Molecular characterization of Brazilian equid herpesvirus type 1 strains based on neuropathogenicity markers

Enio Mori¹,³, Maria do Carmo C.S.H. Lara², Elenice M.S. Cunha², Eliana M.C. Villalobos², Claudia M.C. Mori³, Rodrigo M. Soares⁴, Paulo E. Brandão⁴, Wilson R. Fernandes⁵, Leonardo J. Richtzenhain⁴

¹Instituto Pasteur, São Paulo, SP, Brazil. ²Instituto Biológico, São Paulo, SP, Brazil. ³Departamento de Patologia, Faculdade de Medicina Veterinária e Ciência Animal, Universidade de São Paulo, São Paulo, SP, Brazil. ⁴Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil. ⁵Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Ciência Animal, Universidade de São Paulo, São Paulo, SP, Brazil.

Submitted: February 1, 2014; Approved: October 30, 2014.

Abstract

Partial nucleotide sequences of ORF72 (glycoprotein D, gD), ORF64 (infected cell protein 4, ICP4) and ORF30 (DNA polymerase) genes were compared with corresponding sequences of EHV-1 reference strains to characterize the molecular variability of Brazilian strains. Virus isolation assays were applied to 74 samples including visceral tissue, total blood, cerebrospinal fluid (CSF) and nasal swabs of specimens from a total of 64 animals. Only one CSF sample (Iso07/05 strain) was positive by virus isolation in cell culture. EHV-1 Iso07/05 neurologic strain and two abortion visceral tissues samples (Iso11/06 and Iso33/06) were PCR-positive for ORF33 (glycoprotein B, gB) gene of EHV-1. A sequence analysis of the ORF72, ORF64 and ORF30 genes from three EHV-1 archival strains (A3/97, A4/72, A9/92) and three clinical samples (Iso07/05, Iso11/06 and Iso33/06) suggested that among Brazilian EHV-1 strains, the amplified region of the gD gene sequence is highly conserved. Additionally, the analysis of ICP4 gene showed high nucleotide and amino acid identities when compared with genotype P strains, suggesting that the EHV-1 Brazilian strains belonged to the same group. All the EHV-1 Brazilian strains were classified as non-neuropathogenic variants (N752) based on the ORF30 analysis. These findings indicate a high conservation of the gD-, ICP4- and ORF30-encoding sequences. Different pathotypes of the EHV-1 strain might share identical genes with no specific markers, and tissue tropism is not completely dependent on the gD envelope, immediate-early ICP4 and DNA polymerase proteins.

Key words: equid herpesvirus type 1, equid Brazilian herpesvirus, ICP4 gene (ORF64), glycoprotein D gene (ORF72), DNA polymerase gene (ORF30).

Equid herpesvirus 1 (EHV-1) is the major cause of different clinical syndromes in horses, such as respiratory disease, abortion, neonatal deaths, and neurological disorders. It has been recognized as a cause of substantial financial losses to the horse industry throughout the world (Allen and Bryans, 1986; Gryspeerdt et al., 2010).

Until recently, EHV-1 disease outbreaks usually manifested as abortions in late gestation; however, the frequency and severity of EHV-1 neurological diseases throughout North America and Europe have increased in recent years, and EHV-1 is now considered a potentially emerging disease of the horse by the US Department of Agriculture (Vandekerckhove et al., 2010). Research studies have been
performed suggesting that a molecular variation in the EHV-1 genome is playing a role in these changes in the disease behavior, which could indicate evolution of the viral agent (Pagamjav et al., 2005; Nugent et al., 2006). In Brazil, the first isolation of EHV-1 was recorded in 1966 from an equine-abortion fetus (Nilsson and Correa, 1966). After that, several isolates have been recovered, mainly from aborted fetuses; however, only recently a case report of EHV-1-related neurological signs in an adult mare was described (Lara et al., 2008).

Restriction fragment length polymorphism (RFLP) analysis of whole DNA viral has been used to detect molecular variation among EHV-1 isolates. There are at least two electropherotype patterns of EHV-1 detected by restriction enzyme digestion designated EHV-1 P and EHV-1 B (Allen et al., 1983). Based on previous studies, in the 3'-end and downstream of the open reading frame (ORF) 64 gene (infected cell protein 4 - ICP4 gene), natural recombination between EHV-1 and EHV-4 by the exchange of homologous fragments could be associated with the major molecular differences between isolates EHV-1 P and EHV-1 B (Pagamjav et al., 2005). The EHV-1 B genotype should be a result of this recombination between the progenitors of the EHV-1 P genotype and EHV-4. The ICP4 is an important transcriptional activator, essential for progression beyond the immediate-early phase of infection, associated with lytic infection in HSV-1 (Pinnoji et al., 2007). The ICP4 product is involved in the regulation of gene expression and interaction with host factors, and this intertypic recombination could cause some alteration of EHV-1 virulence and neuropathogenicity in hamsters. The abortigenic genotype (EHV-1 B) may have originated from the neuropathogenic (EHV-1 P) after exchange of a fragment in the ICP4 gene between EHV-1 and EHV-4 (Pagamjav et al., 2005).

Glycoprotein D (gD) is responsible for virus entry and spread into a host cell, being major determinant of host cell tropism and may also be a factor involved in the neuropathogenicity of EHV-1 by modulating neurovirulence and neuroinvasion (Mettenleiter, 2003; Whalley et al., 2007; Azab and Osterrieder, 2012).

EHV-1 molecular epidemiology research has identified a single nucleotide polymorphism (SNP) in the catalytic subunit (Pol) of the viral DNA polymerase (ORF30) gene, causing a subsititution of asparagine (N) by aspartic acid (D) at amino acid position 752. This substitution showed a highly statistically significant (p < 0.0001) correlation with paralytic compared with non-paralytic disease outbreaks (Nugent et al., 2006).

To the authors’ knowledge, there have been few published articles on molecular variability of the EHV-1 Brazilian isolates. Despite the fact that ORF37 (similar to HSV-1 UL24) is considered a neuropathogenicity determinant of EHV-1 in the mouse encephalitis model (Kasem et al., 2010), Carvalho et al. (2012) showed no molecular divergences on the partial sequencing of this region derived from two Brazilian EHV-1 isolates (A4/72 and A3/97) with high and low virulence in the mice model, respectively (Mori et al., 2012).

The purpose of this study was to investigate some putative pathogenicity markers (ICP4, gD and viral DNA polymerase genes) in EHV-1 Brazilian strains to form a basis for comparison of partial nucleotide sequences with corresponding sequences of EHV-1 reference strains from DNA databases deposited in the GenBank (NCBI) to gather insights into the validity of such markers.

Three abortogenic (A4/72, A9/92 and A3/97) EHV-1 Brazilian archival strains, provided by the Biological Institute (Department of Agriculture, Sao Paulo State, Brazil), were recovered originally from organs (lungs, spleen and liver) of aborted fetuses. Viruses were propagated in Vero (CRl-1587, ATCC) and E-Derm (CCL-57, ATCC) cell lines and maintained in Eagle’s minimal essential medium (EMEM) supplemented with 5% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO2.

Seventy-four clinical specimens from horses (fragments of visceral tissues, total blood, CSF and nasal swab) were submitted for routine diagnostic tests to the Rabies and Viral Encephalitis subdivision of the Biological Institute (Department of Agriculture, Sao Paulo, Brazil) between 2005 and 2007 (Table 1). Sixty-four horses with an unknown vaccination history, and suggestive findings of EHV-1 infection [abortion (n = 25), neurological disease (n = 29), respiratory disease (n = 9) and perinatal disease (n = 1)] were sampled by private veterinarians from eight different Brazilian states: the Southeastern region [Sao Paulo state (n = 45), Minas Gerais state (n = 13), and Rio de Janeiro state (n = 1)]; the Midwestern region [Goias state (n = 1)]; South region [Parana state (n = 1), and Rio Grande do Sul (n = 1)]; and the Northeastern region [Rio Grande do Norte (n = 1), and Ceara (n = 1)].

Virus isolation (VI) was attempted with clinical samples (20% w/v brain or CSF) collected at necropsy and inoculated onto a monolayer of Vero (CRl-1587, ATCC) and E-derm (CCL-57, ATCC) cells. When these cells exhibited a cytopathic effect (CPE), the identification of isolates was performed according to previously published methods (Mori et al., 2012).

DNA extraction of the EHV-1 Brazilian strains and specimens were conducted following a previously described method (Chomczynski, 1993). PCR screening tests (heminested) were performed using primers that hybridize to highly conserved gB gene regions that differentiate between EHV-1 [P1 forward 5’-CTGTGGAGATCTAACC GCAC-3’/P2 outer reverse 5’-GGGTATAGAGCCTTTC ATGGG-3’ and P1/P3 inner reverse 5’-GGGTATAGCC TATCACGGTC-3’] (Mori et al., 2009) and EHV-4 [P4 forward 5’-CTGTGCTTATGAGCAGGGA-3’/P5 outer reverse 5’-CGTTCTTCTGGAACAGGGTA-3’ and P4/P6 inner reverse 5’-CGCTAGTGTACATCGTCG-3’]
Next, three different sets of primers representing different regions of EHV-1 were used in positive amplification samples: ICP4 gene [P7 forward 5’-ACGCCCCCTTCGCTTCTC-3’/P8 reverse 5’-CGCTCCACCTCGGTCTCTG-3’] (Borchers et al., 1998), gD gene [P9 forward 5’-AATGTCTACCCTGGTC-3’/P10 reverse 5’-TTACGGGAGCGGTTA-3’] (Galosi et al., 2001) and the DNA polymerase enzyme gene (ORF30) [P11 forward 5’-CCACAAACTTTGCACG-3’/P12 reverse 5’-GGGCTACTTCTGAAAAAG-3’] (Nugent et al., 2006). Amplification was performed in a reaction mixture of total volume 50 mL containing 0.5 mg of DNA sample, 0.5 mM of each primer, 0.2 mM of each dNTP mixture, 2.5 units of Platinum Taq DNA polymerase (Invitrogen Brasil Ltda, Sao Paulo, Brazil), 1 X PCR buffer (20 mM of Tris-HCl pH 8.4, 50 mM of KCl), 1.5 mM of MgCl and ultra-pure water QS. Amplification was carried out in a thermal cycler (Eppendorf Mastercycler Gradient PTC-200, Eppendorf AG, Hamburg, Germany) under the conditions reported in Table 2.

A commercial kit (GFX PCR DNA and Gel Band Purification Kit, GE Healthcare, Uppsala, Sweden) was used for the purification of amplified DNA fragments. Then, bidirectional cycle sequencing was performed using the dideoxynucleotide chain-termination method (Big Dye Terminator v.3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, California, USA) according to the manufacturer’s instructions. Sequence reaction products were analyzed on an automatic DNA sequencer (ABI Prism 377 Genetic Analyzer, Applied Biosystems Foster City, California, USA.) The sequence quality analysis was examined by the Phred program (http://asparagin.cenargen.embrapa.br/phph/). The lower threshold of acceptability for the generation of consensus sequences was set at a Phred score of 20 for each base. Next, the final sequences were assembled using the contig assembly program (CAP) of the software BioEdit v.7.0.9.0 (Hall, 1999). Sequences of each EHV-1 strain and homologous sequences retrieved from GenBank were aligned by the ClustalW method using Bioedit v.7.0.9.0 (Hall, 1999). Nucleotide and amino acid identities of the translated sequences were calculated using Bioedit v.7.0.9.0 (Hall, 1999).

Phylogenetic tree using the sequences from the ORF64 gene region were carried out using the neighbor-joining (NJ) algorithm and the maximum composite likelihood (MCL) evolutionary model with software Mega version 6.0.6 (Tamura et al., 2013). The reliability of the NJ phylogenetic trees was evaluated by analyzing 1,000 bootstrap repetitions, and the virus HHV-3 strain Dumas (accession number NC001348) was used as an outgroup. The genomic partial sequences of the EHV-1 strains [Ab4p (accession number AF656713), KyD (accession number AB279610), V592 (accession number A464052), RacL11 (accession number AB279607), KyA (accession number M629230), Ab1 (accession number M60946), HVS25A (accession number M59773), 98c12 (accession number AB183143), 97c5 (accession number AB183141), 97c7 (accession number AB182650) and 97c9 (accession number AB183142)], the EHV-9 strain P19 (accession number NC011644) and the EHV-4 strain NS80567 (accession number AF030027) were obtained from DNA databases that had been deposited in the GenBank (NCBI) and used for comparison purposes.

Virus isolation assays were applied to 74 samples including visceral tissue, total blood, cerebrospinal fluid (CSF) and nasal swabs of farms specimens from a total of 64 animals (Table 1). Only one cerebrospinal fluid (CSF) sample (namely strain Iso07/05) of a mare with a neurological disorder from a riding school in Ribeirao Pires County (Sao Paulo State, Southeastern Brazil) caused herpesvirus CPE after the first passage in ED cells (Lara et al., 1998). EHV-1 Iso07/05 neurologic strain and two abortion visceral strain (Iso11/06 and Iso33/06) were positive by PCR, whereas A9/92 strain was positive only for gD primers. Iso11/06 and Iso33/06 samples were originated from Belo Horizonte (Minas Gerais State, Southeastern Brazil) and Pirassununga County (Sao Paulo State, Southeastern Brazil), respectively.

The use of gD, ICP4 and ORF30 as primers showed that the Brazilian archival EHV-1 strains (A4/72 and A3/97) were positive by PCR, whereas A9/92 strain was positive only for ICP4 primers. ICP4, gD and ORF30 re-

| Group                  | Number of animals | Specimens |
|------------------------|-------------------|-----------|
|                        | VT    | BL    | NT    | CSF   | NS    | PI  |
| Abortion               | 25    | 24 (fetus) | -     | 1 (fetus) | -     | -   | 1   |
| Neurological disease   | 29    | -     | 5     | 9     | 14    | 10  | -   |
| Respiratory disease    | 9     | -     | -     | -     | -     | 9   | -   |
| Perinatal disease      | 1     | 1 (foal) | -     | -     | -     | -   | -   |
| Total                  | 64    | 25    | 5     | 10    | 14    | 19  | 1   |

(-): Not collected; VT: visceral tissues (lung, liver and spleen); BL: total blood; NT: neuronal tissues; CSF: cerebrospinal fluid; NS: nasal swab; PI: placenta.
regions of the EHV-1 Brazilian sequences were deposited in GenBank (accession numbers EU094656, EU094657, EU088186, EU088187, EU410444, EU410445 and EU094655). EHV-1 strains (Iso07/05, Iso11/06 and Iso33/06) were PCR-positive using gD, ICP4 and ORF30 gene primers. The positive PCR amplicons gD, ICP4 and ORF30 genes were partially sequenced (GenBank accession numbers EU052212, EU169121, EU410443, JN390439, EU825794, EU857541, JN390440, EU825795, and FJ755482). The gD gene amplicon lengths were 935 nt and encoded 310 amino acids. At the nucleotide level of the gD gene, the Brazilian EHV-1 isolates and clinical specimens showed 100% identity among them. Comparing Brazilian EHV-1 isolates and clinical specimens with those deposited in GenBank, it was observed that nucleotide and predicted amino acid sequences of the gD gene exhibited high identities (99.6-100% and 99-100%, respectively), differing in few nucleotides and resulting in low rates of amino acid change (Supplementary Figure S3). The results suggested that among Brazilian EHV-1 strains, the gD gene is highly conserved, thus supporting the use of vaccines that contain DNA or subunits related to this region.

Although there are dramatic differences in the virulence and tissue tropism between A4/72 and A3/97 after intranasal inoculation with the same viruses (Mori et al., 2012), the highly conserved region of gD do not explain the pathogenetic differences of the EHV-1 Brazilian isolates. However, a strain with different pathogenicity in mice might have identical gDs, a fact not reported previously.

In contrast to the attenuated EHV-1 strains (KyA, KyD and Racl11) used as vaccine, drastic mutations in gD sequence, such as deletion, inversion, and insertion, were not found in the strains here analyzed (Supplementary Figure S1). A possible explanation for DNA mutations in KyA, KyD and Racl11 strains may be due to serial passage in hamsters and culture cells (Molinkova et al., 2004; Ghanem et al., 2007).

The nucleotide sequence of the ICP4 region was 309nt long and encoded 102 amino acids. At both the nucleotide and the amino acid levels of the ICP4, the Brazilian EHV-1 isolates and clinical samples showed 100% identity among them. Comparison of the ICP4 nucleotide and amino acid sequences obtained from Brazilian EHV-1 isolates and clinical specimens with those from the EHV-1 genotype P strains (Ab4p and V592) exhibited 100% identity, suggesting that these viruses belonged to the same group (Pagamjav et al., 2005). The genealogic tree for the ORF64 gene constructed with the sequences analyzed in this study clustered in only one group named genotype P (Figure 1).

On the other hand, the nucleotide sequences of the ICP4 in Brazilian EHV-1 strains exhibited 69.6% nucleotide identity with EHV-1 genotype B strains (97c5, 97c7, 97c9 and 98c12) and EHV-4 (strain NS80567). In addition, the Brazilian strains exhibited 49% amino acid identity with EHV-1 genotype B strains and EHV-4.

Pagamjav et al. (2005) suggested that the intertypic recombination in the ICP4 gene could cause an alteration in EHV-1 virulence and neuropathogenicity in the hamster model. The EHV-1 P strains were correlated with neuropathogenic behavior in hamster model. However, as occurred with the gD gene region, the involvement of this gene in neuropathogenicity in mice could not be confirmed based on the results of the ICP4 nucleotide sequencing from the EHV-1 Brazilian isolates (Mori et al., 2012).

The ORF30 nucleotide sequences were 426nt long and encoded 141 amino acids. The DNA polymerase gene region of the EHV-1 Brazilian isolates and clinical specimens showed 100% nucleotide identity with the non-neuropathogenic variant (N752) EHV-1 strain V592. Nucleotide and amino acid identity among the Brazilian strains and the neuropathogenic variant (D752) was 99.7% and 99.2%, respectively.

Although strain Iso07/05 was recovered from CSF and classified as a neurotropic isolate, all the EHV-1 Brazilian strains were classified as non-neuropathogenic (N752) in the catalytic subunit (Pol) of the viral DNA polymerase gene (Nugent et al., 2006). Nugent et al. (2006) found that approximately 15% of isolates from cases of EHV-1 neurological disease did not contain the mutation in this gene.

The A9/92 strain could have differences in its DNA composition in comparison with other EHV-1 strains, which could explain the different ways of spreading and the neurological signs A9/92 strain causes in mice model (Mori et al., 2012).

| Step   | EHV-4 | EHV-1 |
|--------|-------|-------|
|        | gB    | gB    | gD    | ICP4 | ORF30 |
| 1      | Initial denaturation | 95 °C (5-min) | 94 °C (5-min) | 94 °C (5-min) | 96 °C (3-min) | 94 °C (4-min) |
| 2      | Denaturation | 95 °C (30-s) | 94 °C (1-min) | 94 °C (1-min) | 94 °C (30-s) | 94 °C (30-s) |
| 3      | Annealing | 60 °C (30-s) | 60 °C (1-min) | 50 °C (1-min) | 64 °C (30-s) | 56 °C (1-min) |
| 4      | Extension | 72 °C (1-min) | 72 °C (1-min) | 72 °C (90-s) | 72 °C (1-min) | 72 °C (2-min) |
| Number of cycles (step 2 to 4) | 35 | 35 | 25 | 35 | 35 |
| 5      | Final extension | 72 °C (5-min) | 72 °C (7-min) | 72 °C (6-min) | 72 °C (6-min) | 72 °C (10-min) |
This is one of the first molecular epidemiological investigations into EHV-1 Brazilian isolates, and it does not reveal any molecular variation in the *ICP4*, *gD* and viral DNA polymerase gene regions among these strains. These results suggest that other factors, such as immune response, could be involved in the neuropathogenicity of EHV-1 in the mouse models (Mori et al., 2012). In conclusion, different pathotypes of EHV-1 might share identical genes with no specific markers, and tissue tropism is not completely dependent on the *gD* envelope, immediate-early *ICP4* and DNA polymerase proteins. Further studies of other potential neurovirulence markers are required to clarify the relationship between molecular variation and enhanced virulence in the mouse model, which may help elucidate the neuropathogenicity of particular strains of EHV-1.

Acknowledgments

This work was supported by the São Paulo Research Foundation (FAPESP), grant numbers 2007/58861-0 and 2005/56819-1, and the National Council for Scientific and Technological Development (CNPq), grant number 473735/2008-3.

References

Allen G, Bryans J (1986) Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infections. Prog Vet Microbiol Immunol 2:78-144.

Allen GP, Yeargan MR, Turtinen LW et al. (1983) Molecular epizootiologic studies of equine herpesvirus-1 infections by restriction endonuclease fingerprinting of viral DNA. Am J Vet Res 44:263-271.

Azab W, Osterrieder N (2012). Glycoproteins D of equine herpesvirus type 1 (EHV-1) and EHV-4 determine cellular tropism independently of integrins. J Virol 86:2031-2044.

Borchers K, Wolfinger U, Schellenbach A et al. (1998) Equine herpesvirus type 1 and trigeminal ganglia of naturally infected horses: detection of DNA and latency associated transcripts. In: Wernery U, Wade J, Mumford J et al. (eds) Proceedings of the Eighth International Conference on Equine Infectious Diseases. R & W Publications, Dubai, pp 147-152.

Carvalho RF, Spilki FR, Cunha EM et al. (2012) Molecular data of UL24 homolog gene (ORF37) from Brazilian isolates of equine herpesvirus type 1. Res Vet Sci 93:494-497.

Chomczynski P (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 15:532-534, 536-537.

Galosi CM, Roza MVV, Oliva GA et al. (2001) A polymerase chain reaction for detection of equine herpesvirus-1 in routine diagnostic submissions of tissues from aborted foetuses. J Vet Med B 48:341-346.

Ghanem Y, Ibrahim El-S, Yamada S et al. (2007) Molecular characterization of the equine herpesvirus 1 strains RacL11 and Kentucky D. J Vet Med Sci 69:573-576.

Gryspeerdt AC, Vandekerckhove AP, Garré B et al. (2010) Differences in replication kinetics and cell tropism between neurovirulent and non-neurovirulent EHV1 strains during...
the acute phase of infection in horses. Vet Microbiol 142:242-253.

Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95-98.

Kasem S, Yu MH, Yamada S et al. (2010) The ORF37 (UL24) is a neuropathogenicity determinant of equine herpesvirus 1 (EHV-1) in the mouse encephalitis model. Virology 400:259-270.

Lara MCCSH, Cunha EMS, Villalobos EMC et al. (2008) First isolation of equine herpesvirus type 1 from a horse with neurological disease in Brazil. Arq Inst Biol 75:221-224.

Mettenleiter TC (2003) Pathogenesis of neurotropic herpesviruses: role of viral glycoproteins in neuroinvasion and transneuronal spread. Virus Res 92:197-206.

Molínkova D, Celer VJ, Jahn P (2004) Isolation and partial characterization of equine herpesvirus type 1 in Czechia. Folia Microbiol 49:605-611.

Mori E, Mori CMC, Massironi SMG et al. (2009) Detection of equid herpesvirus 1 DNA by Polymerase Chain Reaction after experimental inoculation of horses with a Brazilian A4/72 strain. Braz J Vet Res Anim Sci 46:253-261.

Mori CM, Mori E, Favaro LL et al. (2012) Equid Herpesvirus Type-1 Exhibits Neutropotropism and Neurovirulence in a Mouse Model. J Comp Pathol 146:202-210.

Nilsson M, Correa W (1966) Isolamento do virus do aborto equino no Estado de Sao Paulo. Arq Inst Biol 33:23-25.

Nugent J, Birch-Machin I, Smith K et al. (2006) Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic vs. nonneuropathogenic disease outbreaks. J Virol 80:4047-4060.

Pinnoji RC, Bedadala GR, George B et al. (2007) Repressor element-1 silencing transcription factor/neuronal restrictive silencer factor (REST/NRSF) can regulate HSV-1 immediate-early transcription via histone modification. Virol J 4:56.

Tamura K, Stecher G, Peterson D et al. (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725-2729.

Vandekerckhove AP, Glorieux S, Gryspeerdt AC et al. (2010) Replication kinetics of neurovirulent vs. non-neurovirulent equine herpesvirus type 1 strains in equine nasal mucosal explants. J Gen Virol 91:2019-2028.

Varrasso A, Dynon K, Ficorilli N et al. (2001) Identification of equine herpesvirus 1 and 4 by polymerase chain reaction. Aust Vet J 79:563-569.

Whalley J, Ruitenberk K, Sullivan K et al. (2007). Host cell tropism of equine herpesviruses: glycoprotein D of EHV-1 enables EHV-4 to infect a non-permissive cell line. Arch Virol 152:717-725.

Supplementary Material

Figure S1 - Amino acid sequence alignment among EHV-1 Brazilian isolates (A4/72, A3/97, ISO07/05, ISO11/06 and ISO33/06) and Genbank reference EHV-1 strains (Ab4, KyD, V592, RacL11, HVS25A, KyA and Ab1) gD sequences.

All the content of the journal, except where otherwise noted, is licensed under a Creative Commons License CC BY-NC.

Associate Editor: João Pessoa Araújo Junior