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First molecular detection of canine herpesvirus 1 (CaHV-1) in the Eastern Brazilian Amazon

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ABSTRACT

Background: Canine herpesvirus type 1 (CaHV-1) infects dogs and is associated with neonatal deaths and reproductive, ocular, neurological, and respiratory problems. In Brazil, reports of CaHV-1 have been restricted to the southeast and south regions, particularly in municipalities in the state of Rio Grande do Sul.
Objectives: To assess the presence and variability of CaHV-1 in canine populations in the state of Pará, North Brazil.
Methods: Biological samples from 159 dogs from 4 municipalities in the State of Pará were evaluated using polymerase chain reaction and phylogenetic analyses, with the target being the viral enzyme, thymidine kinase.
Results: CaHV-1 was detected in 13 dogs (8.2%), with 2 animals being from the municipality of Santa Bárbara do Pará, 8 from Algodoal Island, 2 from Salinópolis, and one from Capanema. The study sequences revealed 100% identity among themselves and 64% to 100% identity with the other nucleotide sequences from Australia, Brazil, United Kingdom, and United States, including 100% identity with the 2002 isolate from Australia. The 1996 isolate from France was grouped in a branch that was different from the sequence of this study.
Conclusions: This study presents the first molecular detection of CaHV-1 in dogs from the Amazon region in northern Brazil. The nucleotide identity between the strains and cytosine insertion in the sequences isolated in this study suggests at least 2 strains of CaHV-1 circulating in Brazil (Pará and BTU-1).

Keywords: Canine herpesvirus; molecular diagnosis; epidemiology; the Amazon

INTRODUCTION

Carmichael et al. [1] first isolated *Canid alphaherpesvirus 1*, canine herpesvirus 1 (CaHV-1), as responsible for causing fatal hemorrhagic disease in puppies. There virus is described as a monotypic virus [2], a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Varicellovirus* [3].

Viral transmission occurs through direct or indirect contact with secretions from the oronasal, ocular, and genital mucosa of infected dogs. Puppies may be infected through the

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Conflict of Interest

The authors declare no conflicts of interest.

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uterus or during birth [4]. Animal shelters are the main locations for disseminating CaHV-1 because of the large number of dogs, constant turnover of animals, and breeding [5, 6].

The symptoms and progression of herpesvirus depend on the age of the dog and the moment of primary infection [4]. Infected neonates generally do not survive infection and die from progressive multifocal hemorrhagic necrosis of several organs [7, 8]. Adult dogs and puppies over the age of 2 wks who survive the primary infection develop subclinical infections associated with the establishment of latency [4, 9].

The virus has a worldwide distribution, with more than 80% seroprevalence in some dog populations [10]. CaHV-1 is considered part of the canine infectious respiratory disease complex (CIRDC), together with the canine distemper virus, adenovirus type 2, and canine parainfluenza. Hence, studies reporting infections caused by CIRDC organisms may also indicate the presence of CaHV-1 [11].

In Latin America, the seroprevalence ranges from 6% to 87% [12, 13]. Serological and molecular evidence for CaHV-1 in Brazil is still poorly described and is centered in the southeastern and southern portions of the country [8, 14]. In Rio Grande do Sul, molecular studies indicated a 1.1% to 100% frequency of infection [5, 8], with a 66.9% seroprevalence [15].

Molecular studies in Paraná state reported that 67.9% of dogs studied had a positive diagnosis for CaHV-1 [16]. In Minas Gerais, 10% of females were found to be infected by CaHV-1 [17]. Studies in litters of puppies that died due to a herpes virus infection showed a 100% infection rate [7, 8]. There is no information on the presence of CaHV-1 in the North, Northeast, and Center-West regions of Brazil.

The purpose of the study was to detect CaHV-1 in blood samples and ocular, nasal, and genital secretions from dogs in 4 municipalities in the state of Pará, northern Brazil using the nested polymerase chain reaction (PCR) technique targeting the thymidine kinase (TK) enzyme. This was an epidemiological, analytical, cross-sectional, and descriptive study.

MATERIALS AND METHODS

Ethical considerations

The study was approved by the Animal Use Ethics Committee (AUEC) of the Universidade Federal do Pará (UFPA) with protocol number 5224181219 and used samples ceded by the "Veterinarians of the Amazon Project: population control of dogs and cats with application in the One Health (VAP)", which was approved by AUEC at the Universidade Federal Rural da Amazônia (UFRA), with the protocol 031/2017 and number 23084.010805/2017-31.

Veterinarians of the Amazon Project: population control of dogs and cats with application in the One Health (VAP)

VAP operates in municipalities in the interior of the State of Pará, through ovariohysterectomy surgery in females and orchiectomies in males, for the population control of dogs and cats. The project also seeks to support research in animal health by donating biological samples (blood and ocular, nasal, and genital secretions) to projects aimed at diagnosing infectious diseases. Therefore, VAP voluntarily provided biological samples from dogs to diagnose CaHV-1. Veterinarian Dr. Maridelzira Betânia Moraes David directed this project.

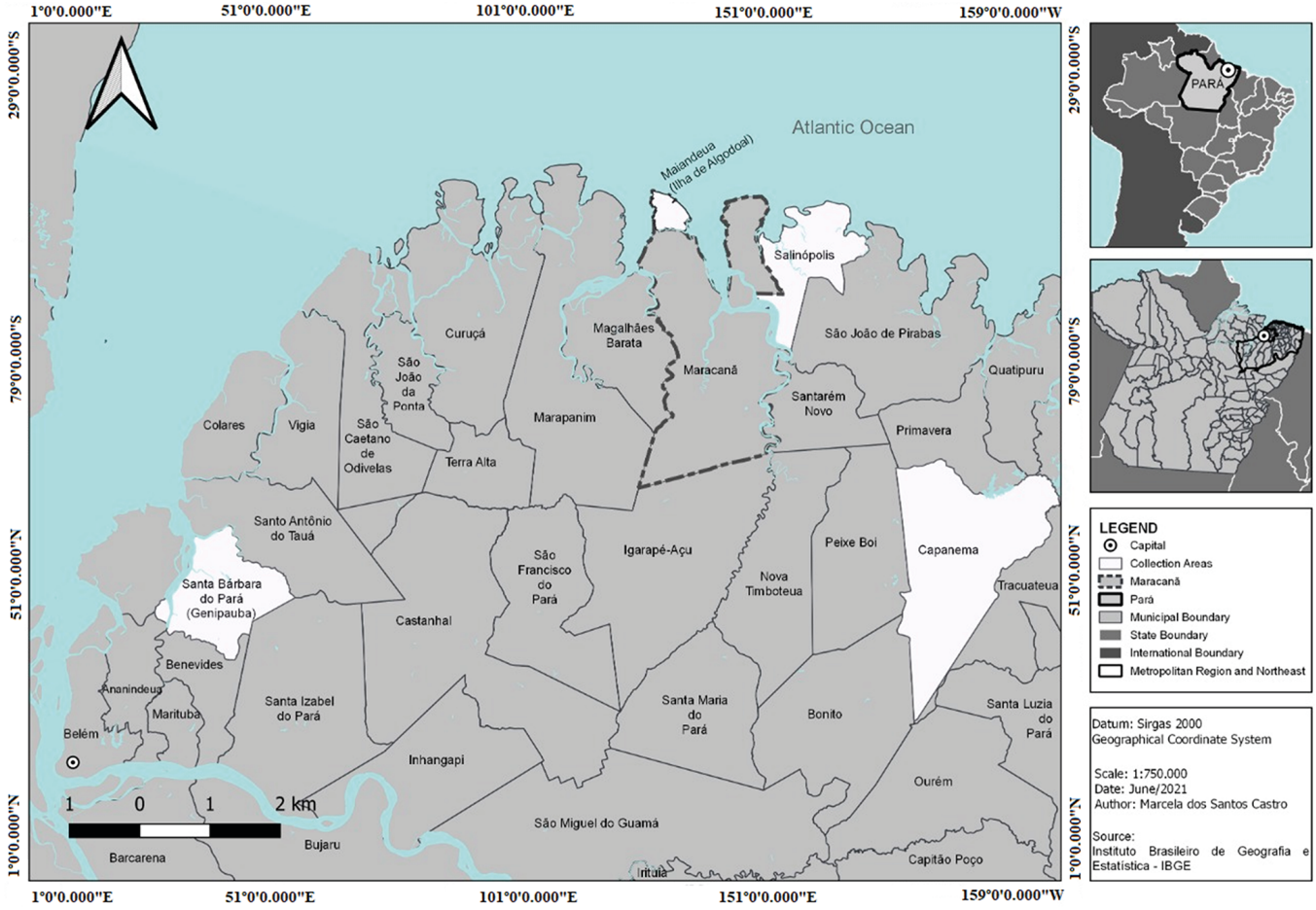

Fig. 1. Location of the municipalities of origin for the samples from dogs obtained for studying infection with canine herpesvirus type 1.

The study population was comprised of dogs from 4 municipalities in the State of Pará: Santa Bárbara do Pará, Maracanã (Maiandeua–Algodoal Island), Salinópolis and Capanema (**Fig. 1**).

Sampling and collection of biological material

The study involved samples obtained (by the VAP) from 159 dogs (**Table 1**) of undefined breeds, aged from 6 to 60 months (puppy [6 to 11 months], young [12 to 24 months], adult [25 to 60 months]) in different classes of housing (shelter resident [dog absent from guardian, living in a space shared with other animals], domiciled [with guardian, living at a fixed residence, with controlled access to the street under guardian supervision during walks], semi-domiciled [with or without guardian, with access to the street without human supervision] and street dogs [without guardians, free living]).

Table 1. Characterization of the dogs in the study and the biological material used for detecting the canine herpesvirus type 1

Municipality	Animals			Blood samples	Secretion samples				Total samples donated
	Female	Male	Total		Ocular	Nasal	Vaginal	Preputial	
Santa Bárbara do Pará (Genipauba)	12 (7.5)	02 (1.3)	14 (8.8)	0	14 (2.3)	14 (2.3)	12 (2.0)	0	40 (6.6)
Maracanã (Algodoal Island/Maiandeua)	66 (41.5)	16 (10.1)	82 (51.6)	82 (13.6)	82 (13.6)	82 (13.6)	66 (11.0)	7 (1.2)	319 (53.1)
Salinópolis	24 (15.1)	10 (6.3)	34 (21.4)	34 (5.7)	34 (5.7)	34 (5.7)	24 (4.0)	0	126 (21.0)
Capanema	20 (12.6)	09 (5.7)	29 (18.2)	29 (4.8)	29 (4.8)	29 (4.8)	20 (3.3)	9 (1.5)	116 (19.3)
Total	122 (76.7)	37 (23.3)	159 (100)	145 (24.1)	159 (26.45)	159 (26.45)	122 (20.3)	16 (2.7)	601 (100)

Values are presented as number (%).

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First, the dogs were examined by VAP veterinarians before castration surgery. Dogs that had been castrated were within the sample size of the survey. Dogs that showed any clinical signs of disease during this screening stage were not castrated and were excluded from the research sample size.

According to information from VAP, the samples were collected randomly from October 2019 to December 2020. Six hundred and one biological samples were obtained from 159 dogs, which involved blood (145), ocular secretion (159), nasal (159), vaginal (122), and preputial (16) samples. The number of biological samples obtained was different for each municipality (**Table 1**).

Blood was collected (5 mL) through cephalic or jugular venipuncture and stored in tubes with EDTA anticoagulant. The ocular, nasal, and genital secretions were obtained using sterile swabs on the mucosa. Statistical analyses were performed using BioEstat 5.3 software (BioEstat Software, Brazil) [18], and the significance level was set to $p < 0.05$.

Extraction of DNA and nested-PCR

DNA was extracted from blood samples using the phenol–chloroform method [19] with a final volume of 50 μL. The extraction technique with NaCl [20] was used to extract the DNA from secretions with a volume final of 20 μL.

The DNA samples were submitted to the nested-PCR technique with TK as the target (commonly used for the diagnosis of CaHV-1 infections [21, 22]), employing oligonucleotide pairs described previously [23]. The first PCR reaction consisted of 2.5 μL of 10× buffer, 3 μL of $MgCl_2$ 25 mM, 0.5 μL of dNTPs 20 mM, 1 μL of CaHV-1[1] 10 pmol/μL primer, 1 of CaHV-2[1] 10 pmol/μL primer, and 0.1 μL of 5 U/μL of Taq DNA polymerase (Invitrogen, USA) along with 1.5 μL (20–30 ng) of genomic DNA. Water was added to the mixture to obtain a final volume of 25 μL. The nested-PCR reaction contained the same mixture as the first PCR, using 1.5 μL of the product from the first reaction and the CaHV-3[2] and CaHV-4[2] initiator pairs. The nested-PCR products were applied to 2.5% agarose gel in Tris-Acetate-EDTA buffer, stained with Diamond Nucleic Acid Dye (Promega Corporation, USA), and later submitted to electrophoresis and visualized under ultraviolet light [22, 23].

Doubly distilled water was used as the negative control, while DNA from the Botucatu strain (BTU-1) of CaHV-1 was employed as the positive control. The DNA was generously provided by Prof. Dr. João Pessoa Araújo Jr and by veterinarian Dr. Jacqueline Kurissio from Universidade Estadual Paulista (UNESP, Brazil).

Sequencing of the products of the reactions was performed using an ABI 3500XL DNA analyzer (Applied Biosystems, USA). The sequencing obtained from the strip produced with the nested PCR was edited using the BioEdit program version 7.0 (Bioedit Ltd., United Kingdom) [24]. To analyze the nucleotide identity between CaHV-1 isolates in this study (MZ889137) based on the TK enzyme gene, the sequences obtained in the present study were compared with other TK gene sequences deposited in GenBank from Australia (AF361075; KY057364) [25, 26], United Kingdom (KT819633) [2], France (X75765) [27], United States (MW353139) [28], and Brazil (KX828242) [21].

The phylogenetic tree was developed using the Neighbor-Joining method and bootstrap with 1,000 repetitions. The evolutionary distances were calculated using the distance-*p* method. There were 168 positions in the final dataset, and evolutionary analysis was conducted using

the Mega X program [29]. The FHV-1 (M26660) [30] TK enzyme gene sequence was used as an outer group.

RESULTS

The specific gene fragment for the TK enzyme of CaHV-1 was detected in 13 (8.2%) out of 18 biological samples from the dogs analyzed: blood (6/18) and nasal (5/18), ocular (4/18), preputial (2/18), and vaginal (1/18) secretions. The product of the nucleotide sequences presented the expected 168 bp fragments (**Fig. 2**).

Of the dogs infected, 61.5% (8/13) were from Algodoal Island, 15.4% (2/13) from Salinópolis, 15.4% (2/13) from Santa Bárbara do Pará, and 7.7% (1/13) from Capanema (**Table 2**). The highest detection (14.3%) of infection occurred in the municipality of Santa Bárbara do Pará. No significant differences were observed between the municipalities regarding the number of animals infected ($p = 0.574$).

Unfortunately, access was not available to all information about age and the different types of housing for all the dogs participating in the study, but data were collected for 54.1% (86/159) of the animals, of which 68.6% dogs were domiciled (n = 59); 25.6% were semi-domiciled

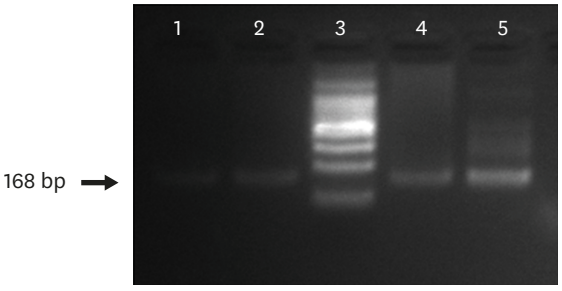

Fig. 2. Electrophoresis in agarose gel 2.5% of nasal secretion samples positive for the canine herpesvirus type 1. 1: nasal secretion sample from dog ID:15; 2: nasal secretion sample from dog ID:22; 3: molecular marker for 100 bp; 4 and 5: positive control; black arrow: size of amplicon of nested-polymerase chain reaction; gray arrows: molecular marker for 100 bp.

Table 2. Detection of CaHV-1 using nested-polymerase chain reaction in dogs from Pará state municipalities

Municipality	No. (%)	Animal ID	Sex	Type of housing	Age, yr (mo)	Detection of CaHV-1				
						Blood	Secretions			
							Ocular	Nasal	Vaginal	Preputial
Santa Bárbara do Pará (Genipauba)	2/14 (14.3)	2	F	Domiciled	3 (36)	–	–	+	–	NA
		8	F	Domiciled	5 (60)	–	+	–	+	NA
Maracanã (Algodoal Island – Maiandeua)	8/82 (9.8)	15	F	Semi-domiciled	5 (60)	–	–	+	–	NA
		22	F	Semi-domiciled	3 (36)	–	–	+	–	NA
		34	F	NI	NI	+	–	–	–	NA
		35	F	NI	NI	–	+	–	–	NA
		36	F	NI	NI	+	+	+	–	NA
		37	F	NI	NI	–	+	+	–	NA
		41	M	NI	NI	–	–	–	NA	+
		44	M	NI	NI	+	–	–	NA	+
Salinópolis	2/34 (5.9)	62	F	Domiciled	2 (24)	+	–	–	–	NA
		63	F	Domiciled	1 (12)	+	–	–	–	NA
Capanema	1/29 (3.4)	74	F	Semi-domiciled	4 (48)	+	–	–	–	NA

Animal ID: identification of dog; + (positive sample); – (negative sample).
NI, not identified; NA, not applicable; F, female; M, male; CaHV-1, canine herpesvirus type 1.

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(n = 22); 2.3% were shelter residents (n = 2); 3.5% were street dogs (n = 3). The canine population analyzed consisted of puppies (12.8%, n = 11), young dogs (38.4%, n = 33), and adults (48.8%, n = 42).

Of the total, 84.6% (11/13) of the animals infected were females; only 15,4% (2/13) were males. The only place with females (6/11) and males (2/2) infected by CaHV-1 was Algodoal Island. The 5 other dogs infected were females from the municipalities of Salinópolis (2/11), Santa Bárbara do Pará (2/11), and Capanema (1/11). The infected dogs in Salinópolis were related (mother and daughter).

Among the infected animals, where it was possible to obtain information on the type of housing and age, 7 dogs, 57.1% (4/7) were domiciled, and 42.9% (3/7) were semi-domiciled. The average age of the animals was 39.42 ± 17.95 months, with adult dogs accounting for 85.7% (6/7) of those infected; there was only one case of infection in a young dog. There was no information on the type of housing for 6 of the infected animals.

The amplified sequences of the TK enzyme gene in the 18 biological samples of the study did not show polymorphic sites, meaning that they had 100% nucleotide identity. Only the sequence of one isolate was deposited in GenBank (MZ889137) because the 18 amplified sequences were identical.

A comparison with other sequences taken from GenBank showed that the study isolates had 99.3% nucleotide identity with the strains from Australia, Brazil (BTU-1 strain), United States, and United Kingdom. The 1996 isolate from France showed 98.7% nucleotide identity, with a nucleotide difference at site 91, containing adenine.

The 18 sequences amplified in the present study showed the insertion of cytosine at locus 165. Gaps at locus 165 in the sequences of isolates from Australia, France, Brazil, the United Kingdom, and the United States were formed because there was no complementary nucleotide for the cytokine found in the isolated sequences in the present study (**Table 3**).

All sequences were edited for the same length (168 bp), and the results confirmed close phylogenetic relationships with the worldwide strains of CaHV-1, showing a close relationship between the CaHV-1 strain circulating in the state of Pará and the strains isolated in Australia, Brazil, United States, United Kingdom, and France (**Fig. 3**).

The sequence studied was grouped with the Australian strain isolated from 2002 (AF361075) [25] with 100% reliability, and both were supported by the same branch as the strains from

Table 3. Comparison of the gene sequence of the thymidine kinase enzyme of CaHV-1 found in the samples in this study with other strains of CaHV-1

Local	Reference	GenBank	Identity (%)*	Position of nucleotide	
				91	165
Pará (Brazil)	This study	MZ889137		G	C
Australia	Reubel et al. [25]	AF361075	99.3	G	–
France	Rémond et al. [27]	X75765	98.7	A	–
Australia	Sarker et al. [26]	KY057364	99.3	G	–
São Paulo (Brazil)	Kurissio et al. [21]	KX828242	99.3	G	–
UK	Papageorgiou et al. [2]	KT819633	99.3	G	–
USA	Lewin et al. [28]	MW353139	99.3	G	–

CaHV-1, canine herpesvirus type 1.
*The values are percentages of identities in the nucleotide sequence for 168 bp, determined by pair alignments; (–) indicate gap.

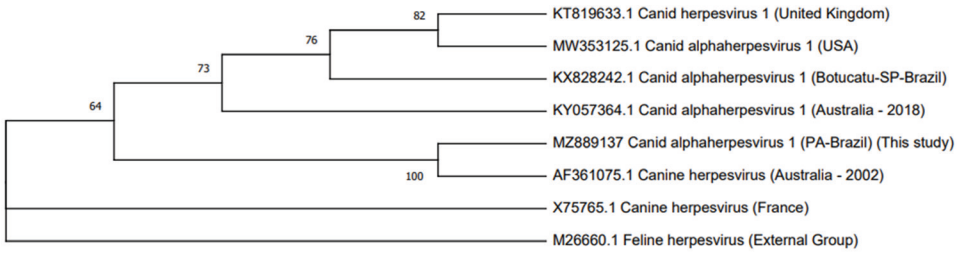

Fig. 3. Phylogenetic tree based on the Neighbor-Joining method for the gene of the Thymidine Kinase enzyme of canine herpesvirus type 1 deposited in GenBank and isolated in this study. Distance-*p* and 1,000 bootstrap (bp) replicas were utilized.

the United Kingdom [2], United States [28], Australia [26], and Botucatu (São Paulo, Brazil) [21] with 64% bootstrap. The isolate from France [27] showed a nucleotide difference at site 91 with adenine insertion (**Table 3**) and was grouped in an external branch (**Fig. 3**).

DISCUSSION

In the canine population analyzed, the molecular method used enabled the detection of CaHV-1 in 8.2% of the dogs. This was the first study reporting the presence of this herpes virus in the canine population of Pará, as well as in the Amazon region in northern Brazil. Furthermore, to the best of the authors' knowledge, this is the first report of CaHV-1 detected in genital samples from dogs in Brazil.

The prevalence of CaHV-1 infections in the Latin American and Brazilian populations is probably underestimated due to the lack of research focusing on CaHV-1 [12]. In the few studies carried out in Brazil using molecular methods, 7% of dogs were infected with CaHV-1 in the state of São Paulo [21].

The present study showed that at least 30.8% (4/13) of infected animals were domiciled, and 23.1% (3/13) were animals with access to the street (semi-domiciled). In terms of age, 46.2% (6/13) were adult dogs, and only one young infected dog was found. Even without information on the type of housing and sex of the remaining animals, infection detection was higher in adult dogs, as reported elsewhere [6, 21]. The reproductive behavior of adult dogs, venereal transmission, and greater contact with other dogs play an important role in CaHV-1 transmission between the animals [4, 16, 17].

Most of the studies related to the detection of CaHV-1 focused on samples of dogs residing in kennels because of the relationship between CaHV-1 and reproductive disorders and neonatal mortality and symptoms that affect the dynamics of breeding kennels [6, 14]. On the other hand, a focus on samples from dogs living in kennels underestimates the frequency of the disease in domiciled dogs, those in shelters, or those living on the streets [6, 21]. The present study used samples from dogs from different origins and regions, where at least 30.8% (4/13) of dogs were domiciled.

As previously mentioned, in this study, the virus was detected in only one young animal from the municipality of Salinópolis, whose mother was also infected. This suggests possible vertical transmission and the existence of maternal anti-CaHV-1 antibodies transferred to puppies during nursing or intrauterine protection, which may have ensured that the

herpesvirus did not progress toward systemic neonatal infection, but instead was kept at the level of infection associated with latency [9, 31].

In the present research, the dogs needed to be clinically healthy because they were included in the castration campaigns promoted by VAP. Nevertheless, CaHV-1 was detectable in 18 biological samples from 13 dogs, with 33.3% (6/18) positive biological samples of peripheral blood, 27.8% (5/18) nasal secretions, 22.2% (4/18) ocular samples, 11.1% (2/18) preputial samples, and 5.6% (1/18) vaginal samples. Overall, the research results suggest that the infected animals were possibly already latent carriers undergoing a period of viral reactivation.

Despite the findings of only a small 168 bp conserved fragment of the TK enzyme, 2 polymorphic sites were found (locus 165 and 91) when comparing the nucleotide identity of the isolates in the present study with strains described in other countries (Australia, United States, United Kingdom, and France) [26-28] and in Brazil (São Paulo) [21], with 100% identity with the 2002 isolate from Australia (AF361075) [25]. The 18 sequences isolated in the study showed 100% nucleotide identity.

The nucleotide identity found in the sequences isolated in the study, the insertion of cytosine at locus 165 in all these isolates, and the nucleotide identity relationship of these isolates with worldwide strains (Australia, United States, United Kingdom, and France) indicate the existence of at least 2 strains of CaHV-1 circulating in Brazil (Pará and BTU-1).

The 1995 isolate (X75765) from France [27] was grouped into an external branch due to the nucleotide difference found at site 91. Thus, the comparison of isolates from different regions of the world and Brazil in the same clade reinforces the evidence of relationships between the CaHV-1 isolates.

This study reported the first molecular detection of the virus in the Amazon region in the northern portion of the country. Overall, CaHV-1 is circulating in the Amazon in canine populations living in the municipalities of Santa Bárbara do Pará, Maracanã, Salinópolis, and Capanema. Nevertheless, more study will be needed to elucidate the epidemiological behavior of this virus in northern Brazil.

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