First detection of the equine herpesvirus 1 neuropathogenic variant in Brazil

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Summary
This report describes the first detection of an equine herpesvirus 1 (EHV-1) neuropathogenic variant (G_{2254}/D_{752}) in Brazil from a case of fatal equine herpesvirus myeloencephalopathy (EHM) in a mare. The results of nucleotide sequencing of the EHV-1 ORF30 gene showed that two other Brazilian EHV-1 isolates from EHM cases are representatives of the non-neuropathogenic variant (A_{2254}/N_{752}), suggesting that other unidentified factors are probably also involved in the neuropathogenicity of EHV-1 in horses. These findings will contribute to the epidemiological knowledge of EHV-1 infection in Brazil.

Keywords
Brazil – DNA polymerase gene – Equine herpesvirus 1 – Neurological disease – Neuropathogenic.

Equine herpesvirus type 1 (EHV-1) is an important and ubiquitous pathogen, which causes extensive economic losses in the horse industry. The virus induces respiratory disease in young horses, abortion in pregnant mares, perinatal foal mortality, and neurological disease. Infection may involve a single animal or an entire herd (8, 11).

In recent years, the occurrence of equine herpesvirus myeloencephalopathy (EHM) among horse populations in North America and Europe has increased both in frequency and severity, leading the United States Department of Agriculture to designate EHM as an emerging infectious disease (2). Nugent *et al.* (9) showed that a single nucleotide polymorphism within the gene encoding the catalytic subunit of EHV-1 DNA polymerase (ORF30) is associated with clinical cases of EHM. The substitution of adenine (A) for guanine (G) at position 2,254 (A_{2254} to G_{2254}) causes a replacement of asparagine (N) by aspartic acid (D) at amino acid position 752 (N_{752} to D_{752}). During the leukocyte-associated viraemia that follows EHV-1 infections in horses, the neuropathogenic variant (G_{2254}/D_{752}) replicates at higher levels and for a longer duration than the non-neuropathogenic variant (A_{2254}/N_{752}) (1). However, the mechanism by which this leukocyte-associated viraemia leads to EHM, and when, is not yet understood. Although the first isolation of EHV-1 (from an equine aborted fetus) was recorded in Brazil in 1966, the first EHM case report occurred only in 2005. This fatal EHM case was of a single mare, housed in an equestrian facility (7).

Recent studies using real-time polymerase chain reaction (PCR) showed that 7% (4/54) of the EHV-1 strains isolated in Argentina from abortion outbreaks were associated with the neuropathogenic variant (G_{2254}/D_{752}), and only two of those four cases were associated with simultaneous

neurological disease (15). The ORF30 genotyping of Brazilian EHV-1 isolates derived from abortions or neurological cases has not been reported. This paper is a report of three EHM cases, including the first reported neuropathogenic (G_{2254}/D_{752}) EHV-1 isolate from Brazil.

Brain tissues were fixed in 10% formalin, processed routinely, embedded in paraffin, sectioned at 5 µm thickness, and stained with haematoxylin and eosin (HE).

Virus isolation was attempted with clinical samples (20% w/v brain or 1 ml of cerebrospinal fluid [CSF]) collected at necropsy and inoculated separately onto monolayers of Vero (CRL-1587, ATCC) and E-derm (CCL-57, ATCC). When these cells exhibited a cytopathic effect, the identification of isolates was performed according to previously published methods (7).

DNA extraction of the clinical samples was also performed following a method previously described (3). Polymerase chain reaction was performed using a pair of specific oligonucleotide primers (forward primer 5´-CCACAA ACTTGATAAACACG-3´ and reverse primer 5´-GCGCTA CTTCTGAAAACG-3´) derived from an EHV-1 ORF30 gene region (9).

After purification of the amplified DNA fragments (GFX PCR DNA and Gel Band Purification Kit, GE Healthcare Limited), bidirectional cycle sequencing was performed with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The sequence reaction products were analysed on an automatic ABI Prism 377 DNA sequencer (Applied Biosystems).

Case report 1

An outbreak of EHM occurred in November 2007 on a farm located in Indaiatuba County (São Paulo State, south-eastern Brazil). Initially, a five-year-old Brazilian Sport Horse (*Brasileiro de Hipismo*) broodmare was anorexic and febrile. After four days, pelvic and thoracic limb paresis, ataxia and *cauda equina* syndrome, including urinary incontinence with urine dribbling, were observed. Due to progressive evolution with lateral recumbency, the mare was euthanised five days after the onset of neurological signs.

Cerebrospinal fluid from the atlanto-occipital cistern had increased protein levels (109 mg/dl) and xanthochromia. At necropsy, the main gross abnormalities observed were brain and spinal cord congestion and haemorrhagic foci in the spinal cord. The main histopathological findings in the spinal cord were chromatolytic and necrotic neurons, spheroids, gliosis, satellitosis, demyelination and mononuclear perivascular cuffs affecting the meninges. In the brain and cerebellum, haemorrhagic foci, mononuclear perivascular cuffs, chromatolytic neurons and demyelination were observed (Figs 1 & 2).

In contrast to the success with positive PCR using EHV-1 ORF30 gene primers, attempts to recover infectious EHV-1 from brain, lung and CSF by inoculation on Vero and E-Derm cells were unsuccessful. Employing ORF30 primers, brain samples (named BR07_1_2 isolate) were positive by PCR. The amplified ORF30 region was sequenced (GenBank accession number FJ793925). The ORF30 sequence of the BR07_1_2 isolate showed 100% identity with the corresponding sequence of EHV-1 reference strain V592 (GenBank accession number DQ172359), a representative of the non-neuropathogenic variant (A_{2254}/N_{752}).

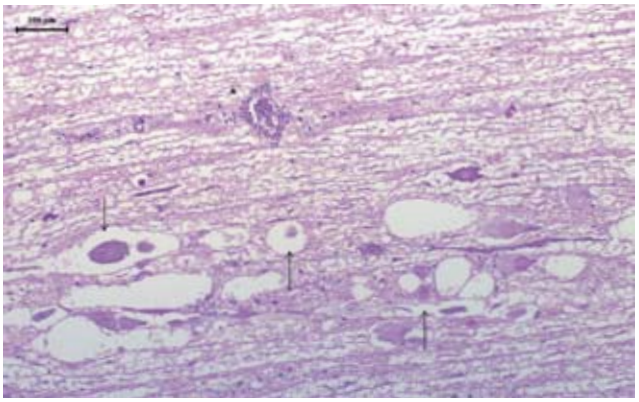

Fig. 1
Longitudinal section of the spinal cord of a five-year-old Brazilian Sport Horse broodmare: spheroids (arrows) and mononuclear perivascular cuffing (asterisk)
Haematoxylin and eosin stain

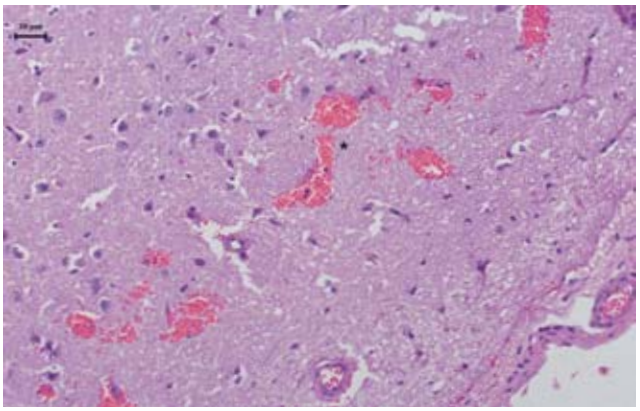

Fig. 2
Brain of a five-year-old Brazilian Sport Horse broodmare: multifocal cortical haemorrhage
Haematoxylin and eosin stain

Case report 2

On 7 February 2009, a six-year-old Paint Horse broodmare from Botucatu County (São Paulo State, south-eastern Brazil) was referred to the veterinary hospital of São Paulo State University in Botucatu.

At that time, the animal showed ataxia affecting the pelvic and thoracic limbs (grade 3), depression, dysphagia, hind limb hypometria, mild left head tilt, horizontal nystagmus, and circling gait. The clinical course of the disease did not improve and recumbency developed. Four days later, the horse died.

Histopathology of the brain and spinal cord suggested EHM, and lesions were characterised by: damage to the microvasculature of the central nervous system (CNS) due to initiation of an inflammatory cascade, vasculitis, thrombosis with resultant ischaemic neuronal necrosis, perivascular mononuclear cuffing, congestion, haemorrhage, diffuse gliosis, and perineuronal and perivascular oedema (Fig. 3).

After the first passage on E-Derm cells, EHV-1 isolate BR09_1_2 was recovered from the brain and CSF (atlanto-occipital), and its identity was confirmed by PCR. Samples from the brain and CSF were also positive by PCR. The amplified regions were sequenced (GenBank accession number HM475132) and showed 100% identity with EHV-1 reference neuropathogenic (G_{2254}/D_{752}) strain Ab4 (GenBank accession number DQ180669).

Case report 3

Case report 3 was already published by the authors in 2008; this was the first EHM report from Brazil (7).

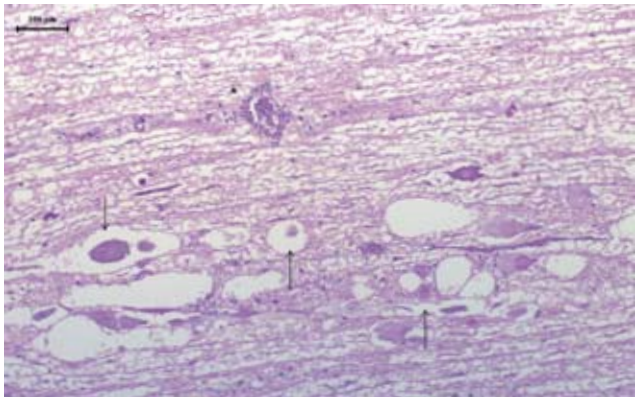

Fig. 3
Medulla oblongata of a six-year-old Paint Horse broodmare: mononuclear perivascular cuffing
Haematoxylin and eosin stain

Using ORF30 primers, both the original unprocessed CSF sample and cultured EHV-1 isolate BR05_1_2 were positive by PCR. This amplified region was sequenced (GenBank accession number EU410443) and showed 100% identity with the corresponding sequence of EHV-1 non-neuropathogenic (A_{2254}/N_{752}) reference strain V592 (GenBank accession number DQ172359).

The results presented here show that two of three recent Brazilian EHV-1 strains corresponded to the non-neuropathogenic variant (A_{2254}/N_{752}), and one of them matched the neuropathogenic variant (G_{2254}/D_{752}). According to some authors (5, 14), the EHV-1 non-neuropathogenic variant (A_{2254}/N_{752}) shows similar shedding and transmission in horse populations when compared with the neuropathogenic one. As evidenced in retrospective surveillance studies, the increasing occurrence of the neuropathogenic variant (G_{2254}/D_{752}) has been a recent event (2,9), which may explain the high occurrence of the non-neuropathogenic variant (A_{2254}/N_{752}) in EHM outbreaks in Brazil.

Despite the recent increased impact of EHM in North America and Europe (2), the authors believe that EHM outbreaks in Brazil are still extremely rare. Between 2005 and 2009, EHV-1 was found in only three of 58 horses that had neurological signs and had tested negative for Western or Eastern equine encephalomyelitis virus (using Vero cultures) and rabies virus (by fluorescent antibody test and intracerebral inoculation of juvenile mice) (data not yet published).

However, expansion of international trade in horses greatly increased the risk of dissemination of the neuropathogenic variant (G_{2254}/D_{752}) among horse populations in other countries. Therefore, the need for further studies of the molecular epidemiology of EHV-1 within the global equine population is strong (6).

The finding of a specific mutation in the amino acid sequence of the EHV-1 polymerase gene may not be the only determinant of neurological disease. The characteristic lesions in horses infected with EHM are vasculitis of small vessels and thrombosis, resulting in ischaemic damage to the CNS. Although EHV-1 is not considered primarily neurotropic, it has been reported that the virus can induce lesions in neurons and astrocytes (12). Moreover, the present study found positive samples from the CNS or CSF by virus isolation and PCR. These findings indicate the presence of virus in nervous tissue and suggest active EHV-1 infection in neurons. Recently, Yamada *et al.* (16) reported that the D/N_{752} difference in ORF30 might not be related to replication ability in neurons. It remains unclear how direct damage to neurons may contribute to EHM development.

Experimental inoculation of horses with the EHV-1 ORF30 neuropathogenic variant (G_{2254}/D_{752}) was unable to reproduce EHM on a large scale (4). The EHV-1 ORF30 neuropathogenic variant (G_{2254}/D_{752}) was also detected in abortion cases without causing EHM (13). This neuropathogenic hallmark (G_{2254}/D_{752}) may be relevant, but not definitive, for the occurrence of EHM in horses. It was estimated that between 14% and 24% of the isolates from cases of EHM harboured the non-neuropathogenic variant (A_{2254}/N_{752}), suggesting that other unidentified factors are probably also involved in the neuropathogenicity of EHV-1 in horses (9, 10).

This report is the first report of the molecular characterisation of EHV-1 strains in Brazil. This information and additional studies will contribute to the knowledge of the epidemiology of EHV-1 infection in Brazil. More samples will be necessary to evaluate the role of the EHV-1 neuropathogenic variant (G_{2254}/D_{752}) in EHM cases in Brazil.

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Première détection du variant neuropathogène de l'herpèsvirus équin de type 1 au Brésil

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Résumé

Le variant neuropathogène (G_{2254}/D_{752}) de l'herpèsvirus équin de type 1 (EHV-1) a été détecté pour la première fois au Brésil chez une jument ayant succombé à une myéloencéphalite à herpès équin (EHM). Le séquençage nucléotidique du gène EHV-1 ORF30 a révélé que deux autres isolats brésiliens du virus EHV-1 provenant d'équidés atteints d'EHM appartenaient au variant non neuropathogène (A_{2254}/N_{752}), ce qui semble indiquer que l'apparition de la forme neuropathogène du virus EHV-1 chez les chevaux est probablement influencée par d'autres facteurs qui restent à déterminer. Ces résultats contribueront à élucider l'épidémiologie de l'infection par le virus EHV-1 au Brésil.

Mots-clés

Brésil – Forme neuropathogène – Gène ADN polymérase – Herpèsvirus équin de type 1 – Maladie neurologique.

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Detección, por primera vez en el Brasil, de la variante neuropatógena del herpesvirus equino 1

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Resumen

Los autores dan cuenta de la detección, por primera vez en el Brasil, de la variante neuropatógena (G_{2254}/D_{752}) del herpesvirus equino 1 (HVE-1) en una yegua muerta de mieloencefalopatía por herpesvirus equino. Los resultados de la secuenciación de los nucleótidos del gen ORF30 del HVE-1 demostraron que otras dos muestras brasileñas de HVE-1 aisladas en animales enfermos corresponden a la variante no neuropatógena (A_{2254}/N_{752}), lo que parece indicar que seguramente hay otros factores no identificados que intervienen en la neuropatogenicidad del HVE-1 en el caballo. Estas conclusiones ayudarán a conocer mejor la epidemiología de la infección por el HVE-1 en el Brasil.

Palabras clave

Brasil – Enfermedad neurológica – Gen de la ADN polimerasa – Herpesvirus equino 1 – Neuropatógeno.

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