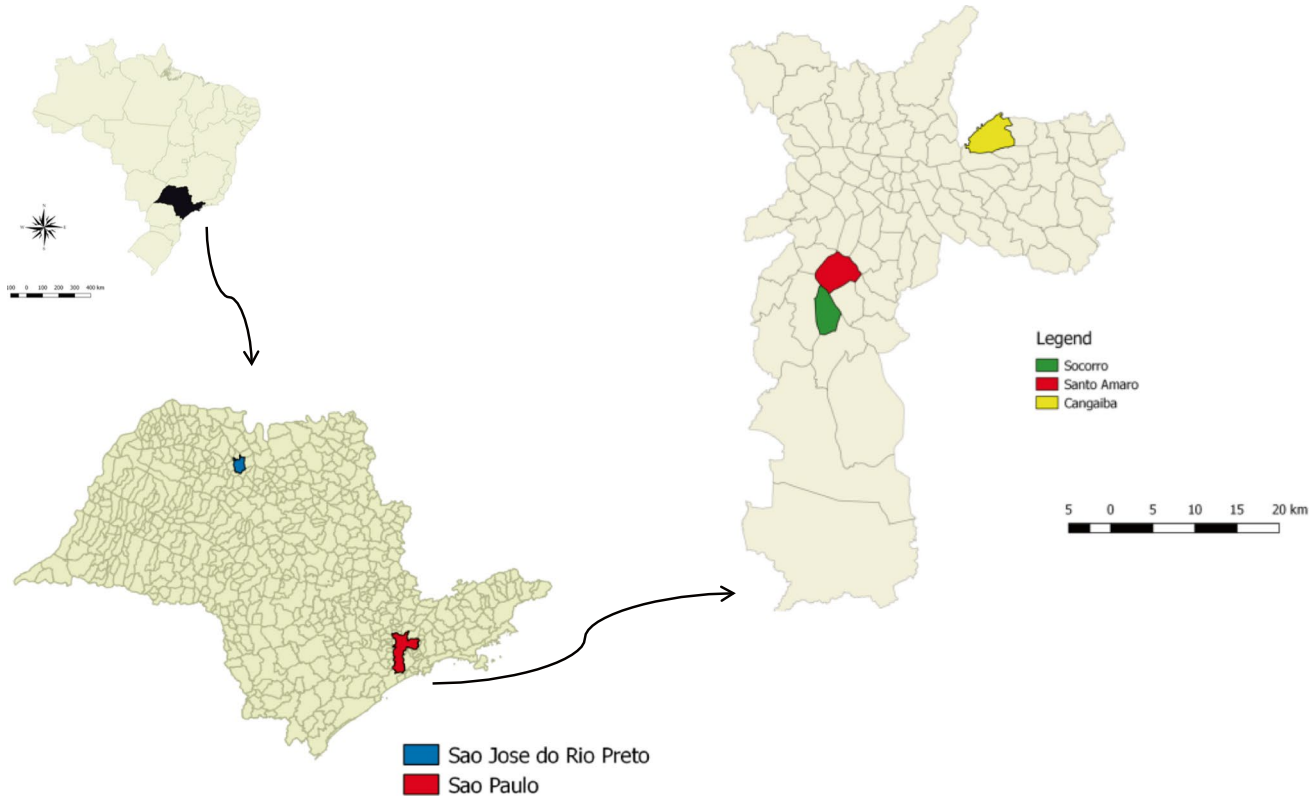

Fig. 1 Map of São Paulo state, Southwestern region, Brazil; highlighting municipalities (in red and blue) and São Paulo municipalities' districts (in red, green, and yellow) from which samples were collected from free-living capybaras in urban parks between 2018 and 2020. Up left: Map of Brazil stressing São Paulo state. Down: Municipalities surveyed: São Paulo and São José do Rio Preto. Up right: Districts surveyed at São Paulo municipality. Map was generated with QGIS software v2.14.9 (https://www.qgis.org/pt_BR/site/about/index.html)

manufacturer's instructions. Qualitative (RT-) PCRs were used for screening RVA and HAdV in seeded specimens. For RVA detection, the extracted dsRNA was subjected to G and P typing by semi-nested multiplex reverse transcription–polymerase chain reaction (RT-PCR) with specific primers for G and P types following protocol previously described by Gouvea et al. [15]. The presence of HAdV was detected by amplifying the Hexon gene, using primers AdVFhex(+)/(−) (292 bp fragment) according to the protocol reported by Primo et al. [16]. All PCR products were visualized by GelRed™ (Biotium, Fremont, CA) staining and UV transillumination, following electrophoretic separation (1 h, 140 V) on 1.5% agarose gels. The G2 and P[4] genotypes were assigned using agarose gel analysis of second-round PCR products, 652 pb and 483 pb, respectively.

The 337 fecal specimens were screened for 12 different fecal-borne viruses: Rotavirus, Picobirnavirus, Human Bocaparvovirus, Bufavirus, Tusavirus, Cutavirus, Enterovirus, Norovirus genogroup GII, Hantavirus, SARS-CoV-2, Astrovirus, and Adenovirus (Supplement 1). All specimens were screened for RVA by a commercial enzyme-linked immunosorbent assay (ELISA) (RIDASCREEN® Rotavirus, R-biopharm, Darmstadt, Germany), according to the manufacturers protocol. Fecal samples were also tested for non-RVA species and Picobirnavirus (PBV) using polyacrylamide gel electrophoresis (PAGE) followed by silver staining of gels, according to standard procedure previously describe [17].

Viral nucleic acids were extracted from 10% fecal samples using *Bio Gene DNA/RNA Viral* (Quibasa – Química Básica Ltda, Belo Horizonte, BH, Brazil) according to the manufacturer's instructions. Human Bocaparvovirus (HBoV), Bufavirus (BuV), Tusavirus (TuV), and Cutavirus (CuV) were screened by quantitative real-time polymerase chain reaction (qPCR) following protocols previously reported [18, 19]. Human Enterovirus (EV), Norovirus GII (NoV), and Hantavirus were tested by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) using procedures formerly defined by Zhang et al. [20], Kageyama et al. [21], and Nunes et al. [22], respectively. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was evaluated using commercial *Allplex™ 2019-nCoV* (Seegene Inc., Seoul, South Korea) RT-qPCR kit assay. Reverse transcription was carried out with random primers [23], and cDNA was screened for the presence of Astrovirus (AstV) using

heminested PCR targeting the RdRp gene following primers and protocol described by Chu et al. [24]. Nested-PCR amplification of a partial sequence of the adenoviral DNA polymerase gene was performed for Adenovirus (AdV) testing following primers and protocol described by Li et al. [12].

Of the 35 stool samples tested using MALDI-TOF MS, a total of 25 bacterial and 5 fungi species were identified (Table 1). MALDI-TOF MS is a rapid, simple, inexpensive, and high-throughput proteomic technique for identification of both bacteria and fungi, but it is important to highlight that its application is dependent on the reference strains included in the database [25]. Therefore, for some fungi or bacteria species, their identification by MALDI-TOF MS may be failed during the sample screening conducted here. *Enterococcus hirae* (20%, 7/35), *Escherichia coli* (14.3%, 5/35), and *Escherichia hermannii* (5.7%, 2/35) were identified in the feces (Table 1). The presence of the enterobacterias in capybaras stool has been previously investigated in Brazil [8], and a high inter-individual bacterial variation was observed. This data was also consistent with a previous study conducted in Venezuela [26]. The successfully microbial identification in these 35 selected samples assures that the biological samples were of consistent quality for the intent analysis and study goals.

Table 1 Frequency of bacterial and fungi species identified in the fecal samples of 35 free-ranging capybaras (*Hydrochoerus hydrochaeris*), São Paulo state, Brazil, 2018–2020

Bacterial	Positive individuals	% of strains
Acinetobacter lwoffii	2	5.7%
Acinetobacter schindleri	1	2.8%
Acinetobacter seifertii	1	2.8%
Arthrobacter gandavensis	1	2.8%
Bacillus altitudinis	1	2.8%
Bacillus cereus	7	20%
Bacillus koreensis	1	2.8%
Bacillus licheniformis	1	2.8%
Bacillus marisflavi	10	28.6%
Bacillus megaterium	5	14.3%
Bacillus pumilus	10	28.6%
Bacillus spp.	2	5.7%
Bacillus thuringiensis	1	2.8%
Cellulosimicrobium cellulans	1	2.8%
Enterobacter bugandensis	2	5.7%
Enterobacter kobei	2	5.7%
Enterococcus hirae	7	20%
Enterococcus mundtii	1	2.8%
Escherichia coli	5	14.3%
Escherichia hermannii	2	5.7%
Klebsiella variicola	1	2.8%
Kurthia gibsonii	1	2.8%
Microbacterium arborescens	1	2.8%
Pantoea séptica	1	2.8%
Serratia marcescens	1	2.8%
Total	**35 samples**	**100%**
Fungi	Positive individuals	% of strains
Cryptococcus diffluens	2	18%
Cryptococcus laurentii	3	27%
Cryptococcus liquefaciens	1	9%
Rhodotorula mucilaginosa	4	36%
Torulaspora delbrueckii (Saccharomyces delbrueckii)	1	9%
Total	**11 samples**	**100%**

Bold means the total of samples and the percentage obtnaied

The qualitative PCR was able to detect RVA G2P[4] and HAdV-F40 in all 20 seeded capybaras stool samples. Virus detection in stool samples could be hampered by PCR inhibitors that may lead to false-negative results [27], especially considering feces from animal origin that can be a very heterogeneous biological material. Several PCR inhibitors have been characterized including phenolic compounds, glycogen, fats, cellulose, constituents of bacterial cells, heavy metals [28], and, more recently, dietary factor and gut microbiota [29]. All DNA/RNA extracts in seeded specimens amplified efficiently and did not differ from results of DNA/RNA extracted from the original reference strains used. Although viral titer was not determined prior to seeding experiments, the qualitative (RT-) PCRs protocols applied here successfully recover virus from the capybaras specimens. Thus, the stool samples employed in the present study were suitable for viral testing. This investigation emphasizes the need for PCR inhibitor evaluation when clinical samples obtained from animal origin are analyzed.

All samples tested negative for RVA, non-RVA species, HBoV, NoV GII, AstV, and AdV. These viruses are well known recognized as important cause associated with acute gastroenteritis in human and animals worldwide, as well as involved in interspecies transmission [30–34]. RVA, non-RVA species, HBoV, NoV GII, AstV, and AdV infections in rodents have been reported in the past, especially in rats [1, 11, 35, 36]. However, no capybara has tested positive for these viruses so far [4–6], in agreement with the data obtained here. There is also no serological evidence of viral gastroenteric infections [8, 37]. Collectively, capybaras' role in the epidemiological chain of enteric viral infections remains unknown. It is important to mentioning that besides anthropogenic influences, local water bodies are also contaminated with sewage, sustaining the continuous exposing of capybaras to enteric viruses and to a potentially zoonotic-transmission route as these animals are living at the human-animal interface environments.

PBV is ubiquitous in feces and gut contents of humans and animals with or without diarrhea but also detected in invertebrates and environmental samples [38]. The true host(s) and the role of these viruses as causative agents of

gastroenteritis and respiratory infections or as opportunistic enteric pathogens remain unclear [39]. Although PBV has been frequently detected in a wild range of animal feces [40], there is no evidence of PBV infecting capybaras in viral surveillance studies [4–6] as also demonstrated in the present investigation.

Hantaviruses are considered infectious agents of great importance for public health and has been known to circulate throughout the Americas in the rodents [41]. The growing number of reports on hantaviruses in other vertebrate animal species, including bats and shrews, has stimulated the interest in search for discovering potential new reservoirs, and Brazil is not an exception [42, 43]. Most rodent hosts shed Hantavirus in their saliva, urine, and feces [44]. In a controlled experiment conducted with bank voles, Hardestam et al. [45] demonstrated the excretion of Puumala hantavirus in these three clinical samples, corroborating that these transmission routes may occur in nature and eventually able to reach humans. None of the capybaras tested here were positive for viral hantavirus RNA in feces. Nevertheless, one limitation of the present study is the lack of serum samples collected from the animals in order to verify serological evidence of previous infections by hantavirus.

BuV, TuV, and CuV are among the newest parvoviruses described in humans [46]. These viruses have been also reported in wild and domestic animals, including in rodents, but information on their epidemiology is rather limited [47–49]. BuV, CuV, and TuV have been described circulating in feces of human and animal origin in Brazil [50, 51]. Here we investigated the capybaras potential to play a role as harboring new and poorly surveyed viruses in the country. No positivity for BuV, CuV, and TuV were detected in capybaras feces, excluding them as potential hosts or reservoirs of these new parvoviruses in the investigated areas.

In the same way, we aimed to determinate whether capybaras could be sheltering EV strains. EV are classified into twelve species EV-A to EV-L (https://talk.ictvonline.org/taxonomy/), where species EV-A to EV-D commonly infects humans, species EV-E to EV-G and EV-K causes diseases affecting livestock industry, including cattle and swine's, species EV-H and EV-J was identified in monkeys, and species EV-I and EV-L were discovered in fecal samples collected from dromedaries and goats, respectively [52–55]. Given the evidence that EV naturally circulate in animals and that human EV have been detected in distinct mammalian species, including in Brazil [56–58], it must be considered its potential for interspecies transmission [59]. Our molecular tests detected no evidence of EV infection among capybaras populations living in the Sao Paulo state, reducing concerns about rodent reservoirs as a potential source of emerging EV infections.

Humans have infected a wide range of animals with SARS-CoV-2 [60–62]. Potential establishment of new reservoirs, like occurred in white-tailed deer populations in USA, rises critical concerns about SARS-CoV-2 evolution and epidemiology but mostly brings serious implications for public health issues, especially considering the risk of spillback of novel variants to humans [61]. Continuous monitoring of SARS-CoV-2 in animals is essential for risk management, particularly considering capybaras as they are water-loving rodents found in areas with abundant water sources, incredibly tolerant to anthropogenic environments and present in large urban centers such as Sao Paulo and São José do Rio Preto cities where water bodies are contaminated, mainly with domestic sewage [63–65]. Several studies have demonstrated the presence of the SARS-CoV-2 in human stool samples [66, 67]. Therefore, viral RNA is shed and disposed in wastewaters, highlighting a potential indirect infection pathway to humans or other susceptible mammals' species [68]. No specimen tested positive for SARS-CoV-2 in the present study, indicating that although the capybaras are in close contact with contaminated effluents, they are not being infected, and they probably do not constitute a reservoir for SARS-CoV-2 in the studied regions. It is important to mentioning that enteric coronavirus has been previously detected in capybaras feces from Sao Paulo state using transmission electron microscopy technique [4].

The knowledge about the presence and diversity of fecal-borne viruses in capybaras is so far limited, and a better understanding of a potential role of enteric infections is that animals are essential to develop preventive measures. The present study reports a molecular surveillance of various viral pathogens in Brazilian capybaras and adds further evidence that the fecal-borne viruses is a minor public health issue, at least during the surveillance period and surveyed areas. Continuous monitoring of sylvatic animals is essential to prevent and control the emergence or re-emergence of newly discovered virus as well as viruses with known zoonotic potential, especially in anthropogenic environments. Capybaras are extremely tolerant to environmental changes and exhibit high vagility along waterways, thus configuring a potential to trigger emerging zoonosis.

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Author contribution AL conceived and designed the study protocol; LSA, YF, EV, RSM, SGM, RG, CDR, HRV, KMBN, MSC, JMG, DMCS, VBDF, ELLA, SRSF, CFB, GCDS, MLN, CMA, RCM, LC, VSM, and ACC participated in the conduct of the study; LSA, DMCS, and VBDF conduct the MALDI-TOF MS assays; LSA conducted the PCR inhibition evaluations; LSA, EV, KMBN, MSC, SRSF, CFB,

GCDS, and AL acquired samples and data; LSA and RG performed the ELISA tests and conduct PAGE screening; LSA, YF, EV, RSM, SGM, RG, CDR, HRV, MSC, JMG, CMA, LC, VSM, ACC, and AL performed the RNA/DNA extraction and the PCR tests; LSA, MSC, JMG, ELLA, MLN, RCM, ACC, and AL analyzed, interpreted the data, and drafted the manuscript; all authors critically revised the manuscript for intellectual content and approved the final version. AL is guarantor of the paper.

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Declarations

Competing interest The authors declare no competing interests.

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