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Dissertação

Detecção e identificação genética de herpesvírus equídeo em equinos na região sul do Rio Grande do Sul

Tamires Ellen Tomio

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Tamires Ellen Tomio

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Banca examinadora:

Prof. Dr. Marcelo de Lima (Orientador)
Doutor em Medicina Veterinária pela Universidade Federal de Santa Maria

Prof. Dr. Geferson Fischer
Doutor em Biotecnologia Agrícola pela Universidade Federal de Pelotas

Prof. Dr. Mario Celso Sperotto Brum
Doutor em Medicina Veterinária pela Universidade Federal de Santa Maria

Prof. Dr. Charles Ferreira Martins
Doutor em Medicina Veterinária pela Universidade Estadual Paulista Júlio de Mesquita Filho

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Aos meus pais

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Resumo

TOMIO, Tamires Ellen. **Detecção e identificação genética de herpesvírus equídeo em equinos na região sul do Rio Grande do Sul.** 2020. 36f. Dissertação (Mestrado em Ciências) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2020.

Os herpesvírus de equídeos são endêmicos e reconhecidos mundialmente pelos prejuízos econômicos nos rebanhos acometidos. Entre os principais herpesvírus que afetam equinos, destacam-se os alfaherpesvírus equídeo tipos 1 e 4 (EHV-1; EHV-4), associados a enfermidade respiratória, reprodutiva ou neurológica, e os gamaherpesvírus equídeo tipos 2 e 5 (EHV-2; EHV-5), que, apesar de associações com enfermidade respiratória, têm seu real impacto nos rebanhos ainda sob investigação. Estudos sorológicos realizados no Brasil indicam uma ampla disseminação do EHV-1/EHV-4 na população equina. Por outro lado, com exceção de um trabalho recente, no qual os EHV-2 e -5 foram identificados em animais assintomáticos na região sul do país, estudos demonstrando a identificação e circulação dos outros tipos de herpesvírus de equinos são escassos em nível nacional. Assim, este estudo demonstra a identificação e caracterização genética do EHV-1, -2, -4 e -5 em amostras clínicas de equinos distribuídos em 7 diferentes propriedades da região sul do Rio Grande do Sul. De um total de 179 amostras de sangue/leucócitos (BC) e 110 amostras de swabs nasais (NS) testadas por PCR, o EHV-1 foi detectado em 11 amostras clínicas (BC, n=8; NS, n=3), enquanto o EHV-4 foi detectado em 13 amostras (BC, n=4; NS, n=9). O EHV-2 foi detectado em 6 amostras clínicas (BC, n=1; NS, n=5) e o EHV-5 em 13 amostras de sangue. A presença de infecções mistas por 2 vírus foi observada em 6 animais, enquanto 2 animais apresentaram infecções por 3 tipos diferentes de herpesvírus. O sequenciamento dos amplicons revelou um percentual de identidade de 97,8–100% com cepas padrão de herpesvírus equinos depositadas no GenBank. O EHV-1 e -4 foram diagnosticados em duas propriedades com cenários distintos, seja como sugestivo de infecção ativa e excreção viral no momento da coleta das amostras ou como indicativo de infecções latentes em uma parcela dos animais avaliados. O EHV-2 e -5 foram detectados exclusivamente em uma das propriedades. Os achados sugerem replicação ativa e excreção do EHV-2, enquanto infecções latentes pelo EHV-5 podem ter sido estabelecidas após contato e transmissão a partir de um equino, na mesma propriedade, com diagnóstico de EMPF. Este trabalho demonstra a detecção e identificação genética de quatro tipos de herpesvírus equinos em propriedades da região sul do RS e representa uma contribuição para o diagnóstico e o conhecimento da situação epidemiológica desses agentes em nível regional e nacional.

Palavras-chave: herpesvírus equídeo; co-infecção; caracterização genética.

Abstract

TOMIO, Tamires Ellen. **Detection and genetic identification of equid herpesvirus in horses from southern Brazil.** 2020. 36p. Dissertation (Master degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2020.

Equid herpesvirus are widespread in the horse population and represent a major threat to the horse industry worldwide. Both EHV-1 and EHV-4 produce well-documented respiratory, reproductive and neurological disease in horses, whereas the real impact of EHV-2 and EHV-5 is less clearly defined. In Brazil, serological investigations have indicated the circulation of EHV-1/EHV-4 in the country. However, genetic characterization of the circulating viruses are mainly focused on the EHV-1. Except for the recent demonstration of EHV-2 and EHV-5 in asymptomatic horses, studies aimed at identifying other equid herpesviruses are scarce. This study describes the detection and genetic characterization of EHV-1, -2, -4 and -5 in clinical samples from horses located at seven farms from southern Brazil. Virus-specific PCRs were used to detect EHV-1, -2, -4 and -5. From the total of 179 buffy coat samples (BC) and 110 nasal swabs (NS), collected from 179 animals, EHV-1 was detected in 11 clinical samples (BC, n=8 and NS, n=3) whereas EHV-4 was detected in 13 samples (BC, n=4 and NS, n=9). In addition, EHV-2 was detected in 6 clinical samples (BC, n=1 and NS, n=5) whereas EHV-5 specific DNA was detected in 13 buffy coat samples. Additionally, mixed infections by two or three viruses were recorded in 6 and 2 animals, respectively. Nucleotide sequence identity among each other ranged from 97.8% to 100% when compared to reference strains deposited in GenBank. EHV-1 and EHV-4 were found in two farms and may represent distinct scenarios, either as indicative of active infection and viral shedding during sample collection or suggestive of latent infections in some horses. On the other side, EHV-2 and EHV-5 were exclusively detected in a single farm. Results have suggested active viral replication and EHV-2 shedding from the infected animals. Latent infection by EHV-5 in the PCR positive horses might have been established after a close contact in the same farm with a horse diagnosed with EMPF postmortem. In summary, results from this investigation demonstrate the detection and genetic identification of four types of equid herpesvirus in horses from southern Brazil and may represent a contribution to diagnosis and better understanding of epidemiological situation of these viral agents in the region.

Keywords: equid herpesvirus; co-infection; genetic characterization.

Lista de Tabelas

Lista de Abreviaturas e Siglas

DNA	Ácido desoxirribonucleico
EHM	Mieloencefalite equina por herpesvírus
EHV	Herpesvírus equídeo (*Equid herpesvirus*)
IBGE	Instituto Brasileiro de Geografia e Estatística
kbp	Quilopares de bases (*kilobases pairs*)
MAPA	Ministério da Agricultura, Pecuária e Abastecimento
nm	Nanômetros
UL	Região única longa (*unique long region*)
ORF	Fase aberta de leitura (*open reading frame*)
EMPF	Fibrose pulmonar multinodular equina

Sumário

1 Introdução

O rebanho equino brasileiro, segundo dados do IBGE (Instituto Brasileiro de Geografia e Estatística), totalizou 5.751.798 cabeças em 2018. Dessas, 915.347 estão localizadas na região sul do país, sendo 527.881 animais somente no estado do Rio Grande do Sul. O “Complexo do Agronegócio do Cavalo” (termo criado em 2006 para dimensionar a importância econômica e social dessa espécie no país), totalizou R$16,15 bilhões, conforme dados divulgados pelo MAPA (Ministério da Agricultura, Pecuária e Abastecimento) em 2016. Além do rebanho equino brasileiro ser o maior da América Latina e quarto maior do mundo, estando atrás apenas dos Estados Unidos, China e México, este também possui um papel relevante no cenário econômico brasileiro, sendo fonte de aproximadamente 610 mil empregos diretos e 2.430 mil empregos indiretos.

Estes dados refletem a importância econômica da atividade, assim como a necessidade de intensificação de pesquisas, visando a qualificação do setor e, principalmente, o manejo sanitário dos rebanhos. Dentre as enfermidades infecciosas que acometem os equinos, as infecções causadas pelos herpesvírus possuem um papel reconhecidamente importante em nível mundial e estão frequentemente associados com perdas econômicas significativas (Oladunni et al., 2019). Neste sentido, é essencial o conhecimento regional da situação epidemiológica e da circulação dos principais agentes infecciosos associados com prejuízos econômicos à indústria equina.

A ordem *Herpesvirales* é composta por três famílias virais: *Alloherpesviridae*, que engloba vírus de ranídeos e peixes ósseos, *Herpesviridae*, que inclui vírus de mamíferos, aves e répteis, e *Malacoherpesviridae*, que contém os herpesvírus de ostras. A família *Herpesviridae* é subdividida em três subfamílias, de acordo com as suas propriedades biológicas (ICTV, 2018). A subfamília *Alphaherpesvirinae* possui cinco gêneros e 41 espécies, além de uma gama variável de hospedeiros, estabelecendo infecções latentes primariamente em neurônios dos gânglios sensoriais e autonômicos. O gênero *Varicellovirus*, pertencente à esta subfamília possui 18 espécies, dentre elas o Alfaherpesvírus equídeo tipos 1, 3 e 4 (*Equid*

alphaherpesvirus, EHV-1, EHV-3 e EHV-4), conforme apresentado na Tabela 1. A subfamília *Betaherpesvirinae* possui quatro gêneros e 23 espécies, e uma gama restrita de hospedeiros. O vírus pode estabelecer latência em células do sistema imune, principalmente monócitos ou linfócitos T. A subfamília *Gammaherpesviridae* possui quatro gêneros e 36 espécies, e assim como os betaherpesvírus, possui uma gama restrita de hospedeiros. Além disso, estabelecem infecções latentes principalmente em linfócitos B, além de possuírem potencial oncogênico. Os Gamaherpesvírus equídeo tipos 2 e 5 (*Equid Gammaherpesvirus*, EHV- 2 e EHV-5) pertencem ao gênero *Percavirus* (Tabela 1) (Louten, 2016; Franco et al., 2017; ICTV, 2018).

Tabela 1. Herpesvírus que acometem a espécie equina

Família	**Subfamília**	**Gênero**	**Espécie**	**Sigla**
Herpesviridae	*Alphaherpesvirinae*	*Varicellovirus*	*Alphaherpesvirus equid 1*	EHV-1
			Alphaherpesvirus equid 3	EHV-2
			Alphaherpesvirus equid 4	EHV-3
	Gammaherpesviridae	*Percavirus*	*Gammaherpesvirus equid 2*	EHV-4
			Gammaherpesvirus equid 5	EHV-5

Os herpesvírus são vírus envelopados, com cerca de 150 nm de diâmetro, possuem um nucleocapsídeo icosaédrico e DNA linear de cadeia dupla (Murphy, 1999). O sequenciamento genômico completo do EHV-1 indicou que o genoma viral possui 150.223 nucleotídeos, contendo cerca de 80 ORFs (*open reading frames*) (Telford, 1992). O genoma viral contém 76 genes com potencial para codificar 77 proteínas diferentes (Patel, 2005). O EHV-1, 3 e 4 possui aproximadamente 125 kpb, enquanto o EHV-2 e 5 possui, 180 kpb (Louten, 2016).

O EHV-1 e 4 possui genoma do tipo D, onde a UL (*unique long region*) é flanqueada por uma repetição invertida muito pequena e não relacionada. O EHV-2 e 5 possui a estrutura do genoma do tipo A que consiste em uma região única

flanqueada por uma repetição terminal direta nas extremidades. Os genes estão distribuídos nas duas cadeias de DNA em orientações opostas, assim, a expressão dos genes virais envolve a transcrição das duas cadeias (Davison, 2014).

Dois ciclos replicativos com características distintas são descritos e exaustivamente estudados na família *Herpesviridae*: infecção aguda ou produtiva (ciclo lítico) e infecção latente. A replicação produtiva lítica ocorre nos locais de penetração do vírus no hospedeiro, como os epitélios e tecidos adjacentes e se caracteriza pela expressão de todos os genes virais, replicação do genoma e produção de progênie viral infecciosa. O estabelecimento da infecção latente é caracterizado pela interrupção do ciclo replicativo, não ocorrendo expressão gênica significativa, replicação do genoma e produção de progênie viral infecciosa (Davison, 2014). Esse mecanismo de latência dos herpesvírus possibilita a sobrevivência e disseminação do vírus entre hospedeiros suscetíveis (Gilkerson et al., 2015; Gonzalez-Medina; Newton, 2015). A latência do EHV-1 e 4 ocorre primariamente em neurônios do gânglio trigêmeo e linfócitos, enquanto a latência do EHV-2 e 5 é descrita em linfócitos B (Louten, 2016).

Os alfaherpesvírus replicam nas mucosas das vias respiratórias superiores e/ou no trato genital de seu hospedeiro. A replicação primária nas células epiteliais da mucosa é seguida pela disseminação viral nos neurônios e/ou tecidos linfoides onde ocorre o estabelecimento da latência. A reativação, com ou sem recrudescência clínica, pode ocorrer devido a períodos de estresse ou imunossupressão. O vírus reativado retorna às mucosas respiratórias ou genitais por transporte axonal anterógrado ou por células imunes infectadas e se replica de maneira eficiente no epitélio, o que pode resultar na liberação de vírus infeccioso nas secreções respiratórias ou genitais (Cleemput et al., 2017).

A infecção pelo EHV tem início, geralmente, pela inalação de aerossóis, ocorrendo a replicação primária no trato respiratório dos animais infectados. Logo após, o EHV se dissemina sistemicamente, infectando linfócitos T circulantes e células do endotélio, podendo desencadear viremia associada a linfócitos (Paillot et al., 2008). Os sinais clínicos apresentados por animais infectados (quando presentes) pelo EHV tendem a ser inespecíficos, tornando inviável estabelecer o diagnóstico definitivo, uma vez que os sinais clínicos da infecção não são patognomônicos. Contudo, os sinais clínicos associados à infecção por EHV são predominantemente respiratórios (tosse, corrimento nasal seroso ou mucopurulento); relacionados a abortos (normalmente em

éguas no último trimestre de gestação, podendo ocorrer a formação do ‘red bag’); morte neonatal (potros nascem fracos); e neurológicos (varia de ataxia leve de membros posteriores à quadraplegia) (Ivens et al., 2019).

A infecção pelo EHV-1 geralmente resulta em doença do trato respiratório, porém pode estar associada a episódios de abortamento e doença neurológica (Vissani et al., 2009; Negussie et al., 2017; Bannai et al., 2018). A mieloencefalopatia é menos comum do que as outras formas de doença (Mori, 2014). Já a infecção pelo EHV-4 é considerada uma das principais causas de doença respiratória aguda em equinos (Studdert et al., 1984; Negussie et al., 2017). Por outro lado, o real impacto clínico das infecções pelo EHV-2 e EHV-5 ainda não é completamente esclarecido. Além das infecções subclínicas, o EHV-2 tem sido associado à enfermidade respiratória, ceratoconjuntivite e pneumonia em animais afetados (Craig et al., 2005; Dall Agnol et al., 2019).

Apesar de algumas divergências entre os pesquisadores, o EHV-5 tem sido associado a casos de fibrose multinodular pulmonar equina (EMPF) em nível mundial (Williams et al., 2007; Hart et al., 2007). Os achados clínicos mais comuns nesta enfermidade são a ocorrência de febre, perda de peso e sinais predominantemente respiratórios (Wong, 2008).

No Brasil, diversos estudos sorológicos realizados em diferentes estados sugerem uma ampla disseminação do EHV-1/EHV-4 na população equina. Os percentuais de soropositividade variam entre 4,5% a 45,5 % de acordo com a região estudada (Cunha et al., 2002 e 2009; Lara et al., 2003; Diel et al., 2006; Pena et al., 2006; Aguiar et al., 2008; Diaz et al., 2015). Porém, cabe ressaltar que vacinas contra as infecções pelo EHV-1 e EHV-4 estão disponíveis comercialmente no Brasil e que os testes sorológicos convencionais de diagnóstico não permitem a discriminação entre anticorpos vacinais daqueles induzidos pela infecção natural.

Nas últimas décadas, a identificação e caracterização genética do EHV-1, seja em infecções latentes (Carvalho et al., 2000) ou em associação com enfermidade (Costa et al., 2008; Lara et al., 2008; Mori et al., 2015; Estima-Silva et al., 2019) têm sido realizados no país. Estudos referentes a identificação dos outros tipos de herpesvírus que acometem equinos eram inexistentes em nível nacional até meados de 2019, onde foi relatado uma análise de 26 amostras de secreção nasal provenientes de equinos assintomáticos, com a identificação do EHV-2 e EHV-5 (Dall Agnol et al., 2019).

Neste sentido, considerando a escassez de dados, principalmente, no que se refere a caracterização dos diferentes tipos de herpesvírus de equinos na região e em nível nacional, este trabalho teve como objetivo principal a detecção e identificação genética dos herpesvírus equino em amostras clínicas de animais da região sul estado do Rio Grande do Sul (RS).

2 Artigo

Genetic characterization and co-infection of equid herpesviruses in Southern Brazil

Tamires Ellen Tomio, Leonardo Clasen Ribeiro, Amanda de Oliveira Barbosa, Gabriel da Silva Zani, Paulo Ricardo Centeno Rodrigues, Geferson Fischer, Silvia de Oliveira Hübner, Gilberto D'Ávila Vargas, Marcelo de Lima

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Genetic characterization and co-infection of equid herpesviruses in Southern Brazil

Tamires Ellen Tomio[1], Leonardo Clasen Ribeiro[2], Amanda de Oliveira Barbosa[1,2], Gabriel da Silva Zani[1], Paulo Ricardo Centeno Rodrigues[1], Geferson Fischer[1], Silvia de Oliveira Hübner[1], Gilberto D'Ávila Vargas[1], Marcelo de Lima[1a]

[1] Laboratório de Virologia e Imunologia Veterinária, Faculdade de Veterinária, Departamento de Veterinária Preventiva, Universidade Federal de Pelotas (UFPEL), Pelotas, RS, Brazil.
[2] Department of population Medicine and Diagnostic Sciences, College of veterinary Medicine, Cornell University, Ithaca, NY, United States of America.

[a] Corresponding author: mdelima.ufpel@gmail.com

Abstract

Equid herpesviruses are widespread in the horse population and represent a major threat to the horse industry worldwide. This study describes the detection and genetic identification of equid herpesviruses in clinical samples from horses located at seven farms (A to F) from southern Brazil. Virus-specific PCRs were used to detect EHV-1, -2, -4 and -5. From the total of 179 buffy coat samples (BC) and 110 nasal swabs (NS) collected from 179 horses, EHV-1 was detected in 11 clinical samples (BC, n=8 and NS, n=3) whereas EHV-4 was detected in 13 samples (BC, n=4 and NS, n=9). In addition, EHV-2 was detected in 6 clinical samples (BC, n=1 and NS, n=5) whereas EHV-5 specific DNA was detected in 13 buffy coat samples. Additionally, mixed infections by two or three viruses were recorded in 6 and 2 animals, respectively. Nucleotide sequence identity among each other ranged from 97.8% to 100% when compared to reference strains deposited in GenBank. In summary, results from this investigation demonstrate the detection and genetic identification of four types of equid herpesvirus in horses from southern Brazil and may represent a contribution to diagnosis and better understanding of epidemiological situation of these viral agents in the region.

Keywords: EHV, genetic characterization, coinfection, equines.

Statements and Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose

Ethics Declaration

The experiment was approved by Comissão de Ética em Experimentação Animal (CEEA) of Universidade Federal de Pelotas (UFPel), under the protocol 13938-2019

Introduction

Herpesvirus infections were endemic among equids worldwide, causing important economic losses in affected herds [1-3]. All herpesviruses associated with equine infections are classified into the family *Herpesviridae*. Among all equid herpesviruses, are particularly relevant the Equine herpesvirus type 1 and type 4 (EHV-1; EHV-4), and Equine herpesvirus type 2 and type 5 (EHV-2; EHV-5), belonging to the genus *Varicellovirus* and *Percavirus*, respectively [4]. Equine herpesviruses are capable of establish latent infections in lymphocytes and trigeminal ganglion after primary exposure [5,6]. The latency state ensures viral propagation in herds through the intermittent reactivation of viral shedding [7].

EHV-1 infection is commonly associated with respiratory illness, however, is capable of inducing abortion or equine herpesvirus myeloencephalopathy (EHM) outbreaks [8-10]. Differently, EHV-4 infection is considered one of the most important causes of acute respiratory disease in horses [9,11]. Conversely, in addition to subclinical infections EHV-2 has been linked to pneumonia and keratoconjunctivitis [12,13]. Regarding EHV-5, the infection is worldwide associated with equine multinodular pulmonary fibrosis (EMPF) [14,15].

In Brazil, there is a clear predominance of serological studies demonstrating a wide dissemination of EHV-1 in the equine population, and possibly EHV-4, due to a high antigenic similarity between these serotypes. Seropositive indices varied between 4,5% and 45,5 % according to geographic area [16-20]. However, there are numerous reports at national level demonstrating the identification and genetic characterization of EHV-1 either in latent infections [21] or in association with clinical disease [22-25].

In 2019, a study evaluating 26 nasal samples from asymptomatic equines had identified for the first time EHV-2 and EHV-5 in Brazil [13]. Furthermore, the occurrence in the country of the only two cases associating EHV-5 and EMPF were described in the south region [25,26]. In face of the lack of information, especially, referring to characterization of different equine herpesviruses regionally and nationally, this study aimed the detection and genetic identification of equine herpesviruses through clinical samples of animals in the south region of Rio Grande do Sul (RS) state.

Materials and Methods

Study Location and Clinical Samples

Total blood samples and nasal swabs were collected from 179 equines (including both sexes) distributed in seven equine breeding farms localized in the south region of RS (situated in the municipalities of Pelotas, Bagé, Jaguarão and Capão do Leão). Collection of blood

samples was conducted using EDTA tubes. Nasal secretions were collected from both nostrils using sterile swabs through deep intranasal sampling, following immediate conditioning in minimum essential medium (MEM) enriched with antibiotics. Samples were maintained under refrigeration during transport to the laboratory, following direct freezing at -80°C until processing. Collections occurred in 2019, between April and June. Farms and animals were randomly selected, according to disponibility and owner's consent. Exceptionally, we obtained total blood, leukocytes and parafined pulmonary tissue from a case of equine multinodular pulmonary fibrosis (EMPF) reported in property F, a few months before sample collection. In this case, the samples remained in the Regional Laboratory Diagnostic (LRD, UFPEL, RS). All samples were processed at Laboratório de Virologia e Imunologia (LabVir - UFPel).

DNA Extraction and Polymerase Chain Reaction (PCR)

Total DNA from leukocyte layers and/or nasal swabs were extracted using the kit ReliaPrepTM Viral TNA Miniprep (Promega, Wisconsin, EUA), following manufacturer's protocol. On other hand, extraction of paraffined pulmonary tissue was carried out utilizing the kit ReliaPrepTM FFPE gDNA Miniprep System (Promega, Wisconsin, EUA). Polymerase Chain Reaction (PCR) for EHV detection was performed through partial amplification of glycoprotein B (gB) gene using different primers sets (Table 1). The reaction mix contained a total volume of 25µL, consisting of 100-200ng of DNA, 1x GoTaq® Color Master Mix (Fitchburg, Wisconsin, EUA) and 0,4µM of each primer. Amplification products were run on a 1.5% agarose gel, stained with Blue Green Loading Dye I (Cotia, São Paulo, BR) and visualized under UV light. Positive controls of EHV-1, EHV-2 and EHV-4 were kindly provided by PhD. Maria Edith Barrandeguy, from Instituto de Virología, Centro de Investigación en Ciencias Veterinarias y Agronómicas (CICVyA), INTA, Castelar, Buenos Aires, Argentina. Regarding EHV-5, a gBlock® Gene Fragment (Síntese Biotecnologia, Minas Gerais, Brazil) was constructed as a positive control.

Partial Sequencing and Sequence Analysis.

Four positive samples to each EHV were selected for genomic sequencing. Initially, PCR products were purified using the kit PureLink® Quick Gel Extraction and PCR Purification (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. Partial sequencing was performed at the ACTGene Laboratory (Alvorada, RS, Brazil) using BigDye™ Terminator Sequencing Kit (Applied BiosystemsWaltham, MA, USA) through the automatic sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). Electropherograms were analyzed using the program Staden, and aligned through the

software BioEdit, versão 7.0.5.3 [27]. Similarity was assessed by comparison with sequences retrieved from the NCBI GenBank database.

Results

Prevalence of EHV-1 and EHV-4

A total of 179 blood samples/leukocytes (LC) and 110 nasal swabs (NS) were tested for the presence of EHV-1 and EHV-4 DNA (Figure 1). Nasal swabs were only tested on farms 1 and 4, which had a recent history of respiratory and/or reproductive disease in the herd or presented respiratory clinical signs in a portion of the animals at the time of sampling. Among all samples analyzed, 11 were positive for EHV-1, being 8/179 (4,47%) in leukocytes and 3/110 (2,73%) in nasal swabs. Also, EHV-4 was detected in 13 samples: 4/179 (2,23%) leukocytes samples and 9/110 (8,18%) nasal swabs. Propriety A concentrates all EHV-1 positive samples detected from leukocytes, totalizing an internal percentage of 11,43% (8/70). The other three EHV-1 positive samples were derived from nasal swabs collected at property F (3/35 - 8,57%). Regarding EHV-4, all positive samples in LC (4/70 - 5,71%) were also originated from property A. Whereas, the nine positive samples from NS were derived from property F (8/35 - 22,86%) and property G (1/5 - 20%).

Prevalence of EHV-2 and EHV-5

The screening for EHV-2 and EHV5 was performed in 179 blood/leukocytes (LC) samples and nasal swabs (NS), covering all properties (A to G). Among all tested samples, the presence of EHV-2 was noticed in 6 animals, with one positive sample of LC (0,56%) and 5 in NS (2,79%). Meanwhile, EHV-5 was detected only in LC samples, totalizing 13 of 179 (7,26%). Notably, all positive samples to EHV-2 and EHV-5 came from property F, representing an internal prevalence of 17,14% (6/35) e 37,14% (13/35), respectively.

Equine Herpesvirus Co-infections

The presence of mixed infections involving two viruses was detected in one animal from property A (EHV-1/EHV-4) and in five animals from property F (EHV-2/EHV-4, n=2); (EHV-4/EHV-5, n=2) and (EHV-1/EHV-5, n=1). Moreover, was also identified the presence of co-infections by three different types of equine herpesviruses in 2 animals from property F (EHV-1/EHV-4/EHV-5, n=1) and (EHV-2/EHV-4/EHV-5, n=1).

Sequencing

Selected PCR products for each herpesvirus were purified, quantified and submitted to sequencing. After analysis, all consensus sequences demonstrated a high percentage of identity (97,8% -100%) with sequences from reference strains and isolates of EHV-1, EHV-2, EHV-4

and EHV-5, previously deposited in the GenBank. Notably, the sequencing formed from the viral DNA recovered from the paraffined pulmonary tissue (equine diagnosed with EMPF), also resulted in a high identity (100%) with EHV-5 sequences deposited in the GenBank.

Discussion

Equid herpesviruses (EHVs) are important viral diseases of horses, and they are associated with respiratory diseases, sporadic or abortion storms in pregnant mares, neonatal deaths in foals and less frequently, myeloencephatopathy [3,28]. Horses are constantly exposed to EHVs around the world and infections have been documented with genetic characterization of the viruses and its association with subclinical or clinical disease [9,12,29].

In Brazil, although several serological investigations have demonstrated the circulation of EHV-1/EHV-4 in different regions of the country, studies of identification and genetic characterization are, predominantly, focused on EHV-1 [21,24]. The detection of EHV-2/EHV-5 have been described in the upper respiratory tract of asymptomatic horses [13] and in bronchoalveolar lavage fluid from asymptomatic horses [30]. However, both studies were conducted using a limited number of animals and clinical samples.

In this study, we demonstrated the identification and genetic characterization of EHV-1, EHV-2, EHV-4 and EHV-5 from clinical samples (whole blood and nasal secretion) from 179 horses located at seven different farms from Rio Grande do Sul, Brazil. The presence of DNA from EHVs was detected in horses from three farms (farms A, F and G). No positive samples were identified in the other farms (B, C, D and E). In addition, nasal swabs collected from animals located at these specific farms were not tested for EHV-1 and EHV-4, as there was no reported history of respiratory disease in the previous years. Similar results were previously reported suggesting that EHV-1 and EHV-4 are not commonly identified in clinically healthy horses [9,13,29].

It was possible to observe a distinct scenario in those farms with positive diagnostic for the presence of EHV-1 and EHV-4. In the farm A, it was detected 12 positive samples (EHV-1, n=8 and EHV-4, n=4) from 70 analyzed samples (17,14%). All positive samples were derived from blood leukocytes collected from asymptomatic horses. Interestingly, the respective nasal secretions samples from the same horses were found negative in the PCR. According to reports from the attending veterinarian of the farm, routine vaccination against EHV-1 and EHV-4 was a frequent practice to all animals in the herd due to a history of sporadic abortion and respiratory disease in previous years.

The circulation of EHV-1 and EHV-4 in vaccinated mares and their foals have been described elsewhere [31]. It is also important to mention that no clinical signs resembling respiratory illness were observed during the collection of clinical samples in our study. Moreover, the majority of the horses on this farm (Farm A) had neutralizing antibodies against EHV-1 due to previous vaccination or natural infection (data not shown). These findings may be suggestive of latent infections caused by both EHV-1 and EHV-4 in a considerable number of animals from the herd. The ability these viruses to establish latency following infection of blood leukocytes, neurons of trigeminal ganglia and lymphoid tissues has been widely reported in the scientific literature [21,32,33].

Additionally, there are evidence suggesting that pulmonary epithelial cells in naturally infected horses could serve as other sites of latency for EHV-4 [34,35]. However, it is important to consider that prevalence of latent infections by EHV-1 and EHV-4 may vary according to geographic location, horse population, management practices and sensitivity of the diagnostic assays [36]. The detection of EHV-1 DNA in peripheral blood mononuclear cells (PBMCs) from two groups of foals (8 out of 64 positives in group 1 and 6 out of 131 positives in group 2) was demonstrated in a study conducted in Australia. In that same study, all nasal swabs were found to be negative [29].

Conversely, in the farm F, we have detected 11 animals with a PCR positive (EHV-1, n=3; EHV-4, n=8) out of 35 tested horses (31,43%). Interestingly, all positive samples were detected from nasal secretions whereas the corresponding whole blood samples from the same animals tested negative for viral DNA. Although in this study, we did not assess the correlation between viral DNA detection and the presence or absence of respiratory clinical signs at the time of sampling, a discrete nasal discharge (mucous, serous, or muco-purulent) was observed in some horses. Even though viral isolation was not attempted from those samples, the findings of our study suggest active infection and viral shedding at the time of sample collection. Given the available data, a definitive association between respiratory signs and identification of EHV-1/EHV-4 as the primary viral agent causing disease cannot be confirmed. The presence of other viral and/or bacterial pathogens should be considered in this case.

Upon analysis of the findings related to EHV-2 and EHV-5, it was observed that these viruses were detected exclusively on farm F (EHV-2, n=6; EHV-5, n=13). All positive samples for EHV-5 (n=13) were identified from whole blood whereas eight samples were found positive for EHV-2 from nasal secretions. An additional sample of whole blood was positive for EHV-2. Wang et al., [29] observed a similar trend as observed in our study with a higher frequency of positive animals for EHV-5 when compared to EHV-2 and a detection rate of positive

samples greater in whole blood than in nasal secretions. In the same investigation, the authors interpreted the findings as suggestive of latent infection induced by these gamaherpesviruses in blood leukocytes, emphasizing the need of diagnostic assays capable of distinguishing between lytic and latent herpesvirus infections.

Latency induced by EHV-2 can be established in B-cells, macrophages and Langerhans cells [37]. In addition, there is evidence that the trigeminal ganglia serve as an important biological site for EHV-2 latency, considering the innervation of the nasal epithelium by maxillary branch of the trigeminal nerve [38]. A proposed model for the pathogenesis of EHV-5 in horses suggests that infected lymphocytes may undergo apoptosis or remain as viral reservoirs in infected horses [39]. Additionally, it is important to highlight that the true prevalence of latent EHV-5 observed in clinical samples tested by PCR might be underestimated in a given horse population due to a reduced levels of viral DNA [29].

The findings of this study are suggestive of active replication and shedding of EHV-2 in 6 out of 35 animals tested (17.1%), considering the detection of the viral genome almost exclusively in samples of nasal secretion. These results are even more relevant when considering the proportion of positive animals for EHV-1(n=3/35) and EHV-4 (n=8/35) from nasal swabs in the same herd. Additionally, it was possible to detect co-infection and a possible simultaneous EHV-2 and EHV-4 shedding in three animals. The ability of EHV-2 and EHV-5 to infect lymphocytes might suppress host immunity leading to an increase of susceptibility to opportunistic infections [40]. It was also demonstrated that equid herpesvirus type-2 was able to trans-activate the promoter of IE (immediate early) gene of EHV-1 in a mixed infection [41].

It is also important to emphasize the previous occurrence of EMPF (equine multinodular pulmonary fibrosis) in a horse from farm F, previously the sample collection carried out in our study. The animal died after exhibiting chronic respiratory disease unresponsive to multiple therapeutic protocols and conventional treatments. Macroscopic findings after necropsy at Regional Laboratory Diagnostic (LRD, UFPEL, RS) along with histopathological examination in pulmonary tissues were indicative of EMPF. The detection of EHV-5 DNA was carried out from paraffin-embedded lung tissue followed by genomic sequencing (data not shown). Unfortunately, it was not possible to collect clinical samples from this animal ante morten to investigate a possible correlation with the identification of 13 EHV-5-positive animals that had prior contact with this horse.

Equid herpesvirus type 5 is a member of Gammahespesvirinae subfamily and highly adapted to its natural host, resulting in fundamentally asymptomatic infection in horses. Most

of the infected animals shed the virus in nasal secretions or harbor it in circulating lymphocytes; however, a small proportion may develop clinical signs and lesions [39].

Finally, it is important to highlight the occurrence of concurrent infections by equid herpesviruses detected in this study. Co-infection was recorded in six animals (EHV-1/4, n=1; EHV1/5, n=1; EHV-4/5, n=2 e EHV-2/4, n=2). In addition, mixed infections with three different equid herpesviruses were detected in two horses (EHV-1/4/5, n=1 e EHV-2/4/5, n=1). The concurrent infections of horses by equid herpesviruses detected in our study is consistent with other previously reported [1,9,13,29]. Nonetheless, the biological relevance or the synergistic pathogenic effect on the respiratory tract for the infected animals remains to be determined.

In summary, this study provides new insights into the epidemiology of equid herpesviruses infection in horses in Brazil. Further investigation is needed, particularly in other geographic regions of the country, to better understand the distribution of these viruses in the horse population.

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Table and figure Legends:

Table 1. Primer sequences used for the amplification of the partial sequence of the glycoprotein B (gB) gene of equine herpesviruses.

Figure 1. Detection results of equid herpesvirus types 1, 2, 4, and 5 DNA in clinical samples from horses distributed across seven properties in the southern region of Rio Grande do Sul state.

Table 1.

PCR	Specificity	Primers sequences (5'-3')	Final Amplicon (bp)	Reference
Semi-nested multiplex	EHV-1/4 gB	1º *round* FC2: CTTGTGAGATCTAACCGCAC RC: GGGTATAGAGCTTTCATGGG 2º *round* FC3: ATACGATCACATCCAATCCC R1: GCGTTATAGCTATCACGTCC R4: CCTGCATAATGACAGCAGTG	EHV-1 188 bp EHV-4 677 bp	[29]
Nested multiplex	EHV-2/5 gB	1º *round* EHV2L1: GATGGTCTCACCTCTAGCAT EHV2R1: CTGGTGTAACACAGGTCTTC EHV5L1: CCAACACAGAAGACAAGGAG EHV5R1: CACGGTGATACAGTCAGAGA 2º *round* EHV2L2: GGTCTCACCTCTAGCATAAC EHV2R2: GCCACACTCTCTTCCTTAGT EHV5L1: CCAACACAGAAGACAAGGAG EHV5R2: AGTTGACCGTCGTTCTAGTG	EHV-2 817 bp EHV-5 410 bp	[29]
Conventional	EHV-5 gB	F: TGATATGACGGCCAGATCACAC R: CCAACCCCACACCATAGTCT	155 bp	[26]

***** Base pairs

Figure 1.

Property	Leukocytes				Nasal Swabs				Total
	EHV-1	EHV-2	EHV-4	EHV-5	EHV-1	EHV-2	EHV-4	EHV-5	
A	8/70 (11,43%)	0/70	4/70 (5,71%)	0/70	0/70	0/70	0/70	0/70	12/70 (17,14%)
B	0/14	0/14	0/14	0/14	-*	0/14	-	0/14	0/14
C	0/25	0/25	0/25	0/25	-	0/25	-	0/25	0/25
D	0/8	0/8	0/8	0/8	-	0/8	-	0/8	0/8
E	0/22	0/22	0/22	0/22	-	0/22	-	0/22	0/22
F	0/35	1/35 (2,86%)	0/35	13/35 (37,14%)	3/35 (8,57%)	5/35 (14,29%)	8/35 (22,86%)	0/35	21/35 (60,00%)
G	0/5	0/5	0/5	0/5	0/5	0/5	1/5 (20,00%)	0/5	1/5 (20,00%)
Total	8/179 (4,47%)	1/179 (0,56%)	4/179 (2,23%)	13/179 (7,26%)	3/110 (2,73%)	5/179 (2,79%)	9/110 (8,18%)	0/179	

* Samples not tested

Considerações Finais

A detecção e identificação genética de herpesvírus equídeo em equinos da região sul do RS representam uma contribuição para o diagnóstico destas infecções e o conhecimento da situação epidemiológica e distribuição destes agentes em nível regional e nacional.

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Anexos

Anexo A - Documento da Comissão de Ética e Experimentação Animal

UNIVERSIDADE FEDERAL DE PELOTAS

PARECER Nº **57/2019/CEEA/REITORIA**
PROCESSO Nº 23110.013938/2019-66

Certificado

Certificamos que a proposta intitulada **"Herpesvírus equino: Isolamento e caracterização molecular de amostras de campo"**, registrada com o nº 23110.013938/2019-66, sob a responsabilidade de Marcelo de Lima - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 16/07/2019.

Finalidade	(x) Pesquisa ( ) Ensino
Vigência da autorização	19/07/2019 a 30/12/2020
Espécie/linhagem/raça	Equinos
Nº de animais	384
Idade	Variável
Sexo	Machos e Fêmeas
Origem	Propriedades do Sul do Rio Grande do Sul

Código para cadastro nº **CEEA 13938-2019**

M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA

Documento assinado eletronicamente por **ANELIZE DE OLIVEIRA CAMPELLO FELIX, Médico Veterinário**, em 19/07/2019, às 13:25, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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