



Genetic identification, clinical and epidemiological aspects of an equine infectious anemia outbreak in the Rio Grande do Sul State, Brazil

José Conrado dos Santos Jardim¹  Paula Fonseca Finger²  Grazielle Vieira Cristofari³ 
Bruno Leite Anjos^{1,2}  Carolina Kist Traesel²  Mário Celso Sperotto Brum^{1,2*} 

¹Programa de Pós-graduação em Ciência Animal, Universidade Federal do Pampa (UNIPAMPA), 97508-000, Uruguaiana, RS, Brasil. E-mail: mariobrum@unipampa.edu.br. *Corresponding author.

²Curso de Medicina Veterinária, Universidade Federal do Pampa (UNIPAMPA), Uruguaiana, RS, Brasil.

³Secretaria do Estadual de Agricultura, Pecuária e Desenvolvimento Rural (SEAPDR), Inspeção de Defesa Agropecuária (IDA), Uruguaiana, RS, Brasil.

ABSTRACT: The study describes the genetic identification, clinical, and epidemiological characteristics of an outbreak of equine infectious anemia occurring in the state of Rio Grande do Sul, Brazil. Three animals kept in the periurban region of Uruguaiana city tested positive for the AGID test. The serology was performed as a requirement for transit. None of the animals showed clinical signs of infection, one animal was necropsied, and the others were stolen. In the post-mortem examination, no macroscopic changes were observed, and microscopically, discrete hemosiderosis was detected in fragments of the liver and spleen. Amplifying and sequencing a proviral DNA fragment in blood, spleen, and mesenteric lymph node samples confirmed EIAV infection. Phylogenetic analysis of the first sequenced EIAV sample from the Rio Grande do Sul State indicates a high similarity with other Brazilian samples. Results confirmed the viral presence in the state's herds and described epidemiological and virological characteristics of EIA that contribute to the maintenance and dissemination of the virus in herds.

Key words: horses, equine infectious anemia virus, molecular epidemiology, phylogenetic analysis, transboundary disease.

Identificação genética, aspectos clínicos e epidemiológicos de um foco de anemia infecciosa equina no Estado do Rio Grande do Sul, Brasil

RESUMO: O estudo descreve a identificação genética, as características clínicas e epidemiológicas de um foco de Anemia Infecciosa Equina que ocorreu no Estado do Rio Grande do Sul, Brasil. Três equinos criados na região periurbana da cidade de Uruguaiana testaram positivos pela prova sorológica de IDGA. O exame foi realizado como requerimento para trânsito dos animais. Nenhum animal apresentava sinais clínicos da infecção, um cavalo foi necropsiado e os outros dois foram roubados. Na necropsia não observou-se nenhuma alteração e microscopicamente foi constatada hemosiderose discreta em fragmento do fígado e baço. A infecção foi confirmada pela amplificação e sequenciamento de um segmento do genoma pró-viral do EIAV de amostras do sangue, baço e linfonodo mesentérico. A análise filogenética do primeiro EIAV sequenciado no Estado do RS indica similaridade com outras amostras que circulam no Brasil. O resultado confirma a presença do vírus no rebanho equino da região e descreve características clínicas e epidemiológicas que contribuem para a manutenção e disseminação do vírus no rebanho.

Palavras-chave: equinos, vírus da anemia infecciosa equina, epidemiologia molecular, análise filogenética, doença transfronteiriça.

INTRODUCTION

Equine infectious anemia (EIA), also known as swamp fever, is a significant viral disease in horses worldwide (JARA et al., 2020). The first description of the condition occurred in 1843 in France, and in 1904 there was the definition of viral etiology (COOK et al., 2013). In Brazil, with an equine herd of more than 4 million animals, the infection was confirmed in 1968 (MAPA, 1988; IBGE, 2017).

Equine infectious anemia virus (EIAV) belongs to the *Retroviridae* family, subfamily

Orthoretrovirinae, and genus *Lentivirus* (COOK et al., 2013; LEROUX et al., 2004). During the process of infection of the host (horses, donkeys, mules, and zebras), the viral RNA genome is reverse transcribed into DNA and inserted into the cell genome, giving rise to the proviral DNA, especially in monocytes and macrophage cells. Thus, the animals develop persistent infection and are considered the primary source of the virus to susceptible animals (COOK et al., 2013; LEROUX et al., 2004). EIAV is transmitted by blood transfer between animals, with the iatrogenic route or mechanical vectors (*Tabanus*

spp. and *Stomoxys* spp.) being the most common ways of dissemination (COOK et al., 2013, ISSEL & FOIL, 2015). The clinical presentation associated with infection can be classified as acute, chronic, or not apparent. The clinical signs are anemia, edema of the ventral region, mucosal hemorrhages, and progressive weight loss (COOK et al., 2013). Laboratory diagnosis (gold standard) is made by serological agar gel immunodiffusion test (AGID) performed in Brazil by private laboratories accredited by the Official Veterinary Service (OVS) (BRASIL, 2004; COOK et al., 2013). The owner of a positive horse may request a retest, performed from a new blood collection by the OVS and tested in the reference laboratory of MAPA (Ministério da Agricultura, Pecuária e Abastecimento) (BRASIL, 2004).

In 1981, the Brazilian authorities (MAPA) instituted the official control of diagnosis and notification of positive cases, which followed the recommendations of the World Organization for Animal Health (WOAH) (BRASIL, 2004). Infection is widespread throughout the territory at varying levels, with the Pantanal (25%) and northern regions (46%) showing the highest prevalence; conversely, southern states show low levels (> 1%) (ALMEIDA et al., 2006; BORGES et al., 2013; CRUZ et al., 2020; TIGRE et al., 2017). In Rio Grande do Sul (RS), located in the extreme south of Brazil, the estimated prevalence of EIAV is close to 0.3% (SEAPA, 2014; BARZONI et al., 2018; REBELATTO et al., 1992). RS has the State Equine Health Program (PESE - SEAPDR), which follows the guidelines of the national program and is suitable for the state's epidemiological situation (SEAPI, 2018). All properties and animals must be registered in the Agricultural Defense System (SDA-SEAPDR). The movement of equines should only happen upon the presentation of a negative AGID test for EIA and the issuing of an animal transport certificate (GTA) by the OVS. The property that presents at least one positive animal is considered an outbreak; the seropositive horses are sacrificed, following the interdiction and testing of all other susceptible animals on the property (SEAPI, 2018).

The western border region of Rio Grande do Sul is characterized by having a significant population of horses used in cattle and sheep management, breeding, and sports (rodeo, racing, and horse riding) (COSTA et al., 2013). This region corresponds to the extensive Brazil-Argentina-Uruguay triple border area and concentrates a considerable portion of the EIA outbreaks in the state (BARZONI et al., 2018; CARDENAS et al., 2022; MACHADO et al., 2021). These outbreaks are associated with the illegal movement of animals between properties, difficulty

in identifying clinical cases, lack of preventive examinations, and even illegal cross-border movement of animals (BARZONI et al., 2018; MACHADO et al., 2021). The present study described the clinical, epidemiological, and virological characteristics observed in an Equine Infectious Anemia outbreak. Results assist in understanding the maintenance of EIAV in the western region of RS and can serve as a model to understand other situations.

MATERIALS AND METHODS

Case description

Three equines, two males and one mare, mixed breed, were diagnosed positive for EIA by AGID. The animals were on a property located in the peri-urban perimeter of Uruguiana; the Rio Grande do Sul, Brazil (Figure 1). The clinical and epidemiological data were obtained from the official forms for disease investigation; at the time of the first serological examination, no animal presented clinical signs of infection.

Hematology and pathology

Blood samples were collected for hematological analysis. An autopsy was performed on one horse when fragments of different tissues (kidney, lung, liver, spleen, and lymph nodes) were collected for anatomopathological and viral analysis. According to routine protocols, the tissue fragments were fixed in 10% formalin, processed and stained with Hematoxylin and Eosin, and analyzed by light microscopy.

DNA extraction

Whole blood (500µL) and tissue (100mg) samples were subjected to digestion with proteinase K, then extracted total DNA by the phenol-chloroform method. Total DNA was precipitated with sodium acetate (3M) and 2.2 volumes of ethanol (100%) (OAKS et al., 1998). After centrifugation, the pellets were washed with 70% alcohol, solubilized in 32 µl of TE buffer, and stored at -80 °C. DNA concentration and purity were determined by spectrophotometry (GE NanoVue Plus).

DNA Amplification by Nested-PCR

Total DNA (250 - 500 ng) from each sample was subjected to amplification of proviral DNA by Nested-PCR reaction within the LTR region, extending to the trans-activator gene (*tat*) (DONG et al., 2012). In the first reaction, the primers 5'-GTAATTGGGCGCTAAGTCTAG-3' and 5'-CCTCTAATAAATCTTGCTGTC-3' were used, generating a 246 bp product. We used the primers

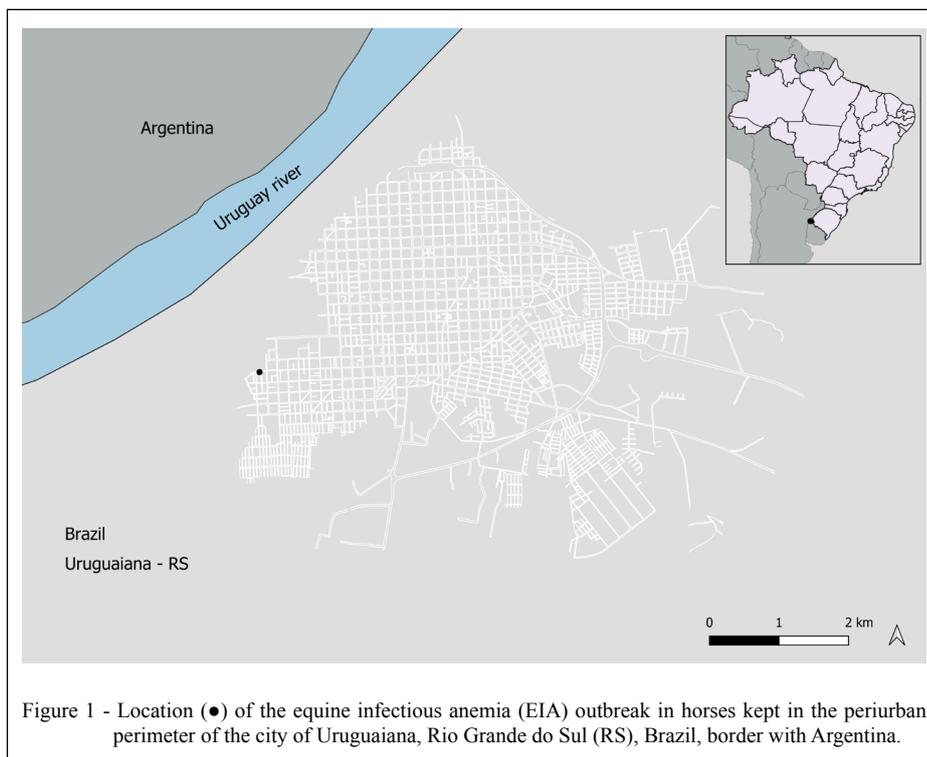


Figure 1 - Location (●) of the equine infectious anemia (EIA) outbreak in horses kept in the periurban perimeter of the city of Uruguaiiana, Rio Grande do Sul (RS), Brazil, border with Argentina.

5'-TGGGTGAATACCATACAGACA-3' and 5'-CCAGTGGAGCATTCCGGTAA-3' in the second round, generating an amplicon between 198 and 203 bp. The products of the reactions were resolved by 1.5% agarose gel electrophoresis and stained with non-mutagenic reagents (Sybr Green Invitrogen) for identification. Positive (DNA from leukocytes from a serologically positive animal) and negative (water) controls were included in all tests.

Sequencing and phylogenetic analysis

Phylogenetic analysis was performed with the program Molecular Evolutionary Genetics Analysis (MEGA) version X 20, using the Joining Tree methodology with bootstrap values calculated using 1000 replicates. Positive PCR amplified samples were purified (PCR Products Purification Kit Mebep Bioscience) and sequenced by the Sanger method using a sequencer AB-3500 (Applied Biosystems) (ACTGene Análises Molecular Ltda, Alvorada, Rio Grande do Sul, Brazil). The BioEdit Sequence Alignment Editor Software suite evaluated and edited the sequences 7.0.5.3 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) to obtain the consensus. Subsequently, the consensus sequences were submitted to the Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/BLAST/>) to compare sequences already deposited in GenBank.

RESULTS AND DISCUSSION

Equine infectious anemia virus infection in the state of Rio Grande do Sul has a low occurrence (BARZONI et al., 2018; CARDENAS et al., 2022; REBELATTO et al., 1992). However, in the last five years, there has been a moderate increase in the notification of cases of infection. At the beginning of the 2010s, there were 2 to 4 outbreaks/per year, and by the end of the decade, there were more than ten outbreaks per year. This situation has drawn the attention of producers and animal health authorities (BARZONI et al., 2018; CARDENAS et al., 2022; MACHADO et al., 2021). Results presented here described the specific characteristics of an outbreak that occurred in the state region that concentrates the most significant record of EIAV infections. This outbreak represents a unique situation that combines clinical and epidemiological aspects that can contribute to the maintenance and dissemination of the virus among animals and herds. Furthermore, knowledge of the different elements involved contributes to understanding the epidemiology of infection. It demonstrated possibilities that should be considered in investigating outbreaks in the region and serve as an alert for other situations.

In Brazil, the infection presents varying prevalence levels among the different states and

regions (ALMEIDA et al., 2006; BORGES et al., 2013; CRUZ et al., 2020; TIGRE et al., 2017). The variations are associated with regional factors such as equine population, the form of breeding, occupation of animals, climate, temperature, the occurrence of mechanical vectors, cultural aspects, and control legislation (BARZONI et al., 2018; CARDENAS et al., 2022). Thus, identifying regional characteristics of equine breeding and the occurrence of outbreaks contributes to understanding the factors that influence viral transmission in a given population.

The animals of this outbreak were identified by AGID serological test for issuing the animal transport permit (GTA). These animals would participate in agglomeration event with more than 5,000 horses (parade), which would have the presence of the inspection of the OVS. For participation, the horses must be moved and undergo the serological examination for EIA, which is mandatory for issuing the GTA. Most EIA diagnoses in Rio Grande do Sul are registered in August, September, and October. This is due to a large number of cultural and sporting events with the presence of horses that exist in this period. Therefore, in this period, the highest number of diagnoses occurs (BARZONI et al., 2018; MACHADO et al., 2021). Thus, passive surveillance plays an essential role in identifying infected animals since the report of clinical cases of EIA is relatively infrequent (BARZONI et al., 2018; REBELATTO et al., 1992).

Animal-to-animal transmission of EIAV occurs mainly through blood transfer between equids by iatrogenic route or mechanical vectors (*Tabanus* spp. and *Stomoxys* spp.) (ISSEL & FOIL, 2015). The form and timing of infection of these animals have not been identified. However, horseflies are present in the region, and sharing injectable material is common (BARZONI et al., 2018). The region's climatic conditions, with cold winter and hot summer, are associated with the seasonality of tabanids, which may limit the role of these insects in the transmission of the agent, different from that observed in the Brazilian Pantanal (CURSINO et al., 2018).

The property where the horses were raised is located in the periurban region of the city of Uruguaiana (Figure 1). The herd consisted of only three horses, and none had clinical signs suggestive of the infection. The animals were slightly thin, which was associated with poor feed quality. The owner did not inform the origin of the animals, but it is believed to be rural (farm). After the positive serology result for EIA (day 0), the OVS interdicted the property (day 11). During the interdiction period, the owner is responsible for the isolation and maintenance of positive animals,

and the transit of equines is prohibited (PESE) (SEAPI 2018). The counter-test (using the same serum sample) conducted by the accredited laboratory confirmed the previous result. The producer requested the retest during the interdiction (day 11). Retesting is allowed by the legislation, and the AGID test is performed in the official laboratory. However, the OVS performs the new blood collection (SEAPI, 2018). The result of the retest (day 28) confirmed the previous findings. On the day the OVS communicated the final retest results, the owner informed thus that the three animals had been stolen and were missing. The disappearance of animals diagnosed with EIAV is a fact described and contributes to the maintenance of a carrier animal in the population (BARZONI et al., 2018; CRUZ et al., 2020).

The breeding and keeping horses in the peri-urban region may contribute to the maintenance and dissemination of EIAV (BARZONI et al., 2018; CRUZ et al., 2020). In this situation, animals are raised on small properties, with inadequate nutritional and sanitary conditions, and without regular veterinary assistance. The eventual contact of these animals with other equids raised on farms or stud farms may contribute to the maintenance of the virus.

On day 78, one of the stolen horses was found and euthanized. At the time of sacrifice, the animal was in good body condition and healthy. The hematological examination showed mild leukocytosis (14,500 leukocytes/ μ L - 5,000 - 11,000) associated with neutrophilia (7,380 cells/ μ L - 2,200 - 6,100). No macroscopic changes were detected during the necropsy. The absence of clinical signs, hematological changes, and visible lesions are compatible with carrier status and are described in the literature as the most common form of infection (BUENO et al., 2020; COOK et al., 2013).

Histopathological evaluation of the spleen revealed moderate hemosiderosis in the splenic parenchyma, both free and in the cytoplasm of reticulo-phagocytic cells. The other organs evaluated showed no noteworthy changes. The spleen and liver are recognized as the main reservoirs of the virus due to the significant presence of macrophages (BUENO et al., 2020). This microscopic change is associated with the accumulation of ferric pigment, especially in resident macrophages, resulting from hemoglobin metabolism triggered after intra and extravascular hemolysis associated with the viral infection and expected in these diseases (BUENO et al., 2020; COOK et al., 2013). The other two stolen horses have remained missing and are probably still being transported illegally. Animals in this situation serve as a source of infection for susceptible equines as

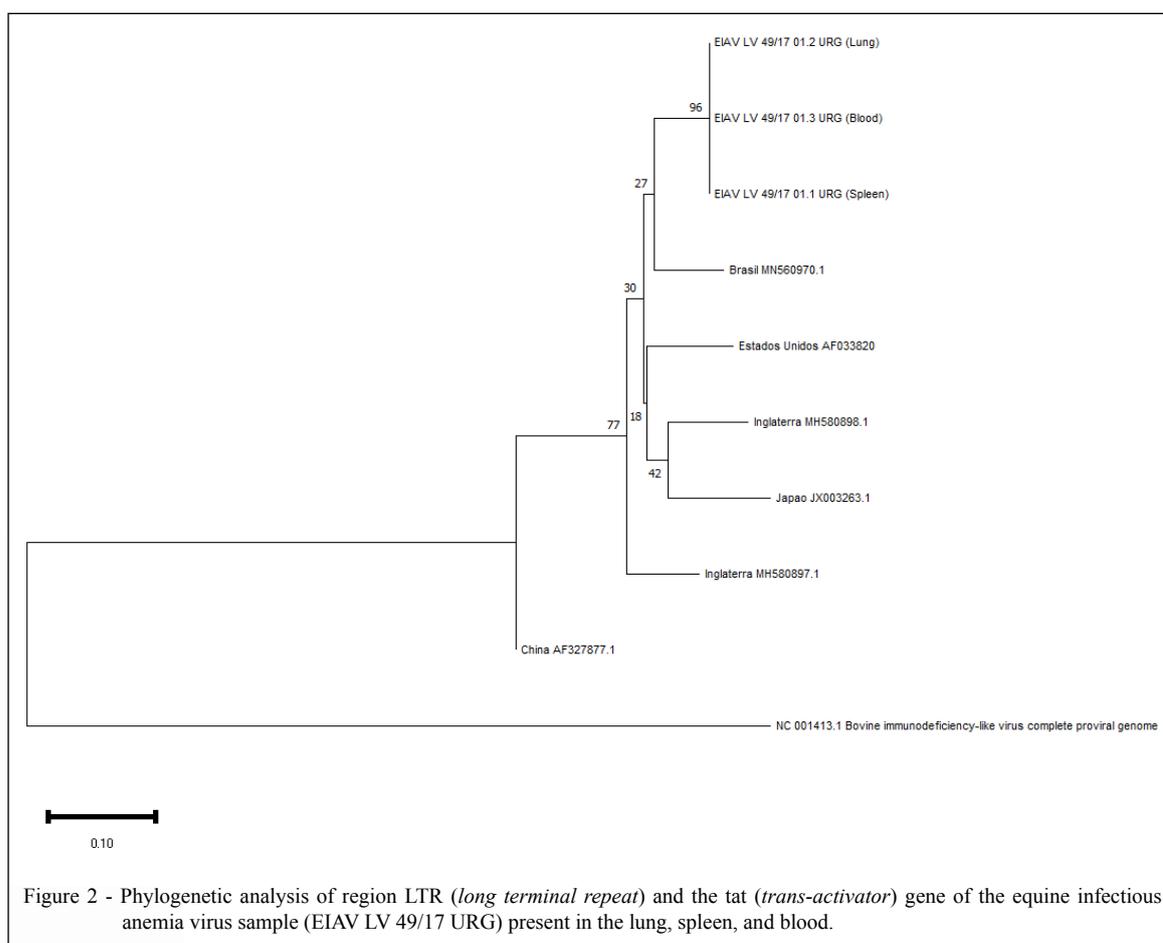
they stay in the herd (BARZONI et al., 2018; CRUZ et al., 2020).

Nested-PCR detected proviral DNA in blood, lung, and spleen samples, and a DNA fragment (~ 203bp) corresponding to that described by DONG et al. (2012) was amplified. Amplifying proviral DNA in tissue samples confirms the serology result and demonstrates that the virus is present in organs with a high concentration of macrophages (BUENO et al., 2020; OAKS et al., 1998). The PCR products were sequenced and the sequences obtained (Submission ID: ON639609) showed 98% to 100% identity with other EIAV samples (Figure 2).

Several genetic analyses of EIA viruses present in Bahia, Rio Grande do Norte, and Pantanal have been performed (BUENO et al., 2020; CURSINO et al., 2018; TIGRE et al., 2017). The Rio Grande do Sul is the southernmost state in Brazil, and this is the first time that an EIAV sample from this region has been partially identified by sequencing. Thus, comparison with other viruses circulating

in RS samples is impossible. However, further genetic identification studies must be performed to characterize the infection's epidemiology. The sequenced region refers to the terminal portion of the LTR (long terminal repeat) and the *tat* (trans-activator) gene (DONG et al., 2012; LEROUX et al., 2004). Despite the high variability of lentiviruses associated with geographic distribution, the LTR region has highly conserved elements, similar to the *tat* gene (DONG et al., 2012; LEROUX et al., 2004, MALOSSI et al., 2020). Thus, the amplified region can be used in phylogeny studies for epidemiological research. However, other regions of the genome or the whole genome may be more suitable for this purpose (BUENO et al., 2020; CURSINO et al., 2018; MALOSSI et al., 2020; OAKS et al., 1998; TIGRE et al., 2017).

The highest concentration of EIAV outbreaks in RS is located in the western border region (BARZONI et al., 2018; CARDENAS et al., 2022). This region is characterized by extensive cattle and



sheep production and concentrates the largest equine herd in the state (~149,000 animals, 14% of the state's herd) (COSTA et al., 2013). In this region, horses are used for work on the farms and eventually participate in sporting events and parades. Besides the animals used for work, there are pure breed's farms (PSI and Crioulo) that move animals to other regions of the state and Brazil (COSTA et al., 2013; IBGE, 2017). On the outskirts of cities, many draft horses and animals are raised on small farms, which EIAV can eventually infect (CRUZ et al., 2020; MACHADO et al., 2021). The illegal movement of animals is frequently reported in the region (BARZONI et al., 2018; CRUZ et al., 2020). The animals diagnosed with EIA were kept on the outskirts of the city; however, the owner did not inform the origin of the animals, nor did he suspect the moment of infection. Most likely, the animals originated from a property located in the interior of the municipality and were transported without examination.

The voluntary testing of animals is relatively low. This may be due to the absence of clinical suspicion, the costs of the examination, and the fear that owners have about having an infected horse sacrificed. The health certificate requirement for transportation and the presence of inspection in events forces producers to perform health examinations, thus increasing the number of tests and; consequently, identifying outbreaks (MACHADO et al., 2021). Therefore, passive surveillance plays a crucial role in identifying infected animals (BARZONI et al., 2018; CRUZ et al., 2020; MACHADO et al., 2021). Another aspect that should be reinforced is building a relationship of trust between the producer and OVS. This type of relationship facilitates epidemiological investigation, clarifying the origin of the infection. It enables measures to prevent new cases and adopt actions that can reduce the circulation of the agent.

CONCLUSION

The results described the characteristics of an outbreak of equine infectious anemia that occurred in a region of Rio Grande do Sul, Brazil. A moderate increase in the registration of cases has been observed in the last five years. The conditions of absence of clinical signs and carrier of the infection, associated with the form of breeding and disappearance of two positive animals, are characteristics that contribute to the maintenance and dissemination of EIAV among animals in the region. These factors alone or together create opportunities for EIAV to perpetuate itself in herds. The molecular identification of a sample circulating in the

Rio Grande do Sul opens the way for the phylogenetic study of samples from other foci, helping to determine the origin of the samples present in the State and can assist in establishing epidemiological links in situations of unavailability of information.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. Funding sponsors had no role in the study design, collection, analysis, and data interpretation during the writing this manuscript and in the decision to publish the results.

AUTHORS' CONTRIBUTION

JCJS (execution, results analysis, writing, review), PFF, GVC, CKT, BLA (execution, results analysis, review), MCSB (conception, results analysis, writing, review, coordinator). All authors critically revised the manuscript and approved of the final version.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

For all due purposes, the authors of the article "Genetic identification, clinical and epidemiological aspects of an equine infectious anemia outbreak in the Rio Grande do Sul State, Brazil" declare that the project that gave rise to the data of the same was not submitted for evaluation to the Ethics Committee of the Universidade Federal do Pampa. However, we are aware of the content of the resolutions of the National Council for the Control of Animal Experimentation - CONCEA "<http://www.mct.gov.br/index.php/content/view/310553.html>" if it involves animals. Thus, the authors assume full responsibility for the data presented and are available for possible questioning, should they be required by the competent bodies.

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