SCIENTIFIC COMMUNICATION

FIRST ISOLATION OF EQUINE HERPESVIRUS TYPE 1 FROM A HORSE WITH NEUROLOGICAL DISEASE IN BRAZIL

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ABSTRACT

This report describes the first Brazilian equine herpesvirus type 1 (EHV-1) isolation from a single fatal equine herpes myeloencephalopathy case in a mare. The isolation of EHV-1 was confirmed from the first passage of cerebrospinal fluid (CSF) sample in Vero cells by PCR and virus neutralization assay. As virus isolation from CSF is unlikely to be successful, as has been shown in several case reports, this circumstantial evidence suggests that the neurological disease was caused by particularly neurovirulent strain of EHV-1.

KEY WORDS: Equine herpesvirus type 1, EHV-1, myeloencephalopathy, cerebrospinal fluid, virus isolation.

RESUMO

PRIMEIRO ISOLAMENTO NO BRASIL DE HERPESVIRUS EQÜINO TIPO 1 EM UM CAVALO COM DOENÇA NEUROLÓGICA. O presente relato refere-se ao primeiro isolamento no Brasil do herpesvírus eqüino tipo 1 (HVE-1) proveniente de um caso clínico de mieloncefalopatia herpética em uma égua, que evoluiu para o óbito. O isolado do HVE-1, denominado 07/05, foi obtido a partir de uma amostra de líquor na primeira passagem em células Vero, confirmando-se sua identidade pela PCR e pela prova de neutralização viral. Como o isolamento viral a partir do líquor geralmente não é bem sucedido, conforme demonstrado em vários relatos de casos, o presente achado sugere que a doença neurológica foi causada por uma amostra particularmente neurovirulenta de HVE-1.

PALAVRAS-CHAVE: Herpesvírus eqüino tipo 1, HVE-1, mieloencefalopatia, líquor, isolamento viral

Equine herpesvirus type 1 (EHV-1) is an important pathogen and has been responsible for causing four recognized clinical syndromes in horses throughout the world: upper respiratory disease in young horses, abortion in late gestation, perinatal foal mortality, and occasionally neurological disorders (Reed & Toribio, 2004). Infection may involve a single animal or be associated with large outbreaks of disease.

The first isolation of EHV-1 in Brazil was recorded in 1966 from an equine aborted fetus (Nilsson; Correa, 1966). Although EHV-1 is ubiquitous in the horse populations, there has been no report of confirmed virus isolation or detection from nervous tissue or cerebrospinal fluid (CSF) of equine neurological cases in Brazil (Carvalho et al., 2000; Goehring et al., 2006).

The EHV-1 neurological disease, also known as equine herpes myeloencephalopathy (EHM), may be a result of hypoxic damage to the central nervous system (CNS) secondary to vasculitis and thrombosis rather than direct neurologic injury by the virus. Consequently, in concordance with the pathophysiology of the disease, attempts to isolate the virus from the CSF or CNS tissue are seldom successful (Pellegrini-Masini; Livesey, 2006).

Nevertheless, a definitive diagnosis of EHM can only be supported by the following laboratory tests: 1) EHV-1 isolation from CSF or brain tissue; 2) identification of viral particles, inclusions, antigen, or nucleic acid in CNS tissue (Reed; Toribio, 2004; Davis; Tyler, 2005).

Interestingly, EHM outbreaks have been reported worldwide with increasing frequency and intensity

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in recent years. The emergence of hypervirulent neuropathogenic EHV-1 strains has been a cause of concern (Studdert et al., 2003; Nugent et al., 2006).

This report describes the first Brazilian EHV-1 isolation from a single fatal EHM case of a mare at an equestrian facility housing 10 horses.

On 5 May 2005, an 8-year-old Mangalarga breed mare from a riding school in Ribeirão Pires County (Sao Paulo State, Southeastern Brazil) was examined by a private veterinarian. At that time, the animal was inappetent, lethargic, depressed, and reluctant to move. There were no reports of respiratory disease, stillborn, abortion or neurological disease in-contact horses. In addition, all the horses in the riding school were vaccinated annually against eastern and western encephalomyelitis, rabies and tetanus; however, none of them was vaccinated against EHV-1 or EHV-4.

Ten days later, the mare showed deterioration with weakness and paresis of the hind limbs. Furthermore, complete paralysis and lateral recumbency developed within the following 24 hours, being the animal unable to stand by itself. Nevertheless, on the further neurological examination, the cranial nerves were normal, as well as there were no signs of cauda equine syndrome, such as, urinary incontinence, hypotonia of tail or anus. Because the prognosis seemed hopeless due to progressive neurologic signs that were not responding to aggressive treatment (supportive nursing, nutritional care, and reduction of CNS inflammation), the animal was euthanized.

The acute progressive nervous signs observed in the present case report could be confused with those observed in equine encephalomyelitis or rabies viruses infection. Nevertheless, further laboratory investigations were negative for equine encephalomyelitis and rabies. At necropsy, no gross abnormalities were observed.

Brain and CSF from atlanto-occipital site were submitted to EHV-1 isolation. Both samples were inoculated on Vero cells. EHV-1 identification was performed by virus neutralization test (Saxegaard, 1966) and by polymerase chain reaction (PCR).

One mL of the clinical samples (20% w/v brain tissue or CSF) was inoculated onto monolayers of Vero cells at 37° C for 1 hour. At the end of the attachment period, cell monolayers were rinsed, refed with 6 mL of maintenance media (Eagle's minimal essential medium - EMEM supplemented with 200 U/mL penicillin, 200 µg/mL streptomycin, and 50 U/mL nystatin) and incubated at 37° C. Cell cultures were examined daily for 7 days for the appearance of characteristic cytopathic effect (CPE) of herpesvirus (rounding, refringent cells, similar to brunches of grapes and syncytia formation). One passage was used routinely for all cases and additional passages (maximum three) were used when primary isolation was unsuccessful. Lack of CPE after three passages in these cultures was interpreted as a negative test.

For seroconfirmation of EHV-1 identity, the neutralization test was performed with a hyperimmune polyclonal antiserum against EHV-1 (titer 1:512) that neutralize 100 $TCID_{50}$ of EHV-1 Brazilian A4/72 strain. Serial twofold dilutions of EHV-1 isolate was prepared and mixed with an equal volume (25µL) containing positive and negative serum controls. The virus and serum mixtures were incubated for 1 hour at 37° C. The mixtures were then added with 100 µL of the prepared Vero cell suspension (25×10^3 cells/well) in MEM with 10% fetal bovine serum. The inoculated plates, after incubation at 37° C in 5% CO_2 atmosphere for three days, were examined daily for presence of CPE. Wells were classified as positive for the virus neutralization test if 100% of the cell monolayer remained intact (no CPE). The endpoint titration was defined as the highest dilution of each sample that inhibited viral CPE.

DNA extraction of the isolate named 07/05 was conducted following a method described elsewhere (Chomczynski, 1993). PCR was performed using a pair of specific oligonucleotide primers (P5 forward primer 5'-ATG TCT ACC TTC AAG CTT-3' and P6 reverse primer 5'-TTA CGG AAG CTG GGT ATA-3') derived from EHV-1 glycoprotein D gene region (Galosi et al., 2001). Amplification conditions were performed in a reaction mixture of total volume 50µL containing 0.5µg of DNA sample, 0.5µM of each primer, 0.2 mM of each dNTP mixture, 2.5 units of Taq DNA polymerase platinum (Invitrogen Brasil Ltda), 1 ´ PCR buffer (20 mM of Tris-HCl pH 8.4, 50 mM of KCl), 1.5 mM of $MgCl_2$ and ultra-pure water QS. Amplification was carried out in a thermal cycler (Eppendorf Mastercycler Gradient PTC-200, Eppendorf AG) under the following conditions: DNA template was initially denatured at 94° C for 5-min followed by 35 cycles of 1-min denaturation at 94° C, 1 min primer annealing at 50° C and 1-min extension at 72° C. Lastly, the reaction was completed in a 6-min final extension step at 72° C. Amplified PCR product was analyzed by horizontal electrophoresis (100V for 25 min) in 1.5% agarose gel and visualized by 0.5 µg/mL ethidium bromide under UV light. A positive control (purified DNA from EHV-4 Brazilian A4/72 strain) and a negative control (ultra-pure water) were included with each PCR assay.

After purification of the amplified DNA fragments (1,208 base pairs) (GFX PCR DNA and Gel Band Purification Kit, GE Healthcare Limited), bidirectional cycle sequencing was performed with BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The sequence reaction products were analyzed on an automatic ABI Prism 377 DNA sequencer (Applied Biosystems).

The sequence quality analysis was examined by *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. After that, the consensus sequence was assembled using CAP contig assembly program and the alignment was obtained with homologous sequence from EHV-1 reference strain Ab4 (GenBank access number AY665713) using the ClustalW method of the software BioEdit version 7.0.5.3 (Hall, 1999).

In the first passage of CSF sample in Vero cells, EHV-1 isolate 07/05 was recovered within 24 hours and its identity was confirmed by PCR and virus neutralization assay; however, the brain material was negative after three passages in Vero cells. In Vero cells, the virus reached an infectivity titer of $10^{2.7}$ $TCID_{50}$/25 µL. The failure to isolate EHV-1 from brain may be due to the lower sensitivity of conventional virological methods (Cinque et al., 2003). Alternatively, neural cells may be low in number and segmental in distribution. It can be assumed that the time lapse between acute virus replication and horse death may be critical for identification of EHV-1 in neural tissues. Other factors, such as the conservation of the material obtained for virus isolation, should play an additional role (**Schultheiss** et al., 1997).

Nevertheless, as virus isolation from CSF is unlikely to be successful, as has been shown in several case reports, this circumstantial evidence suggests that the neurological disease was caused by particularly neurovirulent strain of EHV-1 (Pellegrini-Masini & Livesey, 2006). Nugent et al. (2006) identified a single nucleotide polymorphism in the viral DNA polymerase enzyme gene (ORF30) that showed highly statistically significant correlation with paralytic disease severity during an EHM outbreak. Therefore, additional studies have been needed to indicate whether mutant strains of EHV-1 have occurred in Brazil.

Employing gD primers, both original unprocessed CSF sample and cultured Brazilian EHV-1 isolate 07/95 were positive by PCR. Furthermore, this amplified gD region was sequenced (GenBank accession number EU052212). Glycoprotein D sequences of 07/05 isolate showed 100% of identity when compared with corresponding sequence of EHV-1 reference strain Ab4, suggesting that among EHV-1 strains the gD gene is highly conserved. In fact, and in spite of dramatic differences in the virulence and tissue tropism, the examination of this highly conserved region could not explain the neuropathogenicity of the 07/05 isolate.

Previous EHM reports have not very detailed molecular analysis as here (Thein et al., 1993). In human medicine, the use of molecular techniques has clearly improved the diagnosis and clinical management of viral CNS infections (Cinque et al., 2003). The positive CFS PCR result indicated the presence of viral nucleic acid and was a marker of recent or ongoing active CNS EHV-1 infection (De Biasi et al., 2002). Sequencing DNA techniques have enabled precise characterization of viral genome following their recovery in the CSF. In conclusion, an EHV-1 isolate was obtained from the CSF of a horse with neurological disorders that is identical to strains isolated elsewhere, showing that CSF can be used as a sample for EHV-1 detection.

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