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Distribution of bovine herpesvirus type 1 in the nervous system of experimentally infected calves

M.S. Marin [a,b], M.R. Leunda [b], A.E. Verna [a,b], P.E. Morán [c], A.C. Odeón [b], S.E. Pérez [a,c,d,*]

[a] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Rivadavia 1917, C1033AAJ, Buenos Aires, Argentina
[b] Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria Balcarce, Ruta 226 Km 73.5, Balcarce, Buenos Aires 7620, Argentina
[c] Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Paraje Arroyo Seco S/N, Tandil 7000, Argentina
[d] Centro de Investigación Veterinaria de Tandil (CIVETAN)-CONICET, Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Paraje Arroyo Seco S/N, Tandil 7000, Argentina

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ABSTRACT

Bovine herpesvirus type 1 (BoHV-1) is responsible for respiratory and genital disease in cattle. BoHV-1 encephalitis is only occasionally reported. However, several cases of neurological disease have been recently attributed to BoHV-1. In this study, the distribution and pathological alterations caused by two BoHV-1 strains in the nervous system of experimentally infected calves during acute infection and reactivation are described. Calves were inoculated intranasally with BoHV-1 Los Angeles (BoHV-1.LA) or Cooper (BoHV-1.Cooper) strains. Acutely infected calves were euthanased at 6 days (BoHV-1.Cooper, $n = 2$) and 7 days post-inoculation (BoHV-1.LA, $n = 2$). Latently infected calves that were given dexamethasone to induce reactivation were euthanased at 2 days (BoHV-1.Cooper, $n = 2$) or 5 days (BoHV-1.LA, $n = 2$) after dexamethasone administration.

Both BoHV-1 strains were isolated from the brains of acutely infected calves. Distribution of viral DNA in the neural tissues was similar for both strains. During reactivation, neither BoHV-1.LA nor BoHV-1.Cooper was isolated from any brain section or trigeminal ganglia in infected calves. Macroscopic lesions were not evident in any group. In BoHV-1.LA infected calves, microscopic lesions were found in the brain but not in the trigeminal ganglia. Microscopic lesions in the brain of BoHV-1.Cooper infected calves were not as evident as in BoHV-1.LA infected animals. However, mononuclear infiltrates and neuronophagia were present in trigeminal ganglia. The results of this study demonstrated that respiratory BoHV-1 strains are able to replicate and disseminate within the bovine nervous tissue and provide evidence of the neuroinvasiveness of BoHV-1 strains.

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Introduction

Bovine herpesvirus type 1 (BoHV-1) is an alpha-herpesvirus responsible for respiratory disease, abortions and genital disorders in cattle (Tikoo et al., 1995). BoHV-5, a closely related alpha-herpesvirus, is the causative agent of meningoencephalitis in calves (Pérez et al., 2002), which is highly prevalent in South America. Unlike BoHV-5, BoHV-1 has been reported only occasionally as responsible for bovine encephalitis (Meyer et al., 2001). However, several cases of neurological disease in cattle have recently been attributed to BoHV-1 (Silva et al., 2007; Rissi et al., 2008).

Neurotropic alpha-herpesviruses reach the nervous system via the trigeminal and olfactory pathways. However, it is apparent that BoHV-1 only reaches neural tissues via the trigeminal route (Muylkens et al., 2007). Within the trigeminal ganglion (TG), BoHV-1 probably does not replicate further than first order neurons, where latency is established (d'Offay et al., 1993). Consequently, neurological disease would not be expected to be commonly associated with BoHV-1 infection. Nevertheless, recent reports on the involvement of BoHV-1 in cases of encephalitis in cattle suggest that the virus might be able to replicate in the central nervous system (CNS).

Experimental infections to study the ability of BoHV-1 to replicate in the neural tissue of its natural host have not been conducted. In the present study, the distribution and pathological alterations caused by BoHV-1 in the nervous system of experimentally infected calves during acute infection and reactivation are described.

Materials and methods

Cells and viruses

Madin–Darby Bovine Kidney (MDBK) cells from the American Type Culture Collection (ATCC) were used for this study. MDBK cells were propagated in Minimum Essential Medium (MEM) with Earle salts (E-MEM) (Sigma-Aldrich), supplemented with 10% fetal bovine serum (Bioser) and antibiotic–antimycotic solution

* Corresponding author. Tel.: + 54 249 4439850.
E-mail address: seperez@vet.unicen.edu.ar (S.E. Pérez).

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(Gibco) (100 U/mL penicillin G, 100 μg/mL streptomycin sulfate and 0.025 μg/mL amphotericin B). Cells were incubated at 37 °C in 5% CO_2.

BoHV-1 reference strains Los Angeles (BoHV-1.LA) and Cooper (BoHV-1.Cooper), which are classified as BoHV-1.1 strains (Seal et al., 1985), were used for animal inoculations.

Animals and BoHV-1 challenge

Data from two independent experiments (undertaken for different purposes) were gathered to examine the detection and distribution of BoHV-1 in the nervous system of cattle. One year-old calves, free of detectable antibodies to BoHV-1 or BoHV-5, were inoculated intranasally by aerosol with BoHV-1.LA or BoHV-1.Cooper. The inoculum was evenly distributed in each nostril. To induce an acute infection, calves were inoculated with $10^{6.3}$ tissue culture infective doses ($TCID_{50}$) of BoHV-1.Cooper ($n = 2$) (10 mL inoculum) or BoHV-1.LA ($n = 2$) (25 mL inoculum). The animals were euthanased at 6 and 7 days post-inoculation (dpi), respectively.

To study reactivation from latency, we inoculated calves with 10^3 $TCID_{50}$ of BoHV-1.Cooper ($n = 2$) or BoHV-1.LA ($n = 2$) (10 and 25 mL inoculum, respectively) to induce a latent infection without causing overt disease after primary infection. We then administered dexamethasone (DEX; Schering Plough) as the reactivation stimulus. For the BoHV-1.LA experiment, we gave DEX (0.1 mg/kg/day) IV for five consecutive days, starting at 46 dpi. The calves were euthanased on day 5 following DEX treatment (days post-reactivation [dpr]).

In the BoHV-1.Cooper experiment, cattle received one IV DEX administration at 20 dpi and two IM doses 24 and 48 h later (0.1 mg/kg bodyweight for each dose). Animals were euthanased 2 days after DEX completing DEX treatment (at 25 dpi). Mock infected calves ($n = 2$, for BoHV-1.Cooper, and $n = 2$, for BoHV-1.LA) were inoculated with MEM. They were euthanased at 6 and 7 dpi (control calves for acute infection) and at 2 and 6 dpr (control calf for BoHV-1.Cooper and BoHV-1.LA reactivation, respectively) (Table 1).

Calves were maintained in isolated pens and fed on grass hay, concentrate foodstuff and water ad libitum. They were monitored daily, in the morning, for signs of fever (>39.3 °C), presence of nasal and ocular discharges or any other clinical signs of disease. Calves were deeply anaesthetised and euthanased according to the regulations of the Institutional Committee for Care and Use of Experimental Animals and the Animal Welfare Committee of the University of the Center of Buenos Aires Province, Argentina (file number 08/12, approved on 6 June 2012).

Sample collection

Nasal and ocular secretions and serum samples were collected at time 0, and 4–6 or 7 dpi. For calves that received a low virus dose, samples were collected periodically during the experiment and daily during DEX administration.

At necropsy, the brain was transversally sliced into 1–2 cm-thick sections. Brain samples were collected aseptically and individually for virus isolation, PCR and histopathology. Samples from mock infected calves were collected before sampling from inoculated animals to avoid cross-contamination. We collected samples from the following: the anterior cerebral cortex, including the olfactory cortex (sample 1), frontal cortex (samples 2 and 3) and dorso-lateral cortex (sample 4); the posterior cerebral cortex, including the marginal groove area (sample 5) and the ectomarginal groove area (sample 6); the cerebellum (sample 7), cervical medulla (sample 8), medulla oblongata (sample 9), pons (sample 10) and diencephalon (sample 11) and trigeminal ganglia.

Samples for virus isolation and PCR were transported to the laboratory on ice, within 30 min of collection, and stored at –80 °C.

Table 1
Experimental inoculation of bovine herpesvirus (BoHV)-1.

BoHV-1 strain	Infection	Calf	DEX treatment	Euthanasia
Experiment 1	Acute	1		7 dpi
BoHV-1.LA		2		
	Reactivation	5	46 dpi	6 dpr
		6		
	Mock	9	46 dpi	7 dpi
		11		6 dpr
Experiment 2	Acute	3		6 dpi
BoHV-1.Cooper		4		
	Reactivation	7	20 dpi	2 dpr
		8		
	Mock	12		6 dpi
		10		2 dpr

Experiments 1 and 2 are two independent experiments, in which calves were inoculated with BoHV-1 Los Angeles (BoHV-1.LA) or Cooper (BoHV-1.Cooper) strains. DEX, dexamethasone (initiation of DEX treatment); dpi, day post-inoculation; dpr, day post-reactivation (day of euthanasia after finishing DEX treatment).

Serology

Neutralising antibodies to BoHV-1 or BoHV-5 were evaluated by microtitration (van der Maaten et al., 1985) on MDBK cells. Neutralising antibody titers were determined as the highest dilution that completely inhibited the cytopathic effect (CPE) after 72 h of incubation. Seroconversion was considered to have occurred when a four-fold or a higher increase in the titer of neutralising antibodies was detected.

Virus isolation and identification

Nasal and ocular secretions were diluted (1:5) in MEM supplemented with penicillin–streptomycin (20 μL/mL), gentamicin (2 μL/mL), and amphotericin B (40 μL/mL). Tissues were triturated in 1× Hank's solution (10% w/v) and centrifuged at 1000 *g* for 15 min at 4 °C. Twenty microliters of supernatant was inoculated in triplicate into monolayers of MDBK cells in 96-well plates (GreinerBio-one) and incubated at 37 °C. Samples were monitored daily for CPE and tested by direct immunofluorescence using a polyclonal antibody against BoHV-1 (American BioResearch).

Virus titration

Virus titers in tissue samples were determined by the end-point titration method and expressed as $TCID_{50}$/mL or $TCID_{50}$/g (Reed and Müench, 1938). For determination of virus titers in tissue samples serial 10-fold dilutions of tissue homogenates were performed before inoculation into MDBK cells.

DNA extraction and PCR

Brain (0.5 g) or TG (0.2 g) was homogenised in MEM (10% w/v) and centrifuged for 20 min at 1500 *g* at 4 °C. Supernatants (500 mL) were placed in 1 mL lysis buffer (10 mM Tris–HCl pH 7.4, 25 mM EDTA pH 8, 100 mM NaCl, 0.5% SDS and proteinase K (20 mg/mL) [Promega]) and digested overnight at 37 °C. Total DNA was extracted by the phenol–chloroform method (Sambrook and Russel, 2006). DNA concentration was measured by absorbance at 260 nm (Nanodrop 2000, Thermo Scientific). Detection of BoHV-1 DNA was performed by nested PCRs (Wang et al., 2001; Campos et al., 2009). Tissues from mock infected animals and uninfected MDBK cells were included as negative controls. PCR products were subjected to electrophoresis through 1.2% agarose gels stained with SYBR Safe (Life Technology) and visualised under ultraviolet light.

Histopathology

Tissue samples were fixed in 10% neutral buffered formalin, paraffin-embedded and stained with hematoxylin and eosin (Luna, 1968).

Results

Clinical and serological findings

Calves inoculated with a high dose inoculum were euthanased at 6 dpi (BoHV-1.Cooper, $n = 2$) or 7 dpi (BoHV-1.LA, $n = 2$). The period of primary infection was too short to evaluate seroconversion. For latently infected calves that received DEX treatment, seroconversion was detected at 19 and 22 dpi, for BoHV-1.LA ($n = 2$) and BoHV-1.Cooper ($n = 2$), respectively. Dramatic changes in antibody titers were not observed after DEX administration, particularly in calves inoculated with BoHV-1.LA. The highest neutralising antibody titer after inoculation with any of the BoHV-1 strains was 1:64, which was detectable at the second and third day following initiation of DEX treatment for BoHV-1.Cooper ($n = 2$)- and BoHV-1.LA ($n = 2$) infected calves, respectively.

During the primary acute infection with BoHV-1.LA, nasal secretions were observed at 4 and 6 dpi. Nasal secretions were also evident in both calves inoculated with a low viral dose of BoHV-1.LA, from 4 to 22 dpi. After DEX-induced reactivation, nasal secretions were not observed. Ocular secretions were intermittently detected during acute infection, latency and reactivation. For calves inoculated with a high dose of BoHV-1.Cooper, nasal secretions were detected at 1 and 3–5 dpi. In those given a low dose-inoculum, nasal secretions were observed at 9 and 11 dpi and at 23 and 24 dpi after DEX administration (20 dpi). Ocular secretions were evident during primary infection but not during latency and reactivation.

The highest values for rectal temperatures (39.5 °C–40.5 °C) were recorded at 4–6 dpi for animals inoculated with a high inoculum dose of BoHV-1.LA. Rectal temperatures for mock infected calves varied from 37.8 °C to 38.5 °C during the same period. After initiation of DEX administration, rectal temperatures were in the range

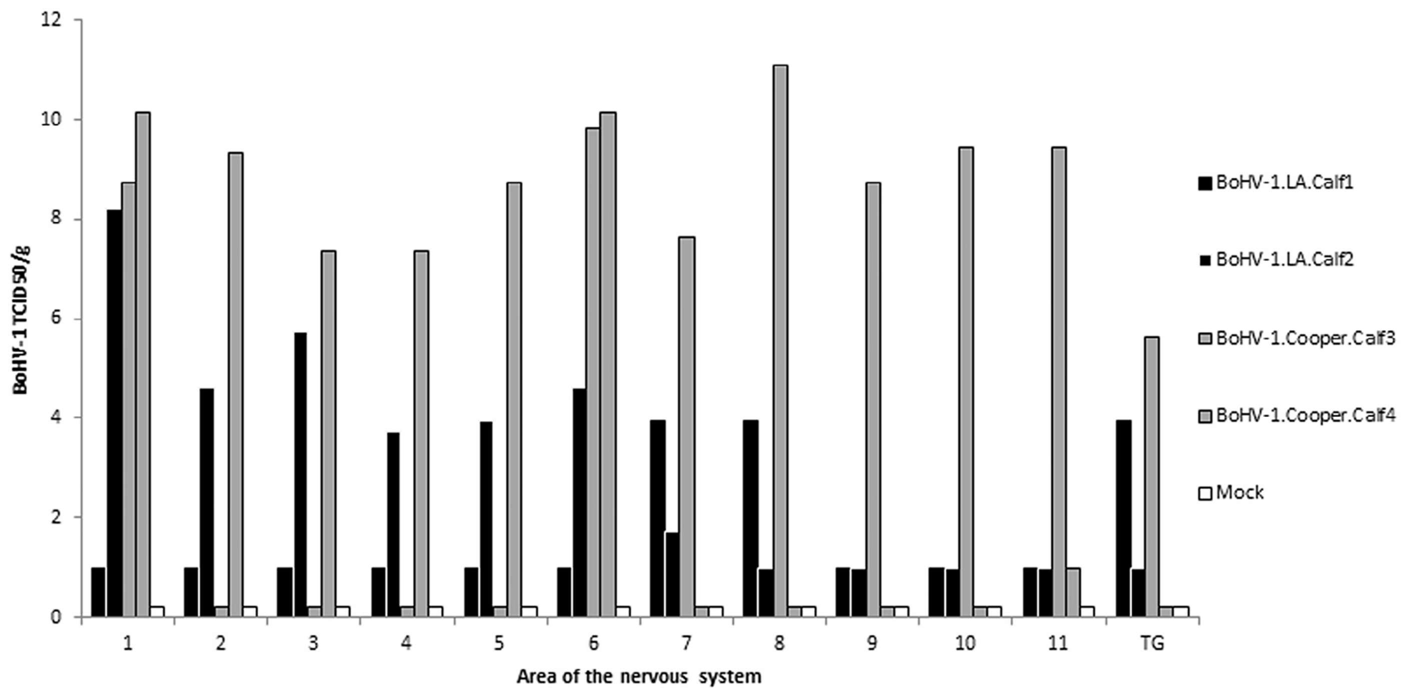

Fig. 1. Titers of BoHV-1 Los Angeles (BoHV-1.LA) and Cooper (BoHV-1.Cooper) strains in the brain and trigeminal ganglion of cattle experimentally inoculated with a high dose (primary acute infection). Results for mock infected calves are represented with a value of 0.2 in order to show the bars. The following areas were evaluated: 1, olfactory cortex; 2–3, frontal cortex; 4, dorso-lateral cortex; 5, marginal groove area of posterior cortex; 6, ectomarginal groove area of posterior cortex; 7, cerebellum; 8, cervical medulla; 9, medulla oblongata; 10, pons; 11, diencephalon. TG, trigeminal ganglion.

of 38.8 °C–39 °C. Rectal temperatures in BoHV-1.Cooper acutely infected animals varied between 39.2 °C and 40.3 °C, and for mock infected animals the values were between 39 °C and 39.4 °C. In calves inoculated with BoHV-1.Cooper that received DEX, the highest rectal temperatures were detected at 24 and 25 dpi (39.3 °C) and were similar to those recorded for mock infected calves. We saw no neurological or any other clinical signs.

Isolation of BoHV-1 from nasal and ocular secretions

BoHV-1.LA was isolated from nasal secretions of both acutely infected calves from 4 to 6 dpi. Virus titers ranged from 1.87 to 3.1 $TCID_{50}$/mL. After inoculation with a low inoculum dose, BoHV-1.LA was isolated in nasal secretions at 6 and 11 dpi and then at day 4 after finishing DEX administration (1.87 $TCID_{50}$/mL for each timepoint). BoHV-1.LA was not isolated from ocular secretions. BoHV-1.Cooper was isolated from nasal secretions of calves inoculated with a high inoculum dose from 1 to 6 dpi (titers from 4.4 to 6.8 $TCID_{50}$/mL). Unlike BoHV-1.LA, at 5 and 6 dpi, BoHV-1.Cooper was also isolated from ocular secretions (4.87 and 5.1 $TCID_{50}$/mL). In calves inoculated with a low dose of BoHV-1.Cooper, the virus was intermittently isolated from nasal secretions from 5 to 11 dpi (titers from 6.07 to 7.5 $TCID_{50}$/mL). At the time of the second IM dose of DEX, the virus titer in nasal secretion was 1.97 $TCID_{50}$/mL and at 2 dpr, the virus was detected in nasal and ocular secretions at 1.73 $TCID_{50}$/mL. BoHV-1 was not detected in swabs from mock infected cattle.

Isolation of BoHV-1 from neural tissue

BoHV-1 was isolated from all neural tissue sections (1–11) of acutely infected calves. In some samples from BoHV-1.LA infected cattle, the amount of virus was too low to determine the virus titer. During acute infection of BoHV-1.LA-inoculated animals, titers in the nervous system were 5–8 log lower than in BoHV-1.Cooper infected calves. The highest titers for both strains were observed in the olfactory cortex, frontal cortex and the ectomarginal groove area. High titers were also detected in the cervical medulla, pons and diencephalon of BoHV-1.Cooper infected animals (Fig. 1).

After DEX-induced reactivation, BoHV-1 was not isolated from the brain or TG of any of the infected calves. Virus was not isolated from mock infected animals. All samples in which CPE was observed were positive for BoHV-1 by direct immunofluorescence.

PCR

During acute infection of calves inoculated with BoHV-1.LA, viral DNA was detected in the olfactory, frontal, dorso-lateral and posterior cortex, cerebellum, cervical medulla and TG. At 6 dpi, BoHV-1.Cooper DNA was detected in the frontal, dorso-lateral and posterior cortex, cervical medulla, medulla oblongata and TG. At 2 dpr, the olfactory, frontal and posterior cortex, cerebellum, medulla oblongata, diencephalon and TG contained BoHV-1.Cooper DNA. During reactivation of BoHV-1.LA, DNA was consistently detected in olfactory and frontal brain sections and TG. Virus genome was not present in samples from uninfected calves or uninfected MDBK cells (Table 2).

Macroscopic and microscopic findings in neural tissue sections

Macroscopic brain lesions were not observed. Microscopic observation of neural tissue from BoHV-1.LA-acutely infected calves revealed focal hemorrhages in all brain areas and mild non-suppurative meningitis. Mononuclear perivascular cuffing (Fig. 2A) and focal gliosis were present in the anterior and posterior cortex and medulla oblongata. Diffuse gliosis was evident in the anterior and posterior cortex and areas of neuronophagia and satellitosis were detected in the anterior and posterior cortex, base of the brain and diencephalon (Fig. 2B). Microscopic lesions were not observed in TG. At 6 dpr, histologically evident lesions were mild and consisted of perivascular cuffing, gliosis and neuronophagia in brain cortex and TG.

In BoHV-1.Cooper infected calves, mild hemorrhages were present in the dorso-lateral cortex and medulla oblongata. Abundant mononuclear infiltrates, neuronophagia and satellitosis were observed in

Table 2
PCR detection of BoHV-1 DNA in neural tissue samples.

Strain	Calf ID	Infection	Neural tissue samples											
			1	2	3	4	5	6	7	8	9	10	11	TG
BoHV-1.LA	1	Acute	+	+	+	–	–	–	+	+	–	–	–	+
	2		+	+	+	+	+	+	+	–	–	–	–	+
BoHV-1.Cooper	3		–	–	–	–	–	–	–	+	+	–	–	+
	4		–	+	+	+	–	+	–	+	+	–	–	+
BoHV-1.LA	5	Reactivation	–	+	+	+	–	–	–	–	–	–	–	+
	6		+	+	–	+	–	–	–	–	–	–	–	+
BoHV-1.Cooper	7		–	+	–	–	–	–	–	+	–	–	–	+
	8		+	+	–	–	+	+	+	–	+	–	+	+
Mock	9		–	–	–	–	–	–	–	–	–	–	–	–
	10		–	–	–	–	–	–	–	–	–	–	–	–
	11		–	–	–	–	–	–	–	–	–	–	–	–
	12		–	–	–	–	–	–	–	–	–	–	–	–

Samples from acutely infected-calves were collected at 6 days post-infection (dpi) (BoHV-1.Cooper) or 7 dpi (BoHV-1.LA). For reactivation studies, samples were collected at 2 or 5 days after finishing DEX treatment for BoHV-1.Cooper and BoHV-1.LA, respectively.
1, olfactory cortex; 2–3, frontal cortex; 4, dorso-lateral cortex; 5, marginal groove area of posterior cortex; 6, ectomarginal groove area of posterior cortex; 7, cerebellum; 8, cervical medulla; 9, medulla oblongata, 10, pons; 11, diencephalon. TG, trigeminal ganglion.

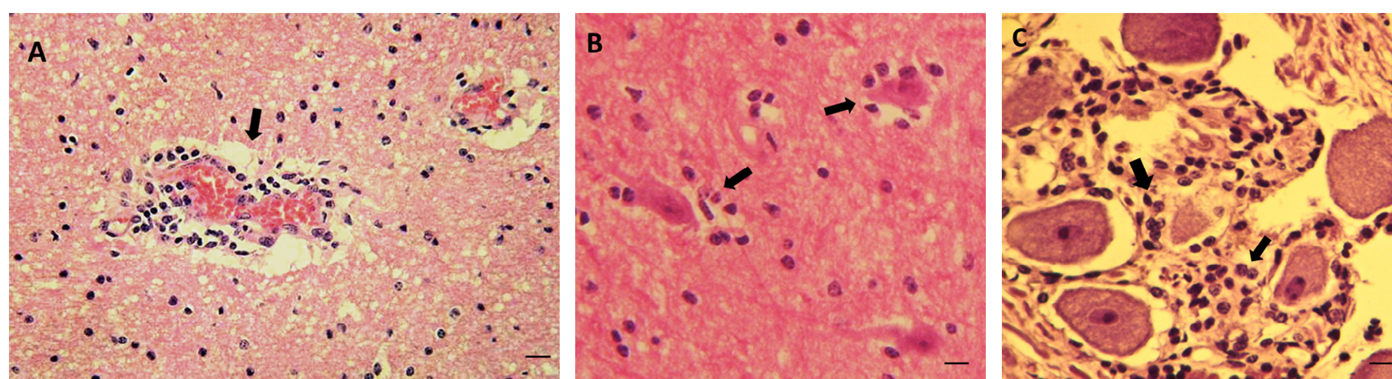

Fig. 2. Microscopic lesions in the brain of BoHV-1 infected calves. (A) Perivascular cuffing in the brain cortex of BoHV-1.LA infected calf euthanased at 7 days post-infection (dpi). (B) Satellitosis in the brain cortex of BoHV-1.LA infected calf euthanased at 7 dpi. (C) Neuronophagia in the trigeminal ganglion of BoHV-1.Cooper infected calf euthanased at 6 dpi (stain, hematoxylin and eosin; scale bar: 5 µm).

the TG (Fig. 2C) and medulla oblongata. At 48 h after DEX-induced reactivation, areas of neuronophagia were present in the frontal cortex. Microscopic lesions were not present in mock infected calves.

Discussion

Although neurological disease associated with BoHV-1 has occasionally been documented (Silva et al., 2007; Rissi et al., 2008), it is considered that BoHV-5 is responsible for typical herpesvirus encephalitis in cattle (Pérez et al. 2002). It is widely accepted that BoHV-1 strains can only replicate focally within the CNS without further spread (Belknap et al., 1994). However, Silva et al. (2007) and Rissi et al. (2008) isolated BoHV-1 from several cases of encephalitis in cattle from southern Brazil. Similar to the findings of the present study, in both reports of natural infections, macroscopic brain lesions were not observed and microscopic lesions were detected in only a few cases.

In the present study, data were collected from two different experiments and direct comparisons between these investigations should therefore be made with caution. However, inoculation of calves with two respiratory strains of BoHV-1 demonstrated that the virus was capable of replicating within the brain, independent of the experimental conditions.

Despite the low number of calves involved in each experimental group, evidence that the virus was detected and/or isolated from several regions of the nervous system during the acute infection and reactivation was consistent. Clinical manifestation of disease was not observed, but it is possible that a longer period is required for calves to show neurological signs. Alternatively, infection of neural tissue by BoHV-1 may not always lead to clinical neurological disease.

Virological and histological data from this study demonstrated that both strains of BoHV-1 tested were able to reach and replicate within the nervous system of calves and induce lesions compatible with encephalitis. Silva et al. (2007) suggested that some BoHV-1 isolates might show an increased ability to replicate in neural tissues and cause neurological disease. Moreover, sporadic cases of encephalitis associated with BoHV-1 have been attributed to individual host susceptibility (Muylkens et al., 2007). It is likely that both factors are involved in the development of neurological disease. In our study, we show that respiratory BoHV-1 strains have neuroinvasive potential. Dix et al. (1983) postulated that viral spread is an important property of the virus to induce acute neurological disease.

Slight differences in the sites of replication in neural tissue and virulence of BoHV-1 strains were evident in this study. The amount of infectious virus in the brain does not reflect the progress of disease directly and the neuroinvasive properties of encephalitic strains are not a function of the rate of replication (Bergström and Lycke, 1990). BoHV-1.LA replicated at lower levels in the brain when compared with BoHV-1.Cooper. However, it induced evident microscopic lesions compatible with encephalitis. Similarly, BoHV-5 grows poorly in cell culture, especially when attempting isolation

from brain samples (Schudel et al., 1986; Pérez et al., 2002). Nonetheless, it is the causal agent of severe cases of necrotising meningoencephalitis in calves.

Our results provide additional evidence to demonstrate that the level of replication of a virus does not always reflect its potential to induce clinical disease. Likewise, BoHV-5 infected cattle do not usually show obvious histological changes of encephalitis at the early onset of disease (Dezengrini et al., 2009). Further studies will be necessary to identify the factors that lead to high levels of virus replication in the brain without causing evident pathological alterations or clinical signs of disease.

Yao et al. (2012) demonstrated that herpes simplex virus type 1 (HSV-1) reactivates from explanted brain and several viral genes appear to contribute to its neurovirulence. During reactivation in the present study, BoHV-l DNA was detected not only in trigeminal ganglia but also within the brain. However, infectious virus was not detected, most likely due to low levels of virus replication at this stage of the alpha-herpesvirus life cycle.

Changes in the distribution of BoHV-1 DNA within the CNS were not detected during reactivation. In contrast, after DEX-induced reactivation of latent BoHV-5, a broader distribution of viral DNA was observed in the brain and slight neurological signs were present (Mayer et al., 2006). The findings on restricted BoHV-1 distribution after a reactivating stimulus are consistent with subclinical BoHV-1 reactivation. Although genome distribution in the brain was restricted, it is important to note that BoHV-1 DNA was also detected in neural sites other than the trigeminal ganglion after DEX-induced reactivation.

It is likely that immune mechanisms at the site of primary replication or at the first stages of replication within the brain play a role in determining the rate of replication and dissemination of BoHV-1 in neural tissue. Further research will be required to understand the mechanism/s that modulate the neuroinvasiveness of BoHV-1 strains.

Conclusions

BoHV-1 does not frequently induce neurological signs. However, it is easily isolated from the brain even when clinical manifestations of encephalitis, macroscopic and histological lesions, are not evident. In this study it was shown that respiratory BoHV-1.1 strains were capable of reaching and disseminating within the CNS of cattle after intranasal inoculation, the most likely route of natural infection.

Conflict of interest statement

Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), which funded the study, played no role in the study design nor in the collection, analysis and interpretation of data, nor in the decision to submit the manuscript for publication. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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