

# Detection of bovine *Herpesvirus* type 5 in formalin-fixed, paraffin-embedded bovine brain by PCR: a useful adjunct to conventional tissue-based diagnostic test of bovine encephalitis

H.F. Ferrari<sup>a</sup>, M.C.R. Luvizotto<sup>b</sup>, P. Rahal<sup>d</sup>, T.C. Cardoso<sup>c,\*</sup>

<sup>a</sup> UNESP, Master in Animal Science, Veterinary School, Araçatuba, São Paulo, Brazil

<sup>b</sup> UNESP, DCCR, Laboratório de Patologia, Veterinary School, Araçatuba, São Paulo, Brazil

<sup>c</sup> UNESP, DAPSA, Laboratório de Virologia Animal, Veterinary School, Araçatuba, São Paulo, Brazil

<sup>d</sup> UNESP, IBILCE, Laboratório de Estudos Genômicos, São José do Rio Preto, São Paulo, Brazil

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## Abstract

The aim of this study was to evaluate the application of PCR technique for the detection of BoHV-5 in routinely formalin-fixed, paraffin-embedded brain tissues in 20 naturally infected calves affected by fatal meningoencephalitis. Brains were divided into two halves, one kept fresh for virus isolation and PCR assay, targeting the glycoprotein C gene from BoHV-5 genome. The other half brain, corresponding to posterior cortex region, was submitted to formalin fixation and embedded into paraffin blocks for microscopic evaluation and total DNA isolation. Most of the slides showed severe multifocal non-suppurative encephalitis with neuronal degeneration, neurophagia, and no acidophilic intranuclear inclusions could be found in neurons and glial. The 20 fresh samples were confirmed, by virus isolation and PCR assay, as having the BoHV-5 virus and, respective glycoprotein C sequence, while 15 of 20 formalin-fixed, paraffin-embedded samples were considered positive for the same analysis. The results revealed the first description of PCR efficiency, applied to formalin-fixed, paraffin-embedded brain collected from naturally infected calves, improving the detection of BoHV-5 from archival samples in South America.

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## 1. Introduction

Bovine *Herpesvirus* type 5 (BoHV-5) is the etiological agent of bovine encephalitis, isolated firstly in Argentina and Australia and later in Canada, Hungary, Italy and the USA (Carrillo et al., 1983; Meltzer et al., 1986). It causes epidemics, affecting calves under 1 year and the mortality rate is 100%, classified as being an enveloped double-strand DNA virus belonging to the family *Herpesviridae*, subfamily, genus *Varicellovirus* (Alegre et al., 2001; Delhon et al., 2003).

Like other members of *Alphaherpesvirus*, it establishes a lifelong latent infection in sensory nerve ganglia after acute infection, whereas the infection is manifested by occasional

reactivation of infection providing an adequate means of transmission and spread, generally only detected by outbreaks. More recently, it has been reported that cross-protection against BoHV-5 and BoHV-1, is provided by compulsory vaccination (Belknap et al., 1994; Diel et al., 2007; Del Médico Zajac et al., 2006).

In South America, this type of encephalitis is diagnosed frequently as *Lyssavirus* infection and the negative tissues are fixed with formalin and embedded in paraffin for veterinary pathology archives latter (Cardoso and Pilz, 2004; Cardoso et al., 2006). Due to significant losses in cattle industry as a result of BoHV-5 infection worldwide, America and European Community have strict sanitary controls of semen and embryos, establishing effective eradication programs, which affect the international meat industry. Brazil represents the first country in the world market as a source of meat to Europe and Asia, which requires revision of sanitary measures (Flôres et al., 2003; Del Médico Zajac et al., 2006; Silva et al., 2006; Diel et al., 2007).

\* Corresponding author at: Departamento de Apoio, Produção e Saúde Animal, Curso de Medicina Veterinária, Rua Clóvis Pestana, 793, 16.050-680 Araçatuba, SP, Brazil. Fax: +55 18 3622 6487.

E-mail address: [tcardoso@fmva.unesp.br](mailto:tcardoso@fmva.unesp.br) (T.C. Cardoso).

In the case of BoHV-5 diagnosis, virus isolation is also undertaken to confirm the etiological agent. However, this procedure is time consuming, requiring expensive laboratory reagents and special technical expertise. Generally, low-yield is often caused by low concentrations of viable virus particles from fresh tissues collected after death. In fact, due to the lack of availability of fresh tissue specimens and acute – and convalescent – phase serum, tissue-based diagnostic methods such immunohistochemistry (IHC) is often the first choice for diagnosis. However, the use of monoclonal antibodies to detect BoHV-5 specific antigens, from autopsy tissues, became uncommon, due to the high cost of the reagents, especially when a large number of samples must be tested (Belknap et al., 1994; Ely et al., 1996; Roehle et al., 1997; Flôres et al., 2003; Vidal et al., 2006; Bhatnagar et al., 2007).

Several PCR-based methods have been developed for rapid detection of BoHV-5 in fresh tissue, in spite of the detection of BoHV-5 from fixed tissues be largely unexplored, particularly for routinely processed bovine autopsy specimens. Recovering nucleic acid from archived formalin-fixed, paraffin-embedded blocks would significantly expand the opportunity for understanding the BoHV-5 epidemiology obtained from negative samples for rabies infection and bovine spongiform encephalopathy disorder (Ely et al., 1996; Belknap et al., 1994; Chan et al., 2001; Cardoso and Pilz, 2004; Claus et al., 2005; Cardoso et al., 2006; Meyer et al., 2001; Vidal et al., 2006).

The aim of this study was to evaluate the applicability of PCR assay for the detection of BoHV-5 in routinely processed bovine formalin-fixed, paraffin-embedded brain and to compare these achieves with those obtained using conventional virological methods.

## 2. Materials and methods

### 2.1. Samples and general procedures

For the present study 20 cattle brains diagnosed as negative for rabies virus and 1 healthy control specimens were used. The brains were obtained from necropsy routine at veterinary school belonging to the University of São Paulo State from 2004 to 2006 years. All the cases studied showed clinical sign at the antimortem inspection of fatal encephalitis and were male cattle with their ages ranged 1 year or less. The whole brain was sampled and sectioned sagittally into two halves; one-half was homogenized by addition of 5 ml of minimal essential medium (MEM Gibco-BRL cat # 61100-061), plus antibiotics centrifuged, filtrated and the supernatant stored at  $-86^{\circ}\text{C}$  until use in different room to avoid cross contamination. The other half was immersed in 10% phosphate-buffered formalin for 1 week or more (Vogel et al., 2003).

### 2.2. Histopathological examination

The posterior cortex region immersed in 10% phosphate-buffered formalin was sectioned into 10–15 mm thick tissue sections, blocked and immersed in 98% formic acid during 24 h and embedded in paraffin wax. Three-micrometer thick sections

were then obtained for routine hematoxylin and eosin (H&E) stain and the respective microscopic lesions were characterized by at least by two different pathologists. Non-stained sections were submitted to DNA extraction (Ely et al., 1996).

### 2.3. Virus isolation

All samples were submitted to virus isolation using the MDBK cells (Madin-Darby bovine cell line ATCC-CCL-22), CRIB cells (derived from Madin-Darby bovine cell line) described as being resistant to BVDV infection and CER cells (hybrid of BHK-21 cell and chicken fibroblast). Cells were cultured on serum-free medium Advanced RPMI 1640 (Gibco-BRL cat # 12633) without fetal bovine serum and/or animal protein. Positive BoHV-5 strain was gently provided from Instituto Biológico, São Paulo, Brazil. Non-infected cells were used as negative control of virus isolation and positive results were determined by the cytopathic effect resulted from cell infection and characterized by immunoperoxidase assay in some uncertain results (Flores and Donis, 1995; Spilki et al., 2006; Cardoso et al., 2007).

### 2.4. DNA extraction and polymerase chain reaction

#### 2.4.1. DNA extraction from fresh brain and cell culture supernatant

Three different methods for DNA extraction were examined using fresh brain suspension. The first was the DNAzol<sup>®</sup> (Invitrogen cat # 10503-027), second Easy-DNA<sup>™</sup> (Invitrogen cat # 45-0424) and last the UltraPure<sup>™</sup> Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (v/v) (Invitrogen cat # 15593-031). For all the methods 200  $\mu\text{l}$  of the samples were used and a primary enzymatic digestion by the addition of an extraction buffer (10 mM Tris, 1 mM disodium ethylene diamine tetraacetic acid-EDTA, 0.5% sodium dodecyl sulphate SDS, 50  $\mu\text{g/ml}$  proteinase K) performed only in the last protocol. The DNA was precipitated with 1 ml of 100% ethanol incubated overnight under  $-86^{\circ}\text{C}$  temperature and centrifuged at  $16,000 \times g$  for 20 min in the next day. The pellet obtained was rinsed with 1 ml of 70% ethanol, dried and re-suspended in 15  $\mu\text{l}$  of DEPEC water and stored at  $-86^{\circ}\text{C}$  until use. The DNA from supernatant of infected cells was extracted using the Easy-DNA<sup>™</sup> protocol.

#### 2.4.2. DNA extraction from formalin-fixed, paraffin-embedded tissues

DNA was isolated from processed material according to Shi et al. (2004) with some modifications. Briefly, 500  $\mu\text{l}$  of 0.1 M NaOH solution was added to each microtube containing three slices of 10  $\mu\text{m}$  of each block and heated at  $121^{\circ}\text{C}$  during 20 min in automatic autoclave. Because, the UltraPure<sup>™</sup> Phenol:Chloroform:Isoamyl protocol was found to be better than the other two procedures after further examination, it was chosen for DNA isolation, just after a cooling time of 5 min according to manufacture's instructions. The upper aqueous phase supernatant, obtained was removed carefully to another clean microtube, adding 0.1 volume of 3 M sodium acetate and again

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