



Detection of bovine *herpesvirus* 5 (BoHV-5) in formalin-fixed, paraffin-embedded bovine brain by nested PCR in Colombian cattle[✉]

Detección de herpesvirus bovino 5 (BoHV-5) en cerebro incluido en parafina y fijado en formalina, por PCR anidada, en bovinos colombianos

Detecção de herpesvírus bovino 5 (BoHV-5) em cérebro incluído em parafina e fixado em formalina, pela PCR, em bovinos colombianos

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Summary

Fifteen cases of viral meningoencephalitis in Colombian cattle were tested by nested PCR analysis for the detection of bovine herpesvirus 5 (BoHV-5). All fatal cases had shown severe neurological signs and had occurred following natural outbreaks of the disease. The neurological infection was histologically characterized by mild to moderate inflammatory changes in the brain and cerebellum, including meningitis, mononuclear perivascular cuffing, gliosis, haemorrhage, and the presence of Gitter cells (macrophages) accompanying large areas of malacia. No intranuclear inclusion bodies were seen in any of the cases. Results from BoHV-5 molecular extraction analyses showed there were five positive cases thus confirming the presence of the virus in Colombia.

Key words: bovine herpesvirus, polioencephalomalacia, viral encephalitis.

Resumen

Quince casos de meningoencefalitis viral en ganado Colombiano fueron analizados por reacción en cadena de la polimerasa (PCR anidada) para la detección del herpesvirus bovino 5 (BoHV-5). Todos

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los casos mostraron severos signos neurológicos antes de morir y ocurrieron como brotes naturales de la enfermedad. La infección neurológica fue caracterizada histológicamente por leves a moderados cambios inflamatorios en el cerebro y cerebelo, incluyendo meningitis, manguitos perivasculares, gliosis, hemorragia y la presencia de células Gitter (macrófagos) acompañando grandes áreas de malacia. No se encontraron cuerpos de inclusión intranucleares en ninguno de los casos. Los resultados del análisis de la extracción molecular de BoHV-5 revelaron la existencia de cinco casos positivos, lo cual confirma la presencia del virus en Colombia.

Palabras clave: *encefalitis viral, herpesvirus bovino, polioencefalomalacia.*

Resumo

Quinze casos de meningoencefalite viral em gado bovino Colombiano foi analisado pela reação em cadeia da polimerasa (PCR) para a detecção do herpesvírus bovino 5 (BoHV-5). Todos os casos mostraram graves sinais neurológicos antes de morrer y aconteceram como surtos naturais da doença. A infecção neurológica foi caracterizada histologicamente por leves a moderados câmbios inflamatórios no cérebro e cerebelo, incluindo meningite, manguitos perivasculares, gliose, hemorragia e a presença de células gitter (macrófagos) acompanhando grandes áreas de malacia. Não se encontraram corpos de inclusão intranucleares em nenhum dos casos. Os resultados da análise da extração molecular do BoHV-5 por PCR revelaram a existência de cinco casos positivos, os quais confirmam a presença do vírus na Colômbia.

Palavras chave: *encefalite viral, herpesvirus bovino, polioencefalomalacia.*

Introduction

Bovine encephalitis caused by Bovine Herpesvirus 5 (BoHV-5) was initially described in 1962 when the virus was isolated following an outbreak of the disease that killed several calves in Australia (Lemos *et al.*, 2002). Initially, the virus was considered identical to the one causing Infectious Bovine Rhinotracheitis (IBR). However, some outbreaks of the disease causing exclusively neurological signs led to suspect there was a variant of such agent exhibiting neurological disease properties (Moretti *et al.*, 1964; Watt *et al.*, 1981; Weiblen *et al.*, 1989). In 1986, through molecular techniques discrimination, such agent was designated 1.3 bovine herpesvirus (Studdert, 1989). In 1992, the International Viral Taxonomy Committee named it BoHV-5 (Roizman, 1992).

Clinically, the condition may be misdiagnosed as rabies, pseudorabies, polioencephalomalacia resulting from thiamine deficiency, lead or salt intoxication, among other conditions (Sanchez *et al.*, 2000; Lemos *et al.*, 2002; Spilki *et al.*, 2003).

BoHV-5 has been reported in Europe (Barenfus *et al.*, 1963; Bartha *et al.*, 1969), Canada (Beck,

1975), the United States (d'Offay *et al.*, 1993), and South America (Carrillo *et al.*, 1983; Colodel *et al.*, 2002; Pérez *et al.*, 2002). For some authors, the occurrence of the disease in the south hemisphere (Australia, Argentina, and Brazil) is more important (Riet-Correa *et al.*, 1989; Lemos *et al.*, 2002; Halfen *et al.*, 2000). This is a sporadic disease involving calves. Morbidity may reach 50%. Neurological signs include depression, anorexia, isolation from herd, ocular and nasal serum discharge, slight sialorrhea, muscular tremor –which is particularly evident on the head and the neck- and hyperesthesia to both touch and noise, followed by loss of sensorial reflexes (particularly visual reflexes although involvement of auditive and dermal reflexes has also been described) (Vasconcelos *et al.*, 1993; Salvador *et al.*, 1998; Lemos *et al.*, 2002).

Circle wandering, ataxia, obstacle crashing, trismus, tongue tone decrease, difficulty for grabbing food and drinking water, nystagmus, teeth grinding (bruxism), decubitus prolong position with difficulty to return to normality, catatonia, and the finally ventral and lateral decubitus prostration, circling movements and death (Beltrao *et al.*, 2000; Colodel *et al.*, 2002; Pérez *et al.*, 2002). The encephalitis by herpes virus involves in a very frequent way the gray substance of the

cerebral cortex, though it could also affect the white substance, being a wide neural necrosis (Jubb *et al.*, 1993; Colodel *et al.*, 2002; Lemos *et al.*, 2002). One can observe macroscopically flattening of the cerebral convolution and the cortical malacia (Colodel *et al.*, 2002; Lemos *et al.*, 2002; Pérez *et al.*, 2002).

The lesions correspond microscopically to an acute necrosis non-suppurative meningoencephalitis, widely distributed, which can vary from mild to severe degree. The lesion is characterized for neural necrosis, gliosis, rupture of the neuropil and monocular perivascular cuff mainly of lymphocytes, macrophages named Gitter cells and occasionally neutrophils in the gray substance of the encephalon (Jones *et al.*, 1997; Salvador *et al.*, 1998; Silva *et al.*, 1999; Pedraza and Alessi, 2006). A leptomeningitis has also been reported lymphocytic histiocytic with perivascular cuff and focal gliosis or diffuse. The lesions are conclusive by the severe citonecrotic changes, which are particularly prominent in the cerebral hemisphere. The encephalitis shows its specificity for the intranuclear eosinophilic inclusion bodies that are presents in the astrocytes and cell core and they can be correlative with different degrees of infection according to the quantity (Salvador *et al.*, 1998; Colodel *et al.*, 2002; Lemos *et al.*, 2002).

The purpose of the present investigation was to detect genetic evident through the molecular extraction of the DNA of the BoHV-5 from nervous tissue of bovines that suspiciously died of herpetic encephalitis and in this way determine the existence of this virus as a possible cause for cows mortality for first time in Colombia.

Materials and methods

Virus and Cell

Sample used as reference strain was BoHV-5 Brazilian isolate EVI-88 - titre of $10^{6.25}$ TCID₅₀/50 ml - provided by Dr. Paulo M. Roehle (FEPAGRO Saúde Animal / Instituto de Pesquisas Veterinárias Desidério Finamor, Rio Grande do Sul State, Brazil). The virus was replicated in the Madin Darby Bovine Kidney (MDBK) cell line. MDBK

cells were grown with minimal Essential Media-Eagle (MEM) supplemented with 8% bovine fetal serum, penicillin (10.000 IU/L), streptomycin (0.2 g/L) and fungizone (2.5 mg/L).

Paraffin-Embedded Brain Tissue

A retrospective study was conducted using 15 formalin-fixed, paraffin embedded brain samples of Colombian cattle with non-suppurative encephalitis. Paraffin blocks came from the National Veterinary Diagnostic Laboratory of the Instituto Colombiano Agropecuario (ICA) in Bogotá, Colombia. We reviewed the histopathological results of the file from 2000 to 2004.

Fifteen samples were selected from two hundred negative cases to the rabies disease (by indirect immunofluorescence test) using a histopathological criteria of compatibility with BoHV-5 infection such as perivascular cuffing, mononuclear cell infiltration, areas of malacia and Gitter cells (macrophages). None of the cases showed inclusion bodies.

The cases analyzed were ten males and five females of the Zebu or Zebu crossed breed, aged between six months and five years, with clinical symptoms of neurological disease. Non-suppurative encephalitis post-mortem diagnosis was possible with the microscopic examination.

Extraction of Viral DNA

The paraffin-embedded samples were cut (5 µm) and mounted on microscope slides. The DNA was extracted according to Ben-Ezra *et al.* (1991) and Greer *et al.* (1991), with the following modifications: the excess paraffin was removed from the slide with a flame, and the resulting material scraped into a 1.5 ml test tube using a needle. The remaining paraffin was removed by soaking in 900 µl of Xilol at room temperature, with vigorous shaking for thirty minutes. The sample was then centrifuged at 1.400 rpm for 20 minutes, the supernatant discarded, and the sediment washed with 100% ethyl alcohol and centrifuged at 13.000 g for 15 minutes. The supernatant was discarded and the sediment dried by vacuum for 15 minutes at room temperature

and resuspended in 100 μ l digestion buffer (Tris 50 mM pH 8.5; EDTA 1 mM; Tween 20 – 0.5%), and Proteinase K (0.2 mg/ml) was added. The samples were incubated at 37 °C for 3 days; each day 3 μ l of proteinase K (20 mg/ml) was added. Afterwards, it was centrifuged at 1.400 rpm for 15 minutes and the supernatant was transferred to a new test tube and heated to 95 °C for 8 minutes, followed by a phenol-chloroform DNA extraction (Invitrogen Corp.). DNA of EVI-88 reference sample was extracted from cell supernatant using phenol-chloroform.

PCR assay

A nested PCR based on coding region of glycoprotein G, US4 gene of BoHV-5 was used (Gomes *et al.*, 2003). The PCR consisted of 35 cycles in total; in the first round each cycle consisted of 60 seconds at 95 °C, 60 seconds at 61 °C followed by 60 seconds at 72 °C. An initial

time of 1 min at 98 °C was included before the first cycle and a final extension of 6 min. at 72 °C was included at the end of the last cycle. For the nested PCR, 1 ml of this product was used with internal primers, using the same program above, except for annealing step (60 seconds at 57 °C). The PCR products were analyzed in 1.2% agarose gel (89 mM Tris-borate, 2 mM EDTA, pH 8.2) and stained with ethidium bromide (0.5 μ l/mL).

Results

The nested-PCR assay employed in this study detected the presence of BoHV-5 as the casual agent in five of affected cases evaluated (33.3%) among the 15 paraffin-embedded samples tested. The external PCR generated a product of 592 bp and the internal PCR a product of 222 bp (sense: TACGGACTGCCGGATTAA, antisense: GTCACCACTACCACCGCCGCCAAC) (Figure 1).

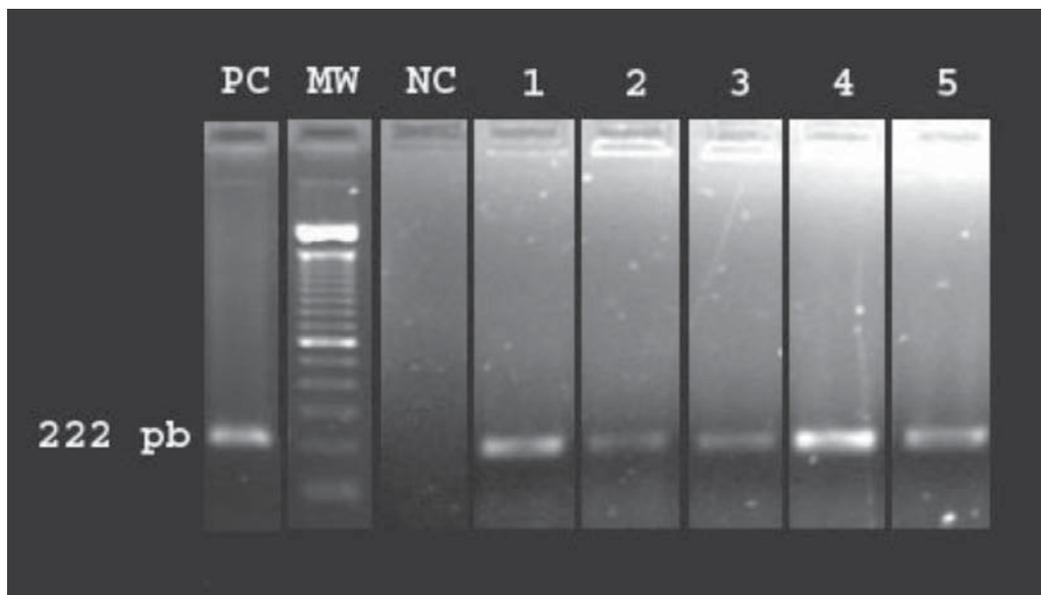


Figure 1. Analysis of PCR-nested products (222 bp). Lane MW: 222 bp 1 KB ladder (Invitrogen). Lane PC (positive control EVI-88 - BoHV-5), lane NC (negative control), lanes 1 through 5 correspond to the positive formalin-fixed, paraffin-embedded blocks from samples of brain to colombian cattle with encephalitis.

All five cases involved patients with previous history of neurological disease. These cases involved both young and adult animals. In all the cases the veterinarians responsible for the submissions requested diagnosis of rabies. In

all cases histological changes were observed in the examined brain sections and showed histological changes typical of herpesvirus-induced meningoencephalitis, notably necrotizing encephalitis (Table 1).

Table 1. Major epidemiological data of the five positive cases to BoHV-5 in Colombian cattle tested by nested PCR.

Protocol number	Age	Sex	Procedence	Microscopic findings
3101	6 m	male	Santander	Meningitis
3241	8 m	male	Cesar	Petechiae
2121	24 m	male	Cordoba	Encephalitis
8781	18 m	male	Cesar	Mononuclear infiltrate
10141	9 m	female	Sucre	Lymphoid infiltrate

The histological lesions were classified mainly as non-suppurative encephalitis with neuronal degeneration, neuronophagia, and without

acidophilic intranuclear inclusions in neurons and glial cells could be observed (Figure 2).

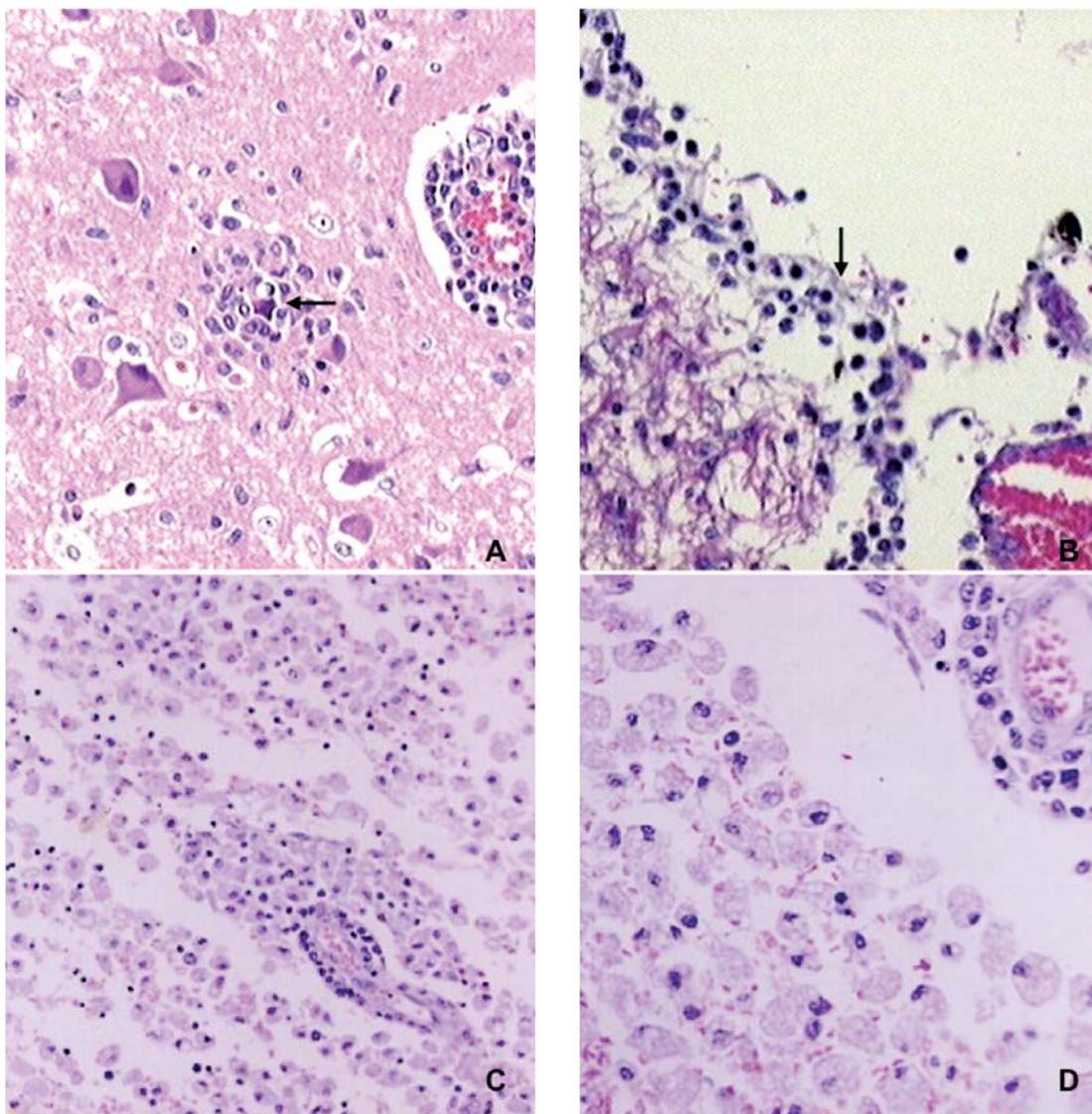


Figure 2. Bovine brain naturally affected by BoHV-5. 2A) Perivascular cuffing with mononuclear infiltration and neuronophagia in a brain cortex (arrows). (Barr=50 mm) HE, objective 10X. 2B) Meningitis (arrow). (Barr=50 mm) HE, objective 10X. 2C) Observed severe perivascular infiltrate of mononuclear cells and malacia areas with macrophages (Barr=50 mm) HE, objective 40X. 2D) macrophages (Gitter Cells) and perivascular cuffing (Barr=50 mm) HE, objective 60X.

Discussion

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 (Ferrari *et al.*, 2007).

The use of PCR for diagnosis of bovine herpesvirus 5 is a useful tool in the identification of the virus using nervous tissue samples kept in formaldehyde (Silva *et al.*, 2007). Up to this moment it had not been possible in Colombia to establish with certainty the presence of the virus. Although it is known that it is a virus spread worldwide, the lack of reports made difficult the research on this type of encephalitis because health authorities did not consider cautious to speculate about the presence of a disease with a big economic impact in other countries. Previous studies performed in herds reporting with nervous symptoms that sometimes recovered (although showing some sequels), reported sera positive to BoHV-5, however, the possibility of a crossed reaction between this virus and BoHV-1 (already identified in the country) did not allow clarity about the presence of the agent causing herpetic encephalitis. One aspect hampering the analysis is

the difficulty to differentiate between antibodies generated after infection and those generated by the commonly used vaccination against infectious bovine rhinotracheitis.

Some brain tissue samples with histological diagnosis compatible with herpetic encephalitis were tested by immunohistochemistry using the technique standardized by Prof. Dr. Eduardo Flores in the Federal University of Santa María (Rio Grande do Sul, Brazil) showing slight positivism in three cases (data not shown) confirmed by PCR in this study, and explaining the difficulty in antigenic recuperation due to the long time interval between fixation and processing (up to three months in some cases). That time interval did not allow the correct staining using monoclonal antibodies. Finally, molecular extraction of BoHV-5 DNA from brain tissue of colombian cattle, and the positive PCR results using primers registered in Gene Bank leaves no doubt about the presence of this virus in this South American country, suggesting its inclusion as a differential diagnosis. This study will allow also the development of research to establish the economic impact of the disease, and the possibility of establishing measures to control a disease apparently present in the country from several years ago.

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