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**Communication**

[*Comunicação*]

# Molecular characterization of neuropathogenic Equine Herpesvirus 1 Brazilian isolates

[*Caracterização molecular de isolados brasileiros de herpesvírus equino 1*]

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Equine herpesvirus 1 (EHV-1) infections cause significant economic losses for horse breeders worldwide due to abortion in pregnant mares, perinatal foal mortality, respiratory illness and neurological diseases in all breeds of horses. Over the past decade, while the prevalence of EHV-1-induced abortions apparently decreased, reports of EHV-1-induced neurological diseases (equine herpesvirus myeloencephalopathy) have been increasing, particularly in Europe and in the United States of America (Pronost *et al*., 2010). There are several commercially available vaccines that are employed for controlling the respiratory disease and abortion caused by EHV-1. However, these vaccines are not reliably protective against EHV-1-induced neurological disease (Goodman *et al*., 2012).

Molecular characterization of abortion or neurological disorders associated with strains of EHV-1 resulted in identification of differences between them. Although 16 genotypes of EHV-1 have been identified, EHV-1B and EHV-1P are associated with more than 90% of abortion in mares worldwide, whereas only the genotype EHV-1P has been associated with EHV-1 neuropathogenicity in horses. This genotype emerged from the recombination of the ICP4 gene (*ORF64*) between EHV-1 and EHV-4 (Pagamjav *et al*., 2005). Furthermore, studies on EHV-1 pathogenesis relating to induction of abortion or neurological disorders have demonstrated differences in the pathogenic potential that are correlated with the ability to replicate and establish infection in leukocytes and endothelial cells (Nugent *et al*., 2006). Infections with neuropathogenic strains are associated with longer and higher level viremia, which interferes with the blood flow to the central nervous systems and the development of neurological diseases (Fritsche and Borchers, 2011). However, the mechanism by which this leukocyte-associated viremia leads to myeloencephalopathy is not yet understood. Further studies indentified a specific mutation ($A_{2254}$ to $G_{2254}$) that results in a replacement of an asparagine (N) by an aspartic acid (D) at amino acid position 752 ($N_{752}$ to $D_{752}$) of the EHV-1 DNA polymerase gene (*ORF 30*). EHV-1 strains carrying this point mutation are considered to have neuropathogenic potential, whereas strains carrying an adenine at position 2254 are considered non neuropathogenic (Nugent *et al*., 2006). However, the mechanism by which this leukocyte-associated viremia leads to myeloencephalopathy is not yet understood.

Molecular characterization of EHV-1 Brazilian isolates is an important step towards the development of vaccine formulations that include viruses that represent the genotypes circulating in the country. In spite of EHV-1 being considered a conserved virus, vaccine formulations based on strains from other countries may result in suboptimal protection. Furthermore, combination of multiple viral strains in a vaccine formulation may result in a broader protection.

In this study, eight EHV-1 strains isolated from the central nervous system (CNS) of horses that died with neurologic clinical signs in the State of Minas Gerais (Brazil) were molecularly

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characterized. Molecular characterization was based on: (i) classifying the isolates as EHV-1B or EHV-1P genotypes; and (ii) identification of a neuropathogenic marker. Molecular characterization of these isolates may provide the basis for the development of vaccines that may be more efficient for preventing EHV-1-induced neurologic disease.

The EHV-1 strains used in this study (n = 8) were obtained in a previous epidemiological survey of encephalitis-causing agents in horses in the State of Minas Gerais (Costa *et al*., 2015), which also resulted in the first isolation of the Saint Louis Encephalitis virus from a horse with neurological disease (Rosa *et al.*, 2013). Nomenclature of these isolates was established as described by Nugent *et al*. (2006), according to the country of origin, year of the outbreak, the unique identifier of the outbreak, and pathogenic characteristics of the outbreak (p: 0 = attenuated vaccine strain, 1 = non neurological isolate, 2 = neurological isolate). Features of the EHV-1 isolates used in this study are summarized in Table 1.

Table 1. Features of EHV-1 strains isolated from horses with neurologic disease in the State of Minas Gerais, Brazil

| | Horse | | |
|---|---|---|---|
| Strain | gender | age (months) | Location |
| BR09_1_2 | Male | 96 | Várzea da Palma |
| BR09_03_2 | Female | 42 | Abaeté |
| BR09_29_2 | Female | 36 | Entre Rio de Minas |
| BR09_30_2 | Female | 02 | Entre Rio de Minas |
| BR09_66_2 | Female | 24 | Itamarandiba |
| BR09_141_2 | Female | 30 | Pompeu |
| BR09_154_2 | Female | 24 | Ganhães |
| BR10_1232_1 | Female | NA | Viçosa |

NA=Not available

Prior to molecular analysis, viral strains were grown in *Madin Darby bovine kidney* (MDBK) cells (ATCC CCL-22) cultured in DMEM (Dulbecco's Modified Eagle Media; Life Technologies, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco). Cultures were sequentially subcultured as described below until reaching approximately 70-90% of characteristic cytopathic effect of EHV-1 (i.e. round cells, detachment of cells, cytoplasmic stranding, vacuolization of the cells, formation of syncytia and lyses plaques) and the isolation was confirmed by PCR after the fourth passage (Varrasso *et al*., 2001). Viral titers were assessed by determining plaque forming units (PFU) according to Desprès *et al*. (1993).

Viral DNA was extracted by phenol/chloroform/isoamyl alcohol according to the protocol described by Sambrook *et al*. (1989). All DNA samples were analyzed for assessing concentration and purity by spectrophotometry. PCRs containing 200 ng of template DNA were performed according to the parameters described in Table 2. PCRs were performed for: (i) differentiation between EHV-1B and EHV-1P genotypes and (ii) identification of the neuropatogenic marker.

Table 2. Primers and restriction enzyme utilized to identify the EHV-1P genotype and the neuropathogenic marker

| Primers | Sequence (5'-3') | Amplicon size | Restriction enzyme |
|---|---|---|---|
| First Reaction: | | | |
| ORF30F8 | GTGGACGGTACCCCGGAC | 380 bp | |
| ORF30R2 | GTGGGGATTCGCGCCCTCACC | | |
| Second Reaction: | | | |
| ORF30F7 | GGGAGCAAAGGTTCTAGACC | 256 bp | *Sal*I* |

* Amplicons from the neuropathogenic strains yield two fragments **(**161 bp and 95 bp), whereas amplicons from non neuropathogenic strains are not digested.

The primers: Pr1 5' (sense – ACGCCCCCTTCGTTCCTC – 3') and Pr2 5' (antisense – CGCTCCACCTCGGTCCTG -3') were used for differentiating between EHV-1B and EHV-1P genotypes (Mori *et al*., 2012). These primers amplify only the *ICP-4* gene of type P, which has not undergone recombination with the natural EHV-4. The conditions of the PCR were performed as described by Mori *et al*. (2012). DNA samples from all eight EHV-1 isolates yielded a 436 base pairs (bp) PCR product, indicating that they all belong to the P genotype (EHV-P).

Identification of the neuropathogenic marker was based on amplification of EHV-1 ORF30 by nested PCR followed digestion with the restriction enzyme *Sal*I (Life Technologies) according to Allen *et al*. (2006). The primer sequences and description of the digested products are detailed in Table 2. The substitution of an adenine by a guanine at the position 2,254 ($A_{2254}$ - $G_{2254}$) introduces a *Sal*I restriction site. DNA samples from all eight EHV-1 isolates yielded a product of 256 bp in nested-PCR, which was digested by *Sal*I, indicating that they all carry this neuropathogenic marker (Figure 1).

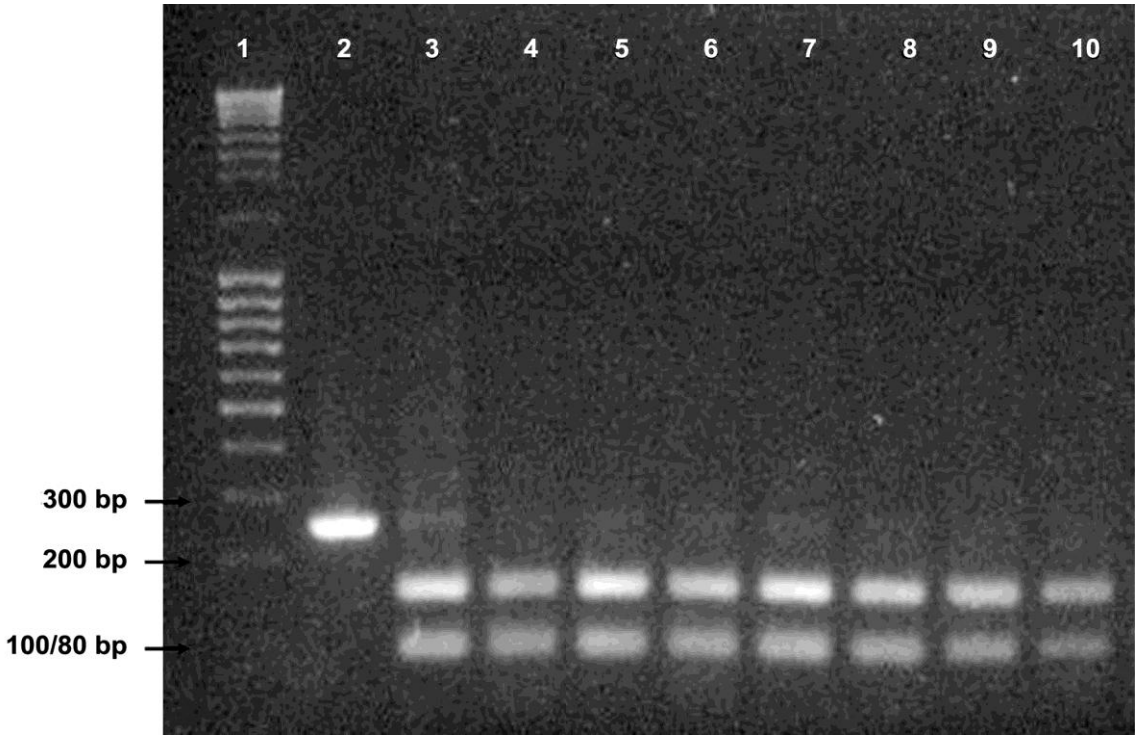


Figure 1. Restriction enzyme *Sal*I digest after nested-PCR amplification. Lane 1: Molecular Marker MassRuler DNA Ladder; lane 2: Isolated BR09_1_2 undigested; lane 3: Isolated BR09_1_2 digested; lane 4: Isolated BR09_29_2 digested; lane 5: Isolated BR09_30_2 digested; lane 6: Isolated BR10_1232_1 digested; lane 7: Isolated BR09_03_2 digested; lane 8: Isolated BR09_66_2 digested; lane 9: Isolated BR09_141_2 digested; lane 10: Isolated BR09_154_2 digested.

To confirm the neuropathogenic marker, PCR products of six isolates of EHV-1 (BR09_1_2, BR09_29_2, BR09_30_2, BR09_66_2, BR09_141_2 and BR10_1232_1) were sequenced by the Sanger method using the automated sequencing system Megabace 1000 (Amersham Bioscience, UK) and the commercial Kit DyEnamic ET Dye Terminator (Amersham Bioscience) according to the manufacturer's instructions. The consensus and quality of these sequences were assessed using the website program http://asparagin.cenargen.embrapa.br/phph/. Consensus sequences were then compared (http://multalin.toulouse.inra.fr/multalin/) with

two sequences of EHV-1 in Genbank (http://www.ncbi.nlm.nih.gov/): AB4 strain (Genbank number DQ180669), reference neuropathogenic strain and V592 (Genbank number DQ172359) reference non neuropathogenic strain. Indeed, isolates that were sequenced had the $G_{2254}/N_{752}$ point mutation, further supporting their classification as carriers of the neuropathogenic marker (Figure 2).

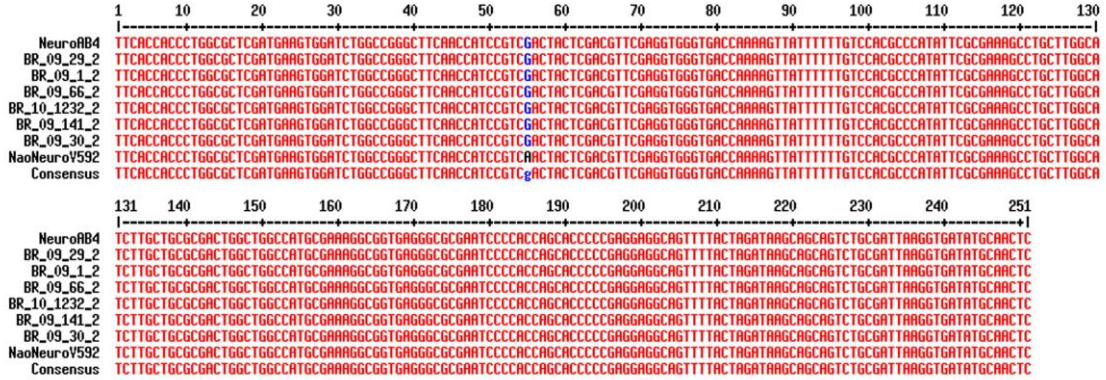


Figure 2. Alignment of partial sequences of EHV-1 ORF30 gene from the field isolates BR09_1_2, BR09_29_2, BR09_30_2, BR09_66_2, BR09_141_2, and BR10_1232_1 with AB4 strain (Genbank number DQ180669), reference neuropathogenic strain and V592 (Genbank number DQ172359) reference non neuropathogenic strain.

The results presented here show that all the EHV-1 strains isolated from central nervous system of horses that died with neurological signs match neuropathogenic variant ($G_{2254}/D_{752}$). The virus with neuropathogenic genotype has been recognized since the 1950's, and the incidence of these isolates increased exponentially every year, leading to an increasing risk for outbreaks of equine herpesvirus neurologic disease (Smith *et al*., 2010).

Furthermore, EHV-1 which possesses this neuropathogenic genotype has been associated with sporadic cases of EHV-1 abortion and was increased in comparison to previous decades in the USA and Europe. This is a concern to researchers and horse breeders worldwide since the vaccine against EHV-1 abortion does not protect against neurological diseases and the association of neurophatogenic strains with abortions can lead to inefficiency of the commercial vaccines. In spite of research efforts worldwide EHV-1 neuropathogenesis is still poorly known.

Keywords: horse, neuropathogenic equine herpesvirus -1, encephalitis

## RESUMO

*Este trabalho descreve a caracterização molecular de oito amostras de herpesvírus equino 1 isoladas do sistema nervoso central de equinos que morreram com sinais neurológicos no estado de Minas Gerais. As amostras de EHV-1 foram isoladas em cultivo celular, e a caracterização molecular foi feita por genotipagem e identificação do marcador neuropatogênico por meio das técnicas de PCR, restrição enzimática e sequenciamento. A caracterização molecular desses isolados pode ser a base para o desenvolvimento de novas formulações vacinais com maior eficácia para a prevenção de doença neurológica causada pelo EHV-1.*

*Palavras-chave: equino, herpesvírus equino 1 neuropatogênico, encefalite*

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