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Short communication

# Serological and molecular investigation of the prevalence of Aujeszky's disease in feral swine (*Sus scrofa*) in the subregions of the Pantanal wetland, Brazil

Rita de Cássia da Silva Paes [1], A.A. Fonseca Junior, L.A.R.C. Monteiro, G.C. Jardim, U. Piovezan, H.M. Herrera, R.A. Mauro, O. Vieira-da-Motta *

*Agência Estadual de Defesa Sanitária Animal e Vegetal – Iagro/MS, Av. Filinto Muller, 1146, CEP 79074-902, Campo Grande, MS, Brazil*

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ABSTRACT

The feral swine (FS) originated from the domestic pig and is present throughout the Brazilian wetland plain (the Pantanal). Aujeszky's disease (AD) was first serologically confirmed in the state of Mato Grosso do Sul (MS) in 2001; however, there was no viral confirmation. The aim of this study was to investigate antibodies against-SuHV-1 in the sera of feral swine in the studied areas, detect SuHV-1 through PCR and classify the viral genome. Among the 218 animals sampled, 186 were analyzed by ELISA, resulting in 88 (47.3%) reactive samples. In the serum neutralization test (SN), 57/179 (31.8%) samples presented antibodies against the AD virus (SuHV-1). By nested PCR, 104 DNA samples were extracted for analysis and confirmed with amplification of a fragment of glycoprotein B (gB) in five samples. The SuHV-1 was detected in 12 samples by using primers for glycoprotein E (gE) and viral genome was classified as Type I by ul44 partial sequencing. The amplification of SuHV-1 glycoprotein fragments in the fetuses of seropositive sows indicate that the vertical transmission contribute to maintain SuHV-1 in a free-living feral swine population. The origin of AD in the feral swine populations of the Pantanal is unknown, however, the determination of viral latency, the vertical transmission of the antigen by the amplification of SuHV-1 glycoprotein fragments in the fetuses of seropositive sows and genome typing contribute to the elucidation of the epidemiology of this disease in the wetlands of MS, Brazil.



## 1. Introduction

The Pantanal wetland is a strong seasonally flooded plain covering approximately 140,000 km$^2$ in the core of South America. This region constitutes an important agro-industry center in Latin America, supporting a cattle population of around 4.5 million that shared same habitats with abundant wildlife. Pantanal region is divided into 10 subregions including Nhecolândia and Rio Negro. The feral swine (*Sus scrofa*) originated from the domestic pig and became wild due to the abandonment of its domestication in the Pantanal during the War of Paraguay. The non-autochthonous species have an estimated population of over one million animals widespread through the Brazilian wetland in more than 10,000 groups (Desbiez et al., 2011).

The etiological agent of Aujeszky's disease (AD), or pseudorabies, is *Suid herpesvirus 1*, (SuHV-1), a multi-host pathogen which infects several animal species. This virus tends to replicate rapidly and can remain in a state of

* Corresponding author at: Universidade Estadual Norte Fluminense – Darci Ribeiro, AV. Alberto Lamego, 2000, CEP 28013-600, Campos dos Goytacazes, RJ, Brazil. Tel.: +55 22 27262053; fax: +55 22 27261654.
*E-mail addresses:* ritinhapaes@yahoo.com.br (Paes), olney@uenf.br, olney.motta@gmail.com (O. Vieira-da-Motta).
[1] Tel.: +55 67 3901 2687; fax: +55 67 3901 2705.

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latency in sensory ganglia and the reactivation occurs in situations of low immunity as a food stress, transport and concomitant infections. Domestic and wild boar are natural hosts of AD, with the existence of healthy carriers. Once infected, the hosts remain carriers for life and can transmit the virus to other animals (Toma and Dufour, 2004 in Müller et al., 2011).

The SuHV-1 in the Pantanal region was first reported serologically from 63 white-lipped peccaries (*Tayassu pecari*), seven collared peccaries (*Tayassu tajacu*) and 17 feral swine (FS) in the Rio Negro subregion. Seventy percent of the FS were seropositive, whereas all of the samples were negative for the white-lipped and collared peccaries (Freitas et al., 2004). In 2005, Monteiro Jr. (personal communication) found 23.3% (27/116) of seropositive samples collected from Aquidauana, a municipal district of Pantanal region and serum neutralization test (SN) resulted in 6.6% (17/116) reactive sera, 14 of which were from adult females and three of which were from adult males. Ribeiro et al. (2006), in a study of SuVH-1 in the feral swine of the Rio Negro and Nhecolândia subregions, found 40/168 (37%) seropositive samples.

The molecular tests are a good tool for evaluate the transmission cycles of pathogens in natural ecosystems by genotyping and identify the distance between isolates by phylogeny. SuHV-1 genotyping using the technique of Restriction Fragment Length Polymorphism (RFLP) was recommended by Herrmann et al. in 1984 grouping the genomic arrangements into four basic groups: Recent genotyping techniques were based on the partial sequencing of gene *ul44*, which encodes glycoprotein C (gC), for the phylogenetic distinction of SuHV-1 (Goldberg et al., 2001; Fonseca et al., 2010a; Müller et al., 2010; Serena et al., 2011; Steinrigl et al., 2012). These techniques facilitate the determination of similarities among strains of the SuHV-1 isolated from populations of domestic and wild boar and suggest that viral transmission may occur in both directions or among other susceptible species (Müller et al., 2011). Previous study reported that the partial sequencing of *ul44* indicated a genetic divergence among Brazilian samples, with isolates of genotypes I and II being grouped into different clades from the phylogenetic trees (Fonseca et al., 2010a). The aim of this study was to investigate antibodies against SuHV-1 in the sera of feral swine in the studied areas, detect SuHV-1 through PCR and classify the viral genome.

## 2. Materials and methods

Blood samples were collected in three trips to the Nhecolândia (NH) and 12 to the Rio Negro (RN) subregions between 2002 and 2009. The feral swine were captured with a lasso with the help of farm workers. The age of the animals was categorized as piglet, subadult, and adult second teething (Sisson and Grossman, 1978). Serum were frozen and sent to the Laboratory for the Diagnosis of Animal Diseases and Food Microbiology (Laddan/Lagro/MS). In order to isolate SuHV-1 and perform the molecular test, tissue fragments were obtained from the tonsils, brain, testicles, trigeminal ganglia and fetuses (in pregnant sows) from animals killed by local people during the hunting practices, common in the region. The competitive ELISA tests (kit PRV GP1 – IDEXX®) and SN used to confirm positive ELISA serological results, were made according to the standards of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (World Organization for Animal Health – WHO, 2012).

Two PCR were used to detect SuHV-1 DNA in the samples, both developed exclusively for diagnose with sensibility and specificity described in previous works. Highly genetic conserved regions were used to avoid false negative results due to problems in primer annealing. All of the PCR tests were performed according to the recommendations of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals from the WHO (2012), with negative controls included to verify the absence of contamination.

The PCR test for the detection of gene *ul27* (encoding for gB) was performed according to Fonseca et al. (2010c). Nested PCR for the detection of gene *us8* (encoding for gE) was performed according to Fonseca et al. (2010b). All reaction conditions for a total volume of 20 μL are listed in Table 1. Results were observed under ultraviolet light in a 1.5% agarose gel stained with ethidium bromide (0.5 $\mu g\,mL^{-1}$).

The phylogenetic analyses were performed by partial sequencing of gene *ul44* as described by Goldberg et al. (2001). All reaction conditions for a total volume of 20 μL are listed in Table 1. Eletropherogram analyses and phylogenetic tree reconstruction were done according to Fonseca et al. (2010c).

The edited sequences and those previously characterized in other studies obtained via GenBank were combined into a two FASTA files one with sequences from SuHV-1 isolated from Brazilian domestic pigs (Fonseca et al., 2010a) and other with sequences from wild boars (Müller et al., 2010; Steinrigl et al., 2012). Phylogeny reconstruction was performed by the maximum likelihood (ML) MEGA 5.0 (Tamura et al., 2011) for the partial *ul44* sequences. The genotyping of the viruses according to the classification defined by Herrmann et al. (1984) was performed using *ul56* genes as a tagged (Fonseca et al., 2011). Positive samples were submitted to a 20 μL reaction using primers listed in Table 1. Enzymatic restriction of the PCR product with *BamHI* was performed according to the manufacturer's instructions (New England Biolabs, USA). The gels were analyzed on agarose gel 2.5% stained with ethidium bromide at 0.5 $mg\,mL^{-1}$ after 1 h of running.

The Chi-squared test was used to assess the level of significance among seropositive animals in the study area and between males and females using the BIOSTAT software version 5.0. The level of significance was set at $P = 0.05$.

## 3. Results

Two hundred and eighteen animals were captured, of which 121 were female and 97 were male. One hundred and fifty two animals were adults, 27 subadults and 23 were piglets. The number of adult animals was considerably higher than the other age categories because the animals were captured with a lasso, thus targeting larger

**Table 1**
Primers and probes used for the detection and sequencing of SuHV-1 from feral swine (*Sus scrofa*) in the Nhecolândia and Rio Negro subregions of the Pantanal wetland of Mato Grosso do Sul, Brazil.

| Gene | Protein | PCR type | Reagents concentration | | | | | Thermocycler program | | | | | Primer | Amplicon (bp) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Taq (U) | DNTP (μmol/L) | DMSO (%) | Primer (nM) | $MgCl_2$ (mmol/L) | Denaturation | | Cycles | # Cycles | Final extension | | |
| *ul44* | gC | Sequencing | 1.5[a] | 200 | 8 | 300 | 1.5 | 95 °C<br>4 min | 95 °C<br>45 s | 59 °C<br>45 s | 72 °C<br>45 s | 40 | 72 °C<br>5 min | GAAGGGCTCACCCAAGAGGAC<br>GTTTCCTGATTCACGCCCACGC | 788 |
| *us8* | gE | Diagnose | 1.5[a] | 200 | 4 | 250 | 1.9 | 95 °C<br>5 min | 95 °C<br>50 s | 58 °C<br>40 s | 72 °C<br>50 s | 35 | 72 °C<br>5 min | CCAACGACACGGGCCTCTAC<br>CCAGCGTCTAGTCCCAGGTG | 558 |
| | | | 1.0[a] | 200 | 4 | 250 | 1.5 | 95 °C<br>5 min | 95 °C<br>50 s | 64 °C[d]<br>40 s | 72 °C<br>50 s | 35 | 72 °C<br>5 min | GCGTTCGTGTGCACCTCCT<br>GGGGACACGTTCCACCTGAT | 317 |
| *ul27* | gB | Diagnose | 2[b] | 200 | 6 | 300 | 1.5 | 95 °C<br>5 min | 95 °C<br>45 s | 60 °C<br>45 s | 95 °C<br>15 s | 35 | 72 °C<br>5 min | GAACCTGACGCTGCTGGAGGACCGACCG<br>AGGCCCTGGAAGAAGTTGCCGATGCATGC | 207 |
| *ul56* | *ul56* | Genotyping | 1.5[c] | 200 | 8 | 300 | 1.5 | 95 °C<br>5 min | 95 °C<br>50 s | 60 °C<br>50 s | 72 °C<br>60 s | 40 | 72 °C<br>7 min | TACCACATCGGTTGATGTGC<br>AACAGGAGCGTCTCCGAGTA | 738 |

[a] Taq Jumpstart (Invitrogen, USA), GoTaq Green Buffer (Promega, USA).
[b] Platinum Taq DNA Polymerase Platinum, Taq Buffer 10× (Invitrogen, United States).
[c] GoTaq Hotstart (Promega, USA), GoTaq Green Buffer (Promega, USA).
[d] Touchdown PCR. The first 15 cycles we used a temperature of 64 °C and the last 20 cycles 60 °C for a total of 35 cycles.

animals. For this reason age was not considered in our analysis. For ELISA test, 186 out of 218 serum samples were processed. Among these, 88 (47.3%) were reactive, 40 (45.5%) from males and 48 (54.5%) from females. Seventy-seven animals were adults, nine were subadults and 2 were piglets. The rates of seropositivity observed among males (28 and 12) and females (27 and 21) in the RN and NH subregions, respectively, showed no statistically significant differences ($\chi^2 = 0.226$ and $P = 0.635$ for RN and $\chi^2 = 0.832$ and $P = 0.362$ for NH).

Where analyzed 179 samples of sera for SN and 57 samples were reactive (31.8%), with 23 males (40.4%) and 34 females (59.6%). None statistically significant difference was observed between males and females for the RN subregion ($\chi^2 = 0.194$ and $P = 0.659$). However, a gender difference was observed in the NH subregion ($\chi^2 = 4.421$ and $P = 0.0355$) where females was more prevalent. Among the 88 samples that were reactive in the ELISA test, the results were confirmed for 49 (55.7%) by the SN.

The extraction of DNA was performed in 104 samples. Fragments of gB were amplified in five samples with conventional PCR and confirmed by nested PCR. Positive amplification was observed in three fetuses and two adult females in the brain and trigeminal ganglia. The amplification of gE fragments was observed in 12 samples from eight fetuses of four different adult sows and in one adult male in tissue samples from the brain, trigeminal ganglia and umbilical cord. Both gB and gE fragments were amplified in the trigeminal ganglia of two fetuses from female 547.

The results of the amplification of the SuHV-1 glycoprotein E gene in samples tested by nested PCR are illustrated in Figs. 1 and 2. Positive samples tested for genotyping using *ul56* gene were classified as belonging to Genotype I. The phylogenetic trees were reconstructed for ul44 by comparing Brazilian samples isolated from domestic and wild boar from various parts of the world. The sequencing of *ul44* presented greater difficulties with the electropherograms, with lower quality peaks. Only one region of the 188 bp was considered of good quality, with a definitive interpretation of the base sequence. This region is a hotspot where most nucleotide substitutions occur in




**Fig. 1.** Agarose gel showing the products of nested PCR amplifications of glycoprotein E from brain and ganglia samples. Samples 7, 10 and 11 are trigeminal ganglia samples, and sample 29 is a brain sample. SuHV-1: Aujeszky's disease virus; P: molecular weight standards (50-bp ladder).

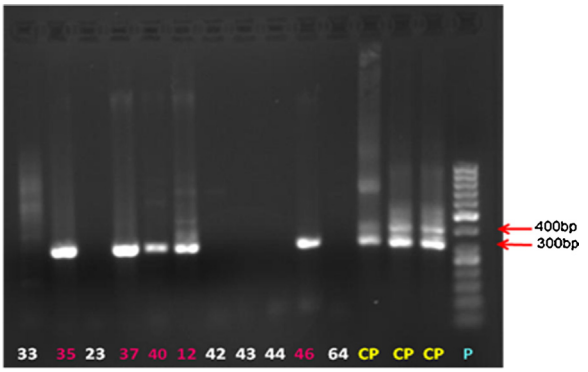


**Fig. 2.** Agarose gel showing the products of nested PCR amplification of glycoprotein E from brain and ganglia samples. Samples 35, 37, 40 and 46 are brain samples, and sample 12 is a trigeminal ganglia sample. PC: positive controls; P: molecular weight standards (50-bp ladder).

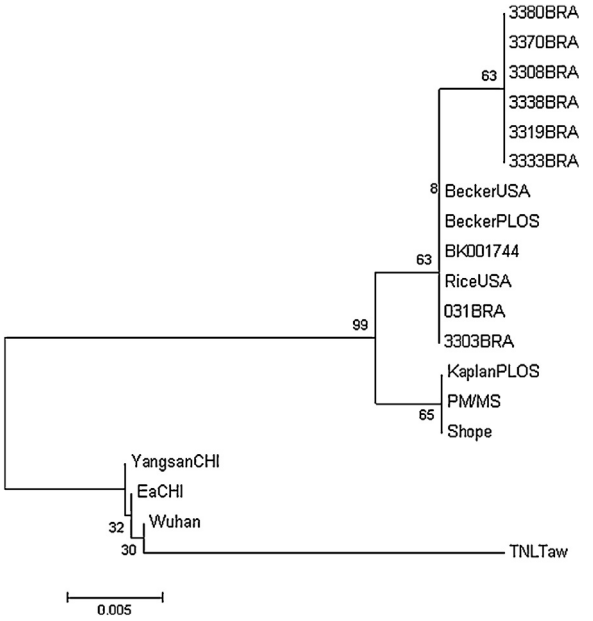


**Fig. 3.** Phylogenetic tree reconstructed from the complete sequences of the *ul14* gene from SuHV-1 isolated from domestic pigs in Brazil. The sequence of SuHV-1 isolated in China (Ea) was used as an outgroup to root the tree. The FS/MS isolate differs from the samples of Brazilian isolates prevalent in Brazil between 1983 and 2003.

*ul44*, so it was sufficient to differentiate the isolates (Figs. 3 and 4). The phylogenetic trees reconstructed for *ul44* were similar to other works even if it used a smaller region of ul4l4 (Fonseca et al., 2010a; Müller et al., 2010). Feral swine/MS did not group with any sequence from Brazilian isolates from domestic pigs (Fig. 3). It was similar to European isolates from wild boar, grouping with sequences originating from viruses isolates in Germany, Austria, Slovakia and Hungary (Fig. 4).

All positive samples were classified as genotype I after anzymatic restriction after partial amplification of *ul56*. Genotype I is the same type found in most isolates from wild boar in Europe (Müller et al., 2010).

## 4. Discussion

Pseudorabies is a disease requiring mandatory notification according to the National Swine Health Program of the Brazilian Ministry of Agriculture, Livestock and Food Supply of Brazil. To avoid the emergence of new outbreaks, several health surveillance rules must be enforced, including the observation of the health of wild animals, which are potential reservoirs of the SuHV-1. Viral detection in wild animals does not change the sanitary status of the country and can demonstrate the lack of a relationship between viruses isolated from domestic and wild pigs (Müller et al., 2011).

The rate of the seropositivity found in this study was lower than that found by Freitas et al. (2004) and higher than those reported by Monteiro Jr. (personal communication) and Ribeiro et al. (2006) for feral swine in the Pantanal of MS. These data showed the temporal instability of SuHV-1 infection under natural environment. The positive serum-reaction of two piglets in this test may be due to the presence of colostral antibodies. The level of protection by this innate immunity is not sufficient to avoid virus replication; as a consequence, great majority of infected born pigs become sick and die. The young animals that overcome the infection develop into carriers hosts. The data reported by previous studies and registered here requires attention because feral swine cohabitate with the second-largest cattle herd in the country, as well as susceptible carnivore species. In fact, dogs and wild carnivorous may consume the meat of feral swine, therefore they are also exposed to infection, via wounds. In north Pantanal (Mato Grosso state), Néspoli et al. (2003) isolated SuHV-1 from three domestic calves that lived with healthy domestic pigs. Nevertheless, Salwa (2004) reported the death of seven cattle, three goats, three sheep, two cats and one dog infected by SuHV-1. In this work, the virus was isolated and detected in tissue from infected animals through PCR.

Since SuHV-1 is a multi-host pathogen, the presence of infected asymptomatic animals in the natural population represents a source of infection for other species. For this reason, the sanitary MS state legislation DECREE/IAGRO/MS No. 607/2003 of September 10, 2003 of the State Agency for Animal and Plant Health (Agência Estadual de Defesa Sanitária Animal e Vegetal, 2003) establishes measures for the intrastate transit of free-living swine from the wet plain. The association between serological and molecular tests for epidemiological studies indicates the prevalence of individuals exposed and infected, respectively. This study confirmed the presence of the virus in its latent form based on the nested PCR results on apparently healthy animals, as demonstrated by PCR-positive animals "female 750" and "male 29/10". The first animal was nonreactive to SN, and no virus was isolated. In the male, viral isolation was also unsuccessful, and no anatomopathological lesions consistent with the disease were observed. The latent infection of SuHV-1 confirmed by PCR in serologically nonreactive pigs was previously reported (Cândido et al., 1999; Ruiz-Fons et al., 2007; Müller et al., 2011).

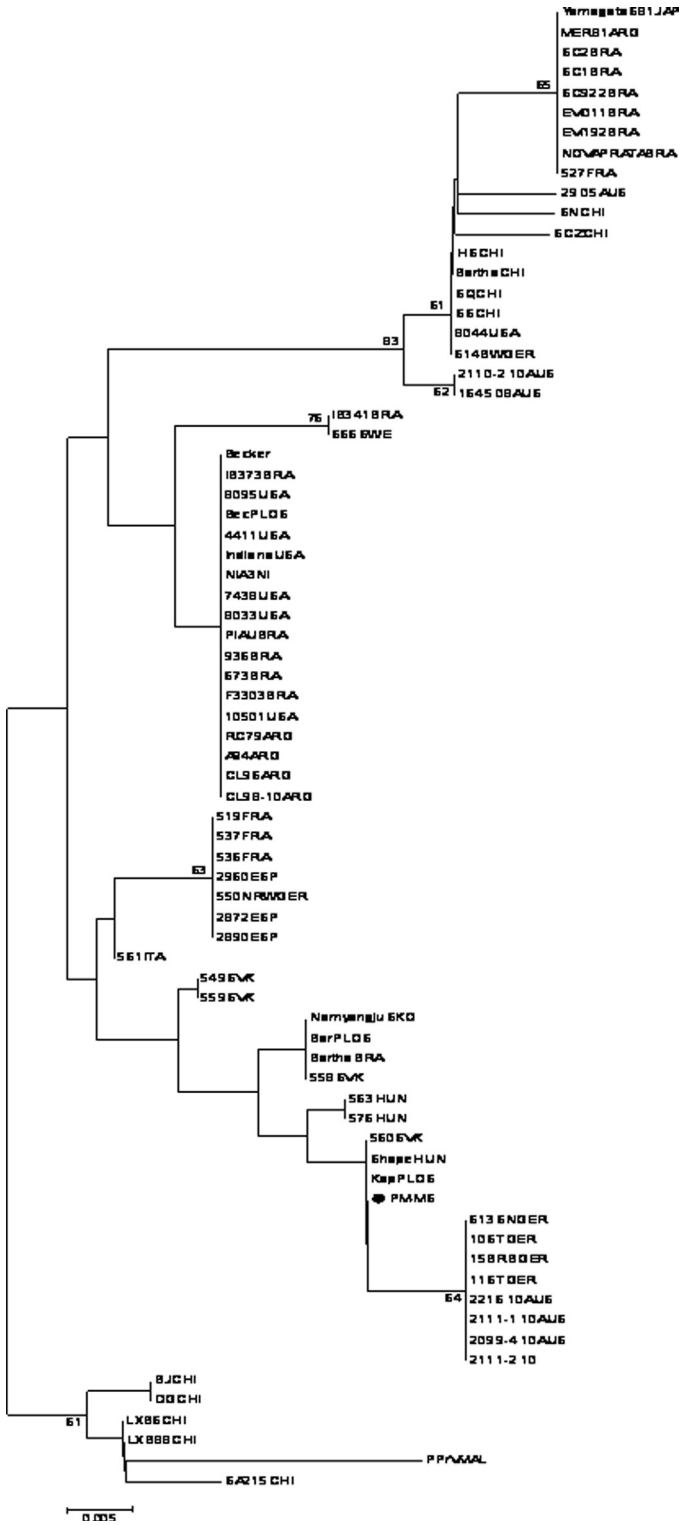


**Fig. 4.** Phylogenetic tree reconstructed from the partial sequences of the *ul44* gene from SuHV-1 isolated from wild boar en Europe. The sequence of SuHV-1 isolated in China (Ea) was used as an outgroup to root the tree. Samples are named with acess number from Geanbank, followed by the named described in the work (Müller et al., 2010; Steinrigl et al., 2012). The last tree letters indicate the country were the virus were isolated: Germany (GER), Austria (AUS), China (CHI), Slovakia (SLK), Spain (SPN), Hungary (HUN). The FS/MS isolate groups with samples from Eastern Europe.

The partial sequencing of *ul44* demonstrated the existence nucleotide substitutions that distinguish FS/MS isolates from viruses isolated from domestic pigs in Brazil until 2003. The genetic similarity varies between sequenced genes, although it is greater than 99% in all of the isolates, in agreement with previously reported results (Goldberg et al., 2001; Müller et al., 2010). SuHV-1 is a highly stable virus sharing several features with the herpes virus, including a low mutation rate of approximately $10^{-8}$ mutations per nucleotide annually (Sakaoka et al., 1994). The few nucleotide substitutions found in SuHV-1 coding regions are significant because they enabled the differentiation of FS/MS from other viruses isolated in Brazil, even when compared with those with the same *BamH*I genotype. The phylogenetic analyses suggest that the outbreaks of pseudorabies in Brazil were not related to the SuHV-1 of feral swine. In fact, feral swine in the Pantanal region live in isolation in the wild environment and have no contact with domestic pigs. In addition, the strong environment selective pressures through three centuries in the Pantanal region (Desbiez et al., 2009) may be selected resistant individuals.

The SuHV-1 that circulates in populations of Brazilian feral swine is genetically similar to the virus isolated from wild boars in Eastern Germany, Austria, Hungary and Slovakia. Studies conducted with viruses from wild boars in Europe indicate that these strains have low virulence and cause no clinical manifestations of pseudorabies (Müller et al., 2011) and in the present work no clinical signs were observed from animals sampled. The genetic similarity with the virus circulating in Europe may be due to an older introduction, different from the one that occurred in domestic pigs from other regions of Brazil, or due to contact with European wild boars. Wild boar were introduced in Brazil at the end of the 1980s and have since expanded throughout the territory and can now be detected in nine states (including MS) and are considered to be an invasive species that is capable of causing high ecological and economic damages (Deberdt and Scherer, 2007).

The present study show that the SuHV-1 circulating among feral swine is not related to the outbreaks recorded in Brazil until 2003, likely due to the geographical isolation of feral swine in Pantanal wetland. The role of feral swine in the epidemiology of SuHV-1 in the Pantanal region may be monitored which attention because (a) swine is a excellent reservoir host for SuHV-1 because it is a asymptomatic carrier; (b) SuHV-1 is a multi-host pathogen and the Pantanal region harbor great diversity of wild mammal's taxa sharing the same habitats with large livestock populations; and (c) the infection by SuHV-1 is an important sanitary national and international barrier for the pig meat trade.

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