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Porcine lymphotropic herpesvirus (*Gammaherpesvirinae*) DNA in free-living wild boars (*Sus scrofa* Linnaeus, 1758) in Brazil

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ABSTRACT

Background: *Suid gammaherpesvirus 3, 4, and 5* (porcine lymphotropic herpesvirus – PLHV-1, -2, and -3) are viruses that infect domestic and feral pigs.

Objectives: This study examined the presence of PLHV DNA in biological samples from free-living wild boars circulating in a Brazilian geographical region with a high density of commercial domestic pigs.

Methods: Lung samples of 50 free-living wild boars were collected by exotic wildlife controller agents between 2017 and 2019 in the state of Paraná, southern Brazil. Lung and spleen fragments were obtained from six fetuses collected by hysterectomy *post mortem* from a pregnant sow. A polymerase chain reaction (PCR) assay using consensus primers (pan-herpesviruses) was performed to detect PLHV DNA. The samples showing positive results for PLHV DNA were submitted to single-round PCR assays with the specific primers for identifying PLHV-1 (213-S/215-As), PLHV-2 (208-S/212-As), and PLHV-3 (886s/886As). The specificity of the species-specific PCR products was assessed by nucleotide sequencing of the amplicons.

Results: Forty-eight (96%) of the 50 lung samples analyzed were positive for PLHV by PCR using pan-herpesvirus primers. In 33 (68.75%) of the positive samples, at least two PLHV species were identified simultaneously. The DNA of PLHV-1, -2, and -3 was found in free-living wild boars of all ages, but not in the fetuses, even though they were from a sow that tested positive for all three viruses.

Conclusion: These viruses are endemic to the population of feral pigs in the Brazilian region evaluated, as well as in domesticated pigs.

Keywords: *Sus scrofa*; swine; *Herpesviridae*; PCR

INTRODUCTION

Porcine lymphotropic herpesviruses (PLHVs) are classified into the viral species, *Suid gammaherpesvirus 3* (PLHV-1), *Suid gammaherpesvirus 4* (PLHV-2), and *Suid gammaherpesvirus 5* (PLHV-3) in the *Herpesviridae* family, *Gammaherpesvirinae* subfamily, and *Macavirus* genus [1]. The subfamily has transformative potential and a preferential association with lymphocytes

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Porto GS, Alfieri AA; Data curation: Porto GS; Formal analysis: Porto GS, Dall Agnol AM; Investigation: Porto GS, Souza TCGD; Methodology: Porto, GS, Dall Agnol AM, Leme RA, Souza TCGDS; Project administration: Alfieri AF; Resources: Alfieri AA; Supervision: Alfieri AF; Validation: Alfieri AA; Writing - original draft: Porto, GS, Dall Agnol AM; Writing - review & editing: Leme RA, Alfieri AA, Alfieri AF.

or lymphoid tissue so that many members of this subfamily are frequently associated with lymphoproliferative disorders [1-3]. In addition, PLHVs are frequently detected in domestic and feral pigs with persistent infections in B lymphocytes [4].

PLHV-1 is associated with post-transplant lymphoproliferative disease (PTLD) in miniature pigs undergoing allogeneic hematopoietic stem cell transplantation because of the accentuated increase in PLHV-1 genomic copies in these animals [5]. Furthermore, sequence analysis and the gene expression of PLHV-1 during PTLD in pigs indicated an active infection by the virus, which might be involved in the etiology of this lymphoproliferative disease [6]. On the other hand, these results are from experimental studies, and there is no knowledge of the clinical diseases caused by natural infections with PLHV [3].

Epidemiological studies of PLHV have been conducted worldwide on swine (Suidae family, Cetartiodactyla order), such as domestic pigs (*Sus scrofa domestica*, Linnaeus, 1758), wild boars (*Sus scrofa* Linnaeus, 1758), bearded pigs (*Sus barbatus* Müller, 1838), and hairy babirusa (*Babyrusa babyrussa* Linnaeus, 1758) [4,7,8]. In Brazil, only a study involving PLHV in domestic swine has been conducted [9]. On the other hand, the epidemiology of PLHV in wild boars has not been investigated in the country.

The original distribution of wild boars includes Europe, Asia, and Northern Africa. In Brazil, free-living species have been recorded since 1980, particularly in the southern region of the country [10,11]. The rapid dispersion of wild boars to other Brazilian regions is a concern because of the environmental, economic, and health problems caused by the invasive species [11]. Considering the dispersion capacity, the history of wild boar invasion in Brazil, and their proximity to pig farms, this study examined the presence of PLHV DNA in biological samples of free-living wild boars circulating in a Brazilian geographical region with a high density of commercial pig farms.

MATERIALS AND METHODS

Sample collection

The management of wild boars in this study was performed by exotic wildlife controller agents properly authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) and registered in the Federal Technical Register of Potentially Pollutive Activity (CTF/APP). This study followed all the guidelines for the care and use of animals by the Ethics Committee on Animal Experiments of the Universidade Estadual de Londrina and was approved under identification number 22831.2017.40.

The lungs of 50 free-living wild boars were sampled in the Northern (six males and 10 females) and Campos Gerais (15 males and 19 sows) regions of Paraná state, from 2017 to 2019. The animals' weight was estimated after capture for further categorization into ages (0–20 kg – piglets/infant individuals; 21–40 kg – juvenile individuals; 41–60 kg – subadults; and > 60 kg – adults) [12]. The animals sampled in this study had weights ranging from 3 to 200 kg, the infant individuals weighed between 3 to 20 kg, juvenile individuals generally weighed 30 kg, while adults had weights ranging from 70 to 200 kg. No subadult wild boars were captured.

Lung and spleen fragments were obtained from six fetuses, which were collected by hysterectomy *post mortem* from a pregnant wild boar were included in the study. The ages of

the fetuses were estimated according to their size in millimeters, as described elsewhere [13]. The fetuses were approximately 150 mm in size, so the estimated age was approximately 65 days of gestation. Tissue fragment samples were collected and stored at -80°C until analysis.

DNA extraction and molecular analyses

The tissue samples were disrupted mechanically using MagNa Lyser Instrument, Roche Diagnostics (Germany), homogenized in 0.01 M phosphate-buffered saline (PBS, 10% w/v) at pH 7.2, and clarified by centrifugation at $2,000 \times g$ for 10 min.

For nucleic acid extraction, 500 μL of tissue suspensions pretreated with proteinase K was submitted to a combination of phenol/chloroform/isoamyl alcohol and silica/guanidine isothiocyanate techniques [14,15]. The extracted nucleic acid was eluted in 50 μL UltraPure diethylpyrocarbonate (DEPC)-treated water (Invitrogen Life Technologies, USA) and stored at -80°C . Sterile ultrapure nuclease-free water was used as a negative control in nucleic acid extraction and all the following procedures.

A polymerase chain reaction (PCR) assay with the consensus primers (pan-herpesviruses) for partial amplification of the herpesvirus DNA polymerase gene [16] was used to survey the presence of the PLHV genome. The PLHV strains detected in this study were classified into species by submitting the samples that presented positive results for PLHV DNA to single-round PCR assays with the specific primers for partial amplification of the DNA polymerase genes of PLHV-1 (213-S/215-AS, 393 bp) [7,17], PLHV-2 (208-S/212-AS, 334 bp) [7,17], and PLHV-3 (886s/886as, 148 bp) [4].

Sequencing analysis

Sequencing analysis confirmed the specificity of the PCR products. One amplified product for each viral species (PLHV-1, PLHV-2, and PLHV-3) was purified using a PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen Life Technologies, USA). The DNA was quantified in a Qubit Fluorometer (Invitrogen Life Technologies, USA) and submitted to nucleotide (nt) sequencing in an ABI3500 Genetic Analyzer sequencer using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with the same primer pairs used for species-specific PCR.

The quality of the sequences obtained was evaluated using Phred software (<http://asparagin.cenargen.embrapa.br/phph/>), and the consensus sequences were obtained using CAP3 software (<http://doua.prabi.fr/software/cap3>). The similarities of the products were compared with the reference and other sequences deposited in public databases (GenBank - National Institute of Health, Bethesda, MD, USA) using BLAST software (<http://www.ncbi.nlm.nih.gov/BLAST>). The sequences were aligned by the CLUSTAL W program (version 1.4), and the similarity percentage was obtained using BioEdit software v. 7.0.26. The phylogenetic tree based on the nt sequences was built using MEGA X software using the Maximum Likelihood method, and p-distance model, with a bootstrap of 1,000 replicates using the Akaike information criterion (AIC). To classify the virus sequences, the pairwise identity was performed using the Sequence Demarcation Tool (SDT) v1.2 software on CLUSTAL W.

RESULTS

Identification of PLHV in wild boars

Forty-eight out of the 50 lung samples analyzed were positive for PLHV DNA. According to the virus species, 48 (96%), 28 (56%), and 22 (44%) samples tested positive for PLHV-1, PLHV-2, and PLHV-3 DNA, respectively. PLHV-1 was single-detected in 15/48 (31.25%) samples. On the other hand, 33/48 (68.75%) lungs showed mixed detections of at least another PLHV species. The PLHV-1 and PLHV-2 combination was observed most frequently (28/48, 58.3%), while PLHV-1 and PLHV-3 were detected simultaneously in 22/48 (45.8%) animals. Triple infections were detected in 17/48 (35.4%) of the wild boars, including in the dam of the fetuses evaluated in this study. However, PLHV-1, PLHV-2, and PLHV-3 DNA were not amplified from any of the fetal lung and spleen samples. **Table 1** lists the wild boars' capture locations, age category, PLHV DNA detection, and GenBank accession numbers from each nt sequence.

Comparative nucleotide analysis

Sequencing analysis confirmed the specificity of the amplicons for the three PLHV species (PLHV-1, PLHV-2, and PLHV-3). The wild boar-derived PLHV strains were called PLHV-1/BRA-UEL/PR-WB53/2018 (GenBank accession number: MW192776), PLHV-2/BRA-UEL/PR-WB6/2017 (GenBank accession number: MW192777), and PLHV-3/BRA-UEL/PR-WBA53/2018. A comparison of the three PLHV strains detected in this study with reference sequences of PLHVs and other gammaherpesviruses available in GenBank showed that each strain in this study was grouped into different clades, according to the nt sequences representative of each viral species (PLHV-1, PLHV-2, and PLHV-3) with bootstrap values of 99% and 100% (**Fig. 1**).

The PLHV strains of the present study showed a high percentage of nt similarity to several gammaherpesviruses strains (**Fig. 2**). Comparative analysis between the PLHV strains herein showed that the PLHV-1/BRA-UEL/PR-WB53/2018 strain had 100% nt identity with the other three PLHV-1 sequences available in the GenBank, including the European PLHV strains (GenBank accession numbers: AF118399 and NC_038264) [7] and a Brazilian strain (GenBank accession number: MN873041) [9]. The PLHV-2/BRA-UEL/PR-WB6/2017 strain also showed 100% nt identity with the PLHV-2 reference strain (GenBank accession number: NC_038265) and with the European (GenBank accession numbers: AY170314, AF118401, and AF191043) and Brazilian (GenBank accession number: MN873042) [4,7,17,9] strains. Finally, the PLHV-3/BRA-UEL/PR-WBA53/2018 strain also showed 100% nt identity with the European (GenBank accession numbers: AY170315 and AY170316) [4] and Brazilian (PLHV-3/BRA-UEL268/2016) [9] strains.

DISCUSSION

The results showed that the three PLHV species are circulating in wild boar populations in the Brazilian region evaluated, with a high rate of positive animals. Previous studies expected high frequencies of PLHV infections for domestic and feral pigs, with values close to 95% [4,7,17]. On the other hand, no epidemiological studies of PLHV focusing on Brazilian free-living wild boars have been published. This is the first study conducted in Brazil to investigate PLHV infections in free-living wild boars.

Although the percentage of positive animals for each species of PLHV varied, PLHV-1 was the most frequent viral species found, followed in order by PLHV-2 and PLHV-3. Other studies reported a higher percentage of domestic pigs and wild boar positives for both PLHV-1

Table 1. Porcine lymphotropic herpesvirus (PLHV-1, PLHV-2, and PLHV-3) DNA detection in 50 wild boars managed in the northern and the Campos Gerais regions of the state of Paraná, Brazil

Wild boar	Origin	Age category	PLHV-1*	PLHV-2†	PLHV-3‡
A1	Campos Gerais	Adult	+	+	-
A2	Campos Gerais	Adult	+	-	-
A3	Campos Gerais	Infant	+	+	-
A4	Campos Gerais	Infant	+	-	-
A5	Campos Gerais	Juvenile	+	-	-
A6 ^a	Campos Gerais	Infant	+	+	-
A7	Campos Gerais	Infant	+	-	-
A8	Campos Gerais	Infant	+	+	-
A9	Campos Gerais	Infant	+	-	-
A10	Campos Gerais	Infant	+	+	+
A12	Campos Gerais	Adult	+	-	-
A13	Campos Gerais	Adult	+	+	-
A14	Campos Gerais	Adult	+	-	+
A15	Campos Gerais	Juvenile	+	+	-
A16	Campos Gerais	Adult	+	-	-
A17	Campos Gerais	Juvenile	-	-	-
A18	Campos Gerais	Juvenile	+	+	-
A19	Campos Gerais	Adult	+	-	-
A20	Campos Gerais	Juvenile	+	+	-
A21	Campos Gerais	Juvenile	+	+	+
A22	Campos Gerais	Juvenile	+	+	+
A23	Campos Gerais	Adult	+	+	+
A24	Campos Gerais	Adult	+	+	+
A25	Campos Gerais	Adult	+	+	+
A26	Campos Gerais	Juvenile	+	+	-
A27	Campos Gerais	Juvenile	+	+	+
A28	Campos Gerais	Adult	+	+	+
A29	Campos Gerais	Adult	+	+	+
A30	Campos Gerais	Juvenile	+	+	+
A33	Campos Gerais	Infant	+	+	+
A34	Campos Gerais	Adult	+	+	+
A35	Campos Gerais	Adult	+	+	-
A36	Campos Gerais	Adult	+	+	-
A37	Campos Gerais	Adult	+	-	-
A40	Northern	Infant	+	-	+
A41	Northern	Infant	+	-	+
A42	Northern	Infant	+	-	-
A43	Northern	Infant	-	-	-
A44	Northern	Infant	+	-	-
A45	Northern	Infant	+	-	-
A46	Northern	Infant	+	-	-
A47	Northern	Adult	+	+	+
A48	Northern	Adult	+	-	+
A49	Northern	Adult	+	+	+
A50	Northern	Adult	+	-	-
A51	Northern	Infant	+	-	-
A52	Northern	Infant	+	+	+
A53 ^{b, c}	Northern	Infant	+	+	+
A54	Northern	Infant	+	+	+
A55	Northern	Juvenile	+	-	+
Total			48	28	22

(+) presence; (-) absence.

Name of sequences and GenBank accession numbers: *PLHV-1/BRA-UEL/PR-WB53/2018 (MW192776); †PLHV-2/BRA-UEL/PR-WB6/2017 (MW192777); ‡PLHV-3/BRA-UEL/PR-WBA53/2018.

followed by PLHV-3 [18] and PLHV-1 followed by PLHV-2 [19] in domestic pigs; studies also noted that there is a higher detection rate of PLHV-2 in feral pigs [17]. High rates of mixed infections with at least two species of PLHV have also been reported in other studies [9,17,18].

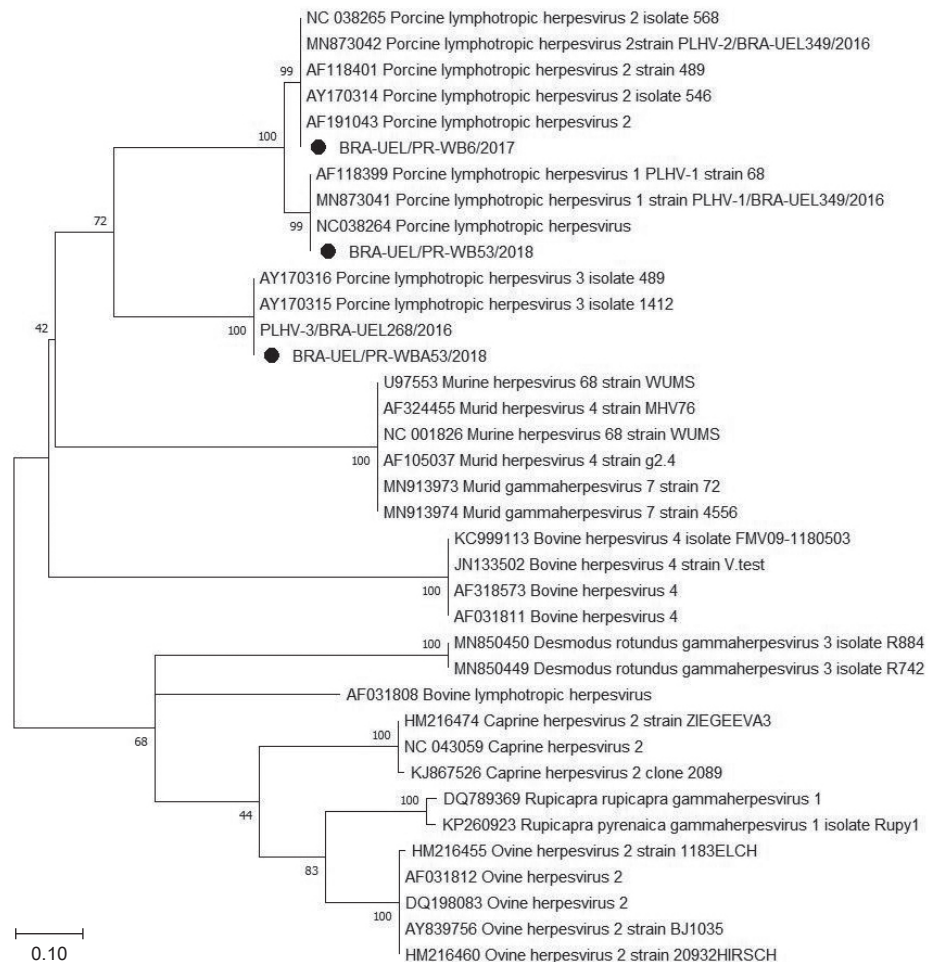


Fig. 1. Molecular phylogenetic analysis using the Maximum Likelihood method. The phylogenetic tree with 37 strains of gammaherpesviruses was based on a 104 nt fragment of the DNA polymerase gene of each strain. Three PLHV strains are from Brazilian wild boar sampled in this study and are highlighted with a black-filled circle in the tree (PLHV-1/BRA-Uel/PR-WB53/2018, GenBank accession number MW192777); (PLHV-2/BRA-Uel/PR-WB6/2017, GenBank accession number MW192777); (PLHV-3/BRA-Uel/PR-WBA53/2018). The tree was constructed using the Maximum Likelihood method and Hasegawa-Kishino-Yano model with gamma correction (I) using the Akaike information criterion (AIC). Bootstrap values were determined for 1,000 replicates. Bootstrap values > 40 are indicated at the internal nodes.

Previous studies have shown that domestic pigs in the first weeks of life are negative for PLHV [9,20]. The two PLHV-negative wild boars in this study were infant and juvenile individuals, weighing 7.5 and 30 kg, respectively. Free-living animals gain weight at a slower rate than farm animals because of the lack of genetic improvement and a specialized diet. Thus, in nature, even animals with lower weight may already be old enough to have been challenged by the horizontal transmission of microorganisms and develop an infection.

The negative result for PLHV DNA of the fetuses descending from a mother testing positive for PLHV-1, PLHV-2, and PLHV-3, suggests that vertical transmission may not occur up to the second third of the gestation period. An experimental study conducted by Tucker et al. [21] examined four PLHV-positive sows. Only 1 (3%) out of the 33 piglets derived from cesarean sections tested positive for PLHV DNA in the spleen tissue. Therefore, experimental studies involving a large number of animals and the combination of different laboratory techniques,

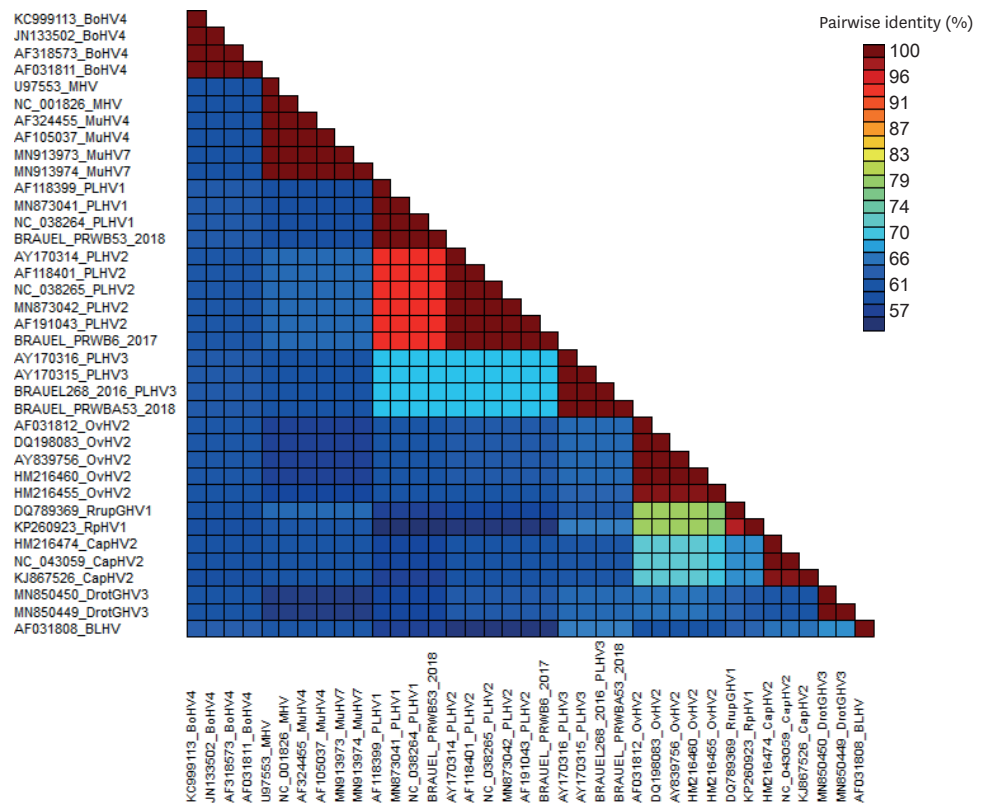


Fig. 2. SDT matrix of the pairwise identity scores generated by alignment of a 104 nt fragment of the DNA polymerase gene of 37 gammaherpesvirus strains. In this color-coded distance matrix, the colored cell represents the identity between two nucleotide sequences. The sequences were aligned by the CLUSTAL W program with Sequence Demarcation Tool version 1.2.

such as quantitative PCR, should be performed to validate the hypothesis that vertical cross-placental transmission of PLHV is less frequent, even in free-living wild boars.

Sequence analysis showed that the PLHV-1, PLHV-2, and PLHV-3 species found in the Brazilian free-living wild boars were 100% nt identical to the PLHV strains from domestic pigs from the same geographical region [9]. These results demonstrate that these viral species circulate in domestic and wild pig populations, as reported previously [17], with domestic pigs being a significant reservoir of the lymphotropic herpesvirus [18]. The PLHV strains in the present study also showed a high percentage (100%) of nt identity with the strains from Europe [4,7]. These results corroborate previous studies based on the comparison of the conserved region (DNA polymerase gene) of the PLHV genome [8]. Furthermore, other regions of the genome can be used for in-depth virus characterization.

Most of the samples (68%) were collected in a region (Campos Gerais) with a high density of commercial pigs. The presence of wild boars close to pig farms has attracted attention to the possible spread of viral agents to commercial pigs. Although PLHV species are probably non-pathogenic, these viruses target the lymphoid tissues and cells for viral replication [19]. Thus, the circulation of PLHV might represent a risk to animal health and possibly negatively affect production because it is unclear if *Macavirus* can favor infections by other pathogens or if other cofactors are necessary for pathogenic expression [19].

Pig farming is an important segment of Brazilian agribusiness, and the country is the fourth-largest pork producer globally [22]. The country has adopted strict biosecurity standards to guarantee the health of this production chain [23]. On the other hand, the expansion of the free-living wild boar populations throughout the national territory is a concern. These animals can act as a reservoir for various microorganisms that can be transmitted to domestic pigs [24]. Monitoring the occurrence of any species of microorganisms in free-living wild boars is a vital surveillance action that can improve biosecurity for national pig production. Keeping a commercial pig herd free of PLHV is very difficult. Among the main reasons are i) the horizontal transmission [20]; ii) the capacity of PLHV to establish a state of latency in infected individuals that allows the virus to spread and infect other susceptible individuals after reactivation [3]; iii) the high rate of infected herds [9,25]; iv) the possibility that the wild boars can also act as disseminators of PLHVs.

In Brazil, the rapid expansion of wild boars to non-colonized areas has been accelerated by translocation and their introduction for hunting or meat production and crossbreeding with free-ranging domestic pigs [26]. Thus, wild boars are free to circulate throughout almost the entire national territory [11], increasing the possibility of contact with Brazilian commercial pig herds. This circulation can make this invader a possible source of PLHV transmission to commercial pig herds and influence the prevalence of infections caused by these viruses.

In conclusion, PLHV-1, -2, and -3 infections are endemic in free-living wild boars, similar to what has been reported in domestic pigs in Brazil. There is no evidence that natural PLHV infections in domestic and feral pigs are accompanied by clinical signs. On the other hand, monitoring the presence of PLHVs, or any other microorganisms, in wild boars has epidemiological and biosecurity importance because these animals may act as reservoirs and potential carriers of pathogens to domestic pigs.

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