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Latency of bovine herpesvirus type 5 (BoHV-5) in tonsils and peripheral blood leukocytes

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ABSTRACT

Bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) can both establish latency in the trigeminal ganglion. Non-neural sites of latency have been described for BoHV-1 but not for BoHV-5. The aim of this study was to determine whether peripheral blood leukocytes and tonsils are targets for BoHV-5 infection and to establish whether all stages of that virus's infectious cycle can occur in those cell types. Comparisons with BoHV-1 infection of these tissues were also made in order to better understand the pathogenesis of both viruses.

BoHV-1 and BoHV-5 were isolated from tonsils of acutely-infected calves. BoHV-5 was also isolated from a tonsil homogenate after dexamethasone-induced reactivation. During latency, infectious virus was recovered from a tonsil explant of one BoHV-5-infected calf. The genomes of BoHV-5 and BoHV-1 were detected in tonsils from acutely-infected calves although were not detected in tonsils from latently-infected calves or from calves treated with dexamethasone. Virus DNA was intermittently detected in leukocytes.

The study has shown that BoHV-5 can establish latency in bovine tonsils and peripheral white blood cells, and that it can be reactivated from latently-infected tonsils, which might contribute to viral transmission. The titres of BoHV-1 and BoHV-5 in tonsils were similar, suggesting that replication at this site is a common feature for both viruses.

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Introduction

Bovine herpesvirus type 1 (BoHV-1) is an alpha-herpesvirus which causes several syndromes in cattle, including respiratory disease, abortions and genital disorders. Acute infection of the respiratory tract by BoHV-1 causes immunosuppression, leading to secondary bacterial infections, pneumonia and death (Tikoo et al., 1995). Another closely-related alpha-herpesvirus, bovine herpesvirus type 5 (BoHV-5), is the causal agent of non suppurative meningoencephalitis in calves (Pérez et al., 2002), a condition which is highly prevalent in South America, particularly Argentina and Brazil. Meningoencephalitis in calves is usually fatal (Rissi et al., 2008). However, mild to moderate (Del Médico Zajac et al., 2010) or subclinical (Cascio et al., 1999) BoHV-5 infections have been described. Additionally, BoHV-5 has recently been associated with genital infection (Kirkland et al., 2009), demonstrating that cell types other than neural tissue might be targeted during infection.

Infection by alpha-herpesviruses is characterized by acute infection, latency and reactivation cycles. The trigeminal ganglion is the main site of latency for both alpha-herpesviruses. Latent herpesvirus infections can be reactivated by natural or glucocorticoid-induced stress and virus shedding during reactivation is responsible for virus transmission (Rock et al., 1992). In contrast to the subclinical reactivation observed in most herpesvirus infections, BoHV-5 reactivation is frequently accompanied by mild clinical signs (Caron et al., 2002; Pérez et al., 2002).

Latency of BoHV-1 in peripheral blood leukocytes (PBLs) (Mweene et al., 1996; Wang et al., 2001), spleen (Mweene et al., 1996), tonsils (Winkler et al., 2000; Pérez et al., 2005), inguinal and sacral lymph nodes (Vogel et al., 2004) has been demonstrated. Unlike BoHV-1, BoHV-5 can establish latency in several areas of the central nervous system (Vogel et al., 2003) although non-neural sites of latency have not been reported for BoHV-5 infection.

Tonsils are lympho-epithelial, immunocompetent tissues, located in strategic anatomical areas of the oral-pharynx and naso-pharynx

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and are the portal of entry for many viruses (Griebel et al., 1990). Winkler et al. (2000) and Pérez et al. (2005) demonstrated that BoHV-1 can infect and persist in the tonsils. In contrast, there is no information available regarding BoHV-5 infection of bovine tonsils and PBLs.

The objectives of the present study were to determine whether PBLs and tonsils were targets for BoHV-5 infection and to establish whether all stages of the virus infectious cycle (primary acute infection, latency and reactivation) could take place in these cell types. In addition, comparisons were made with the infection of lymphoid cells by BoHV-1.

Materials and methods

Viruses

Cooper (BoHV-1) and 97–613 (BoHV-5) strains were used for animal inoculations. BoHV-5 97–613 was isolated from the brain of a 2-year-old cow with neurological signs (Pérez et al., 2002). Titres of virus stocks were determined by the end-point titration method and expressed as $TCID_{50}$/mL (Reed and Müench, 1938).

Cell cultures

For viral stocks, virus isolation and in vitro tests, Madin–Darby bovine kidney (MDBK) cells grown in minimum essential medium (MEM, Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (Internegocios), L-glutamine (10 µL/mL) (L-glutamine Sigma-Aldrich), penicillin (100 µg/L) (penicillin G sodium salt, Sigma-Aldrich) and streptomycin (200 µg/L) (streptomycin sulfate, Sigma-Aldrich) were used. Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Experimental design

All procedures for animal handling and experimentation were performed according to the Animal Welfare Committee of the University of the Center of Buenos Aires Province (Res. 087/02). BoHV-1- and BoHV-5-free and seronegative crossbred, 1-year-old calves were used. Calves were randomly assigned to one of four groups. In group 1 (primary acute infection; $n = 4$) two calves were intranasally inoculated with a high dose ($10^{6.3}$ $TCID_{50}$ in 10 mL) of BoHV-1 and the other two with $10^{6.3}$ $TCID_{50}$ (in 10 mL) of BoHV-5. These calves were euthanased at 6 days post-infection (dpi).

Group 2 (latency; $n = 4$) contained two calves that were intranasally inoculated with a low dose (10^3 $TCID_{50}$ in 10 mL) of BoHV-1 and two with 10^3 $TCID_{50}$ (in 10 mL) of BoHV-5. Calves in this group were euthanased at 24 dpi. In group 3 (reactivation; $n = 4$), two calves were intranasally inoculated with 10^3 $TCID_{50}$ (in 10 mL) of BoHV-1 and two with 10^3 $TCID_{50}$ (in 10 mL) of BoHV-5. At 20 dpi they received an intravenous dose of 0.1 mg/kg bodyweight dexamethasone (Dexametona, Schering Plough) followed by two intramuscular doses 24 and 48 h later (Inman et al., 2002). Calves in this group were euthanased at 25 dpi, 2 days after finishing dexamethasone treatment. Group 4 (mock-infected; $n = 2$) calves were intranasally inoculated with 10 mL MEM as placebo. One calf was euthanased at 6 dpi and the other treated with dexamethasone using the same regime as the calves in group 3. This calf was euthanased at 25 dpi.

Preparation of PBLs

PBLs were separated from 10 mL of unclotted blood (anticoagulant: sodium citrate/EDTA, pH 6.5). Blood samples were obtained from all inoculated groups and mock-infected calves at 6 dpi. Additional blood samples were obtained at 9, 13, 20 and 24 or 25 dpi from calves in groups 2 and 3 and from one mock-infected calf in group 4. Blood samples were centrifuged at 2000 *g* for 15 min and the buffy coat was transferred to tubes containing 10 mL of cold ammonium chloride. The cell pellet obtained after centrifugation at 1000 *g* for 7 min at 4 °C was re-suspended in 1 mL of phosphate-buffered saline solution (PBS) and centrifuged at 10,000 *g* for 2 min. The supernatant was discarded and the cell pellet was stored at –20 °C.

Virus isolation from tonsil homogenates and explants

Tonsil homogenates were prepared as a 10% solution in MEM with antibiotics, namely, penicillin (200 µg/L; penicillin G sodium salt, Sigma-Aldrich) and streptomycin (400 µg/L; streptomycin sulfate, Sigma-Aldrich). Homogenates were centrifuged at 1,000 *g* for 30 min at 4 °C. One hundred microlitres of supernatant was inoculated into MDBK cells in 24-well plates in duplicate. Cultures were observed daily for cytopathic effect (CPE) and the supernatants were passaged every 3 days, for a total of three passages.

Explants were prepared from the tonsils of latently-infected calves (collected at 24 dpi) as well as from latently-infected and mock-infected calves that received dexamethasone treatment (collected at 25 dpi). To prepare the explants, tonsils were minced into 1 mm^3 pieces, washed in PBS and two to three pieces were inoculated into six-well plates. Explants were overlaid with 3 mL MEM with antibiotics (penicillin and streptomycin, as indicated for tonsil homogenates) and 10% FCS. MDBK cells were used as indicator cells for co-cultivation. The culture medium was replaced every 3–4 days, and cultures were observed daily for CPE. Samples were evaluated by direct immunofluorescence using an anti-BoHV polyclonal antibody (VMRD). Virus titres in the supernatants from tonsil homogenates were determined by the end-point titration method, as previously described.

DNA extraction and PCR from PBLs and tonsils

DNA extraction from white blood cell pellets and tonsils was performed by the phenol-chloroform method. Nested PCRs for BoHV-1 and BoHV-5 as described by Wang et al. (2001) and Mayer et al. (2006), respectively, were used for genome amplification. PCR products were run in 1.2% agarose gels stained with SYBRsafe (Life Technology) and visualized under ultraviolet light.

Histopathology

At necropsy, tonsils were collected from each calf, placed in 10% neutral buffered-formalin and processed for histopathology. Sections were cut at 5 µm and stained with haematoxylin–eosin.

Statistical analysis

Virus titres were analyzed under a completely randomized design by ANOVA with repeated measurement (R Core Team, 2013). The experimental unit was the calf. Data are presented as least-squares means ± standard error of the mean.

Results

Primary (acute) infection, latency and reactivation of experimentally-inoculated calves

Virus shedding in nasal and/or ocular secretions was detected until 6 dpi (time of euthanasia) for calves in group 1. Calves in groups 2 and 3 shed virus until 9 dpi. At 24 dpi, BoHV-5-inoculated calves in group 2 (latency) had neutralizing antibody titres of 1:128 (calf 7) and 1:256 (calf 10). For BoHV-1, antibody titres were 1:8 and 1:128 (calves 12 and 13, respectively). Viral DNA was detected by PCR in the trigeminal ganglion of all calves. For calves in group 3, virus re-excretion was detected after dexamethasone administration. Control calves remained seronegative during the experiment and virus shedding was not detected.

Pathological findings

Gross lesions were not observed in mock-infected calves (Fig. 1A). In BoHV-5-acutely-infected calves (group 1), haemorrhages and petechiae were observed in retro-pharyngeal lymph nodes and tonsils, respectively. Retro-pharyngeal lymph node haemorrhages were observed at necropsy of calves inoculated with a high dose inoculum of BoHV-1. Macroscopic lesions in the tonsils of these calves were not seen (Figs. 1B–E). Similarly, gross lesions in lymphoid tissues were not evident in animals in groups 2 and 3 (data not shown).

At 6 dpi, tonsils from BoHV-1- and BoHV-5-infected calves had hyperplastic lymphoid follicles, with large, pale germinal centers (Fig. 2A). Furthermore, BoHV-5-infected tonsils collected at the same time had a moderate number of cells infiltrating the tonsil epithelium (Fig. 2B). For both BoHV-1- and BoHV-5-inoculated calves, tonsils collected during latency or after dexamethasone-induced reactivation had no evident microscopic abnormalities or variable degree of enlargement of the germinal centers, either for BoHV-1- or BoHV-5-inoculated calves (Figs. 2C–F). Microscopic alterations were not detected in mock-infected tonsils (Fig. 2G).

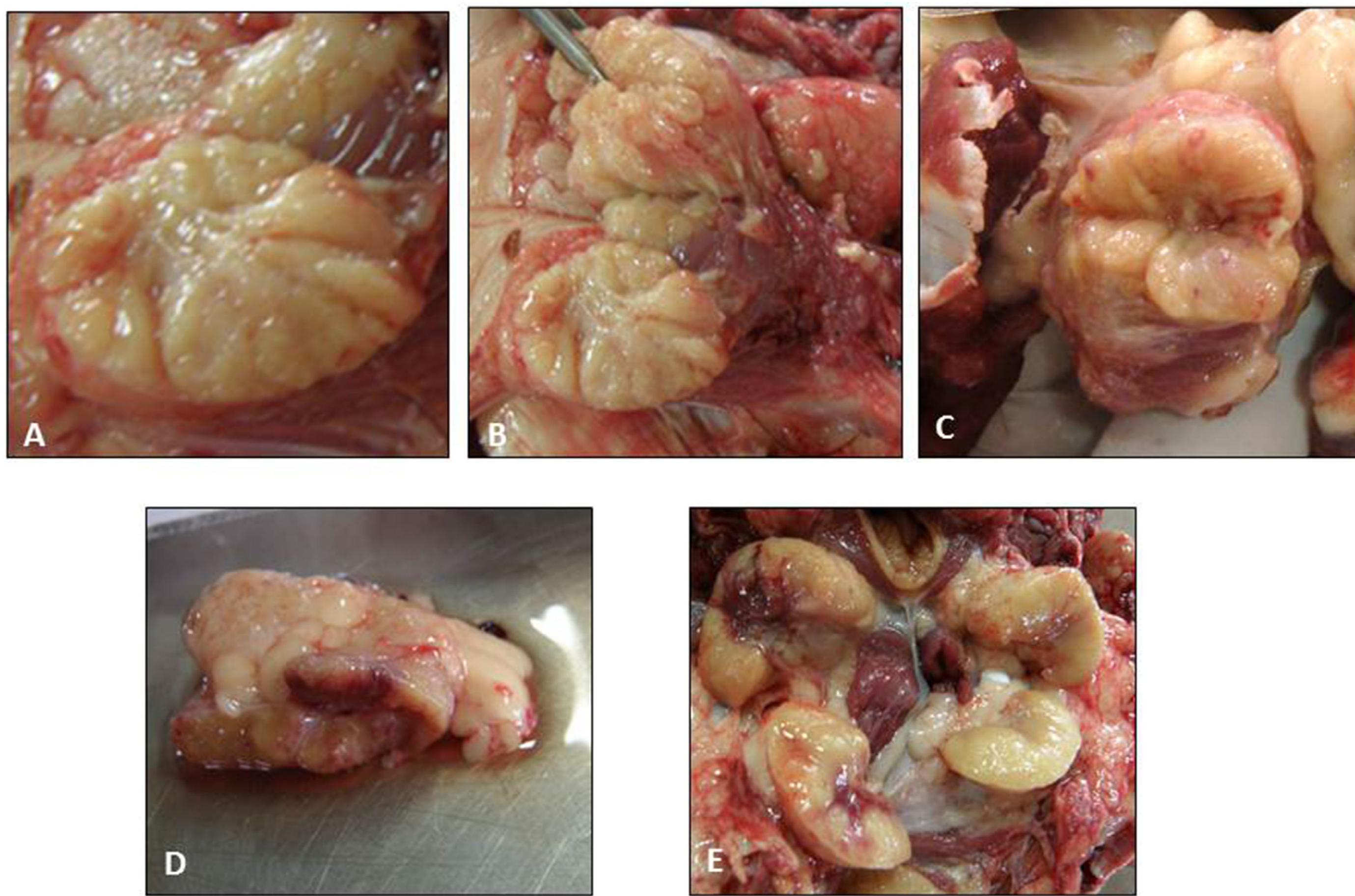

Fig. 1. Macroscopic findings in lymphoid tissues of calves inoculated with a high dose inoculum of BoHV-1 or BoHV-5 (6 days post infection). A: tonsil from mock-infected calf; B: tonsil from a BoHV-1 infected calf; C: presence of petechiae in the tonsil of a BoHV-5-infected calf; D and E: haemorrhages in the retro-pharyngeal lymph nodes of BoHV-1- and BoHV-5-infected calves, respectively.

Virus isolation from tonsil explants and homogenates

BoHV-1 and BoHV-5 were isolated from tonsil homogenates of calves that had been euthanased at 6 dpi. As expected, significant differences were detected ($P = 0.008$) in virus titres of infected tonsils compared to those harvested from mock-inoculated calves. However, differences between virus inoculated groups were not observed ($P = 0.87$) (Fig. 3A). At 24 dpi, infectious virus was not detected in the tonsils of either BoHV-1- or BoHV-5-inoculated calves in group 2. In group 3 calves, 48 h after finishing dexamethasone administration, BoHV-5 was isolated from the tonsil homogenate of one calf. However, significant differences were not detected ($P = 0.5$) in virus titres with respect to mock- or BoHV-1-inoculated calves (Fig. 3B).

After explantation, infectious virus was only recovered from the tonsil of one BoHV-5-latently-infected calf after 7 days of co-culture with MDBK cells. Infectious virus was not recovered from tonsil explants of BoHV-1- or mock-infected calves (Table 1).

Detection of BoHV-5 and BoHV-1 DNA in tonsils and PBLs

The genomes of BoHV-5 and BoHV-1 were detected in the tonsils of calves at 6 dpi. However, BoHV-5- or BoHV-1-PCR products were not detected in the tonsils collected from latently-infected calves or from calves that received dexamethasone or mock-infected calves (Figs. 4A, B, respectively).

The presence of BoHV-1 or BoHV-5 DNA was detected in PBLs of calves inoculated with a high virus dose (group 1). For BoHV-1-infected calves, viral DNA in PBLs was intermittently detected from 2–6 dpi. In one calf inoculated with a high dose inoculum of BoHV-5, virus DNA was detected at 1, 3 and 5 dpi. In the other calf, BoHV-5 was only detected at 2 dpi (Table 2). For calves inoculated with a low viral dose, the genome of BoHV-1 and BoHV-5 was intermittently detected in PBLs collected at different time-points during primary infection, latency and reactivation (Table 3), except for calf 9 (a latently infected calf that received dexamethasone treatment), in which BoHV-5 DNA was not detected in PBLs at any time during the first 6 dpi. However, the BoHV-5 genome was detected in the PBLs of this calf at 13 and 25 dpi. Furthermore, all experimentally inoculated calves had virus DNA in their PBLs by 20 dpi. BoHV-1 DNA was detected earlier (at 1 dpi), than BoHV-5. Viral DNA was not detected in PBLs from mock-infected calves.

Discussion

BoHV-1 and BoHV-5 were experimentally inoculated in calves of the same age, using inocula containing similar infectious doses of each virus. The animals were managed under identical field conditions, thus allowing comparisons to be made between infections of the tonsils by both alpha-herpesviruses.

Previous studies have demonstrated that BoHV-1 infects $CD4^+$ T cells in the tonsils and that this tissue is a site for virus persistence (Winkler et al., 1999, 2000). It has been shown that BoHV-1 present in the tonsils is reactivated after dexamethasone treatment (Winkler et al., 2000; Pérez et al., 2005) and it was

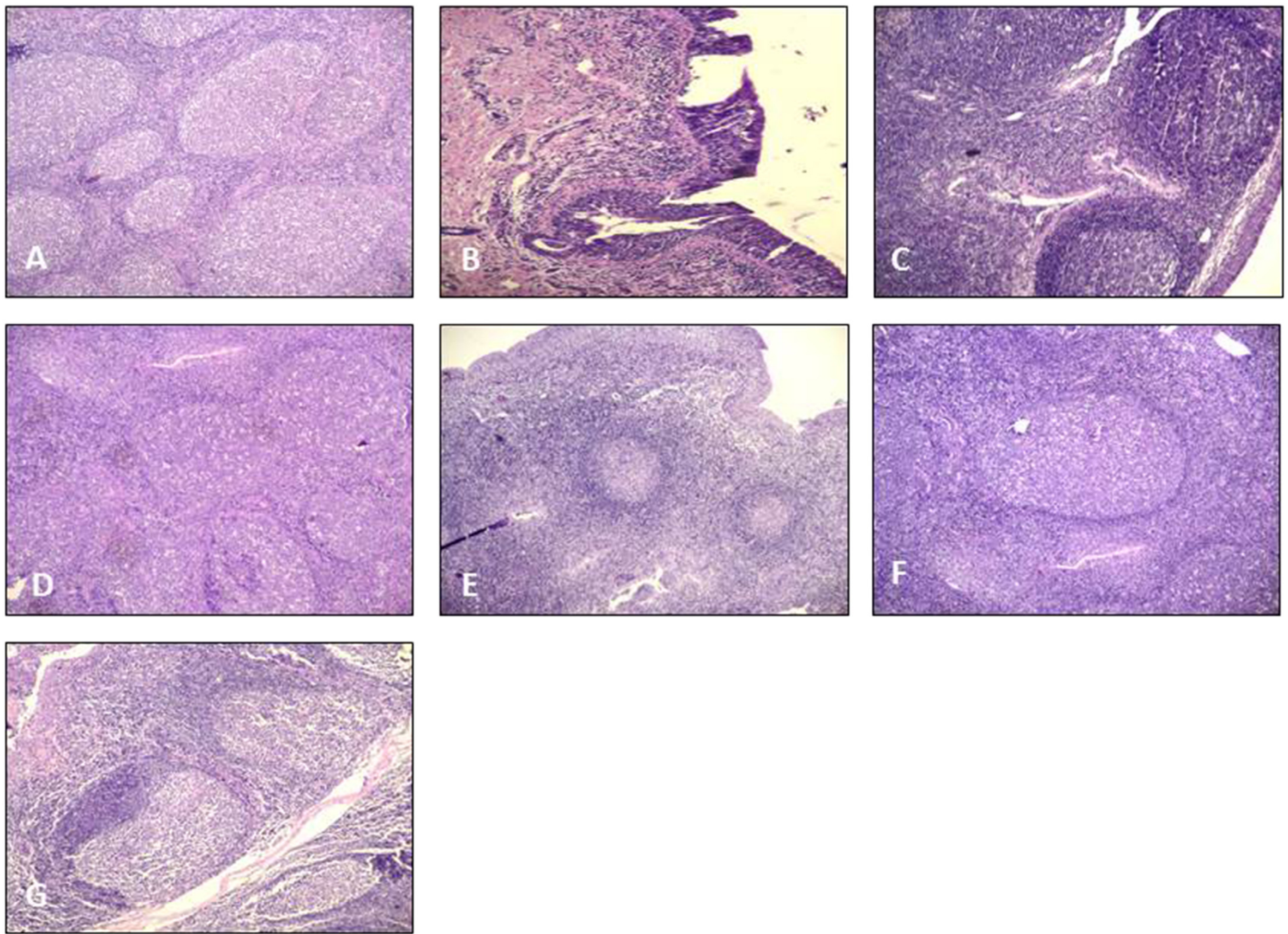

Fig. 2. Microscopic findings in the tonsils of calves inoculated with BoHV-1 or BoHV-5. A and B: tonsils from BoHV-1- and BoHV-5-infected calves, respectively, at 6 days post infection (dpi); C and D: tonsils from BoHV-1- and BoHV-5-infected calves, respectively, at 24 dpi; E and F: tonsils from BoHV-1- and BoHV-5-infected calves, respectively, 48 h after finishing dexamethasone treatment; G: tonsil from mock-infected calf. Magnification: A, C–G: 20×; B: 10×.

suggested that virus shedding from this tissue might contribute to virus transmission. Similarly, Roush et al. (2001) and Sahin et al. (2007) demonstrated that tonsils could be reservoirs for at least two human herpesviruses.

In the present study, the intranasal inoculation of calves with BoHV-5 demonstrated that tonsils are a site for acute virus replication, with BoHV-5 titres similar to those reached by BoHV-1. BoHV-1 and BoHV-5 DNA were not detected in the tonsils of latently-infected calves or calves that received dexamethasone treatment. By using a semi-quantitative PCR, Pérez et al. (2005) demonstrated that very low levels of BoHV-1 DNA were present in the tonsils of latently-infected calves, even when a high dose inoculum was used. It is likely that the low dose inocula used in our study to induce a latent state is responsible for the lack of detection of viral DNA in the tonsils during latency and reactivation, since in the tonsils of animals inoculated with a high viral dose, BoHV-1 and BoHV-5 DNA were readily detected.

BoHV-5 present in the tonsils of latently-infected calves was reactivated after dexamethasone administration resulting in an increased risk of viral spread as virus was present in nasal and ocular secretions 24 h after dexamethasone administration. In these calves virus excretion was intermittently observed until euthanasia at 25 dpi (data not shown). Reactivation of latent virus in the tonsils of BoHV-5-infected cattle would clearly contribute to virus transmission.

Explant-induced reactivation is an ex-vivo process, which is extensively used to show latency. The success of virus recovery from tissue explants is related to several factors, including the absence of the host immune response as a limiting factor for virus growth and spread and the stimulation of virus replication by cellular growth in the explant (Kalter et al., 1973). In the present study, BoHV-5 was recovered from the tonsil of one latently-infected calf when tissue explantation was performed, whereas BoHV-1 was not isolated. Winkler et al. (2000) showed that infectious BoHV-1 was present in the tonsils of calves 48 h after dexamethasone treatment in calves intranasally inoculated with a high dose inoculum (10^7 $TCID_{50}$). In our experiment, latency and reactivation studies were conducted after inoculation with a low viral dose (10^3 $TCID_{50}$). Under these conditions, only BoHV-5 was able to reactivate from the tonsils. This might reflect differences in the pathogenesis of both viruses during latency and reactivation, and might explain why BoHV-5 reactivation is usually accompanied by mild clinical signs (Caron et al., 2002; Pérez et al., 2002), whereas BoHV-1 reactivation remains subclinical (Kahrs, 1977).

Irrespective of the virus inoculum dose, peripheral blood cells were infected. Virus isolation from these cells was not attempted, since our previous experience and data from other authors (Nyaga and McKercher, 1979) demonstrate that herpesvirus isolation from leukocytes is not successful. Although monocytes support

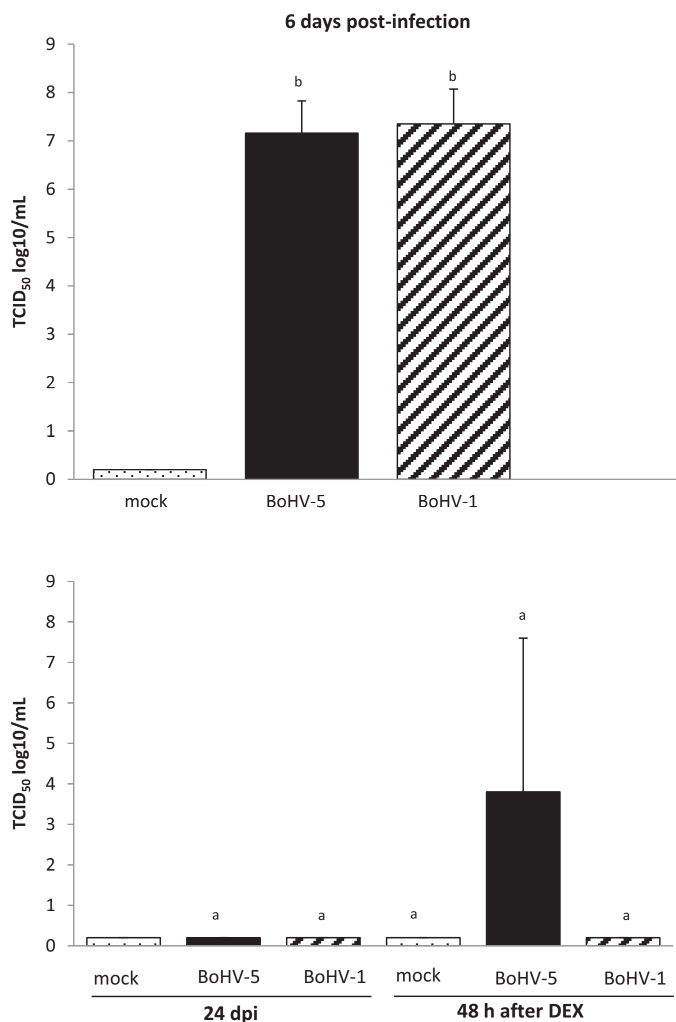

Fig. 3. Isolation and titration of BoHV-1 and BoHV-5 from tonsils of intranasally infected calves. A: virus isolation from tonsils of mock-infected and BoHV-1- or BoHV-5-inoculated calves collected at 6 days post infection (dpi); B: virus isolation from tonsils of mock-infected, BoHV-1- or BoHV-5-latently-infected calves (24 dpi) or from infected calves 48 h after finishing dexamethasone administration. Data represent the average for each group. Negative results are represented by a value of 0.2 in order to visualize the bars. Different letters indicate statistically significant differences between groups ($P < 0.05$).

BoHV-1 replication, they constitute only 5% of the leukocyte population. Lymphocytes allow virus replication only after phytohaemagglutinin stimulation. It is suggested that the small quantities of virus released by these cells would explain why the virus is not regularly isolated from bovine blood (Nyaga and McKercher, 1979).

Abortive infection of peripheral blood mononuclear cells has been demonstrated for herpes simplex virus-1 (Cantin et al., 1994) and BoHV-1 infections (Winkler et al., 2000; Pérez et al., 2005). Abortive infections might contribute to the establishment of latency allowing the infected cell to survive the effects of the immune system (Cantin et al., 1994). The non-productive infection of these cells might also be responsible for the failure to detect infectious virus when attempting virus isolation. In our study, the leukocyte type harbouring BoHV-1 or BoHV-5 DNA was not investigated but the finding that leukocytes are persistently or latently infected is an initial step in understanding the mechanisms of pathogenesis of both viruses. In particular, the ability to demonstrate the presence of herpesvirus genomes in peripheral blood leukocytes would be important for assessing the contribution of viremia to viral dissemination. It is generally assumed that viremia is not an important route for dissemination of herpesviruses in the organism, but systematic studies addressing this issue have not been conducted. In the present work, infection of leukocytes was an early event after virus inoculation via the intranasal route and viral DNA was consistently detected in these cells during latency and reactivation. The use of more sensitive techniques would be valuable to estimate more precisely the viral load in lymphoid cells.

The detection of BoHV-1 and BoHV-5 genomes in PBLs was intermittent, suggesting that viral load in these cells was low. However, the fact that PCR-positive leukocytes were detected when viral excretion in nasal and ocular swabs was no longer detected (data not shown), is indicative that these cells can harbour latent virus. Wang et al. (2001) detected BoHV-1 antigen and DNA in mononuclear cells for 6–8 months after infection.

In this study, BoHV-5 DNA (but not that of BoHV-1) was consistently detected in blood cells after dexamethasone administration. It has been suggested that the activity of corticosteroids on leukocytes may allow virus replication in these cells (Pusterla et al., 2010), so facilitating the detection of viral genome. The results of our study suggest that dexamethasone treatment might facilitate detection of BoHV-5 but not BoHV-1. The fact that BoHV-5 is detected in tonsil explants from latently-infected calves, in tonsil homogenates from calves that received dexamethasone and, at the same time, in peripheral white cells, is suggestive that reactivation of BoHV-5 is more efficient than BoHV-1-reactivation, even after the inoculation of a low dose of virus.

Conclusions

BoHV-5 establishes latency in bovine tonsils and PBLs. Furthermore, the virus reactivates from latently-infected tonsils. The production of infectious virus in tonsil tissue might contribute to virus shedding and transmission during acute infection and reactivation. BoHV-1 and BoHV-5 titres in the tonsils are similar suggesting that replication at this site is a common feature for both viruses. In this study, only BoHV-5 was reactivated from the tonsils, which might reflect differences in the pathogenic potential of each virus at this stage of infection. PBLs harbour viral genomes during latency and it is possible that infection of these cells might provide an additional way to gain access to the nervous system.

Table 1
Detection of infectious virus in explants and homogenates after induction of BoHV-1 and BoHV-5 latency or reactivation.

	Mock	BoHV-5				BoHV-1			
		Latently-infected	Latently-infected + DEX	Latently-infected + DEX	Latently-infected	Latently-infected + DEX	Latently-infected	Latently-infected	Latently-infected + DEX
Calf ID	6	7	8	9	10	11	12	13	14
Tonsil explant	–	+	–	–	–	–	–	–	–
Tonsil homogenate	–	–	–	+	–	–	–	–	–

Latently infected calves were euthanased at 24 days post infection (dpi). Dexamethasone-treated calves (DEX) received the first dexamethasone dose at 21 dpi and were euthanased at 25 dpi (48 h after finishing dexamethasone treatment). –/+ indicate presence or absence of infectious virus, respectively.

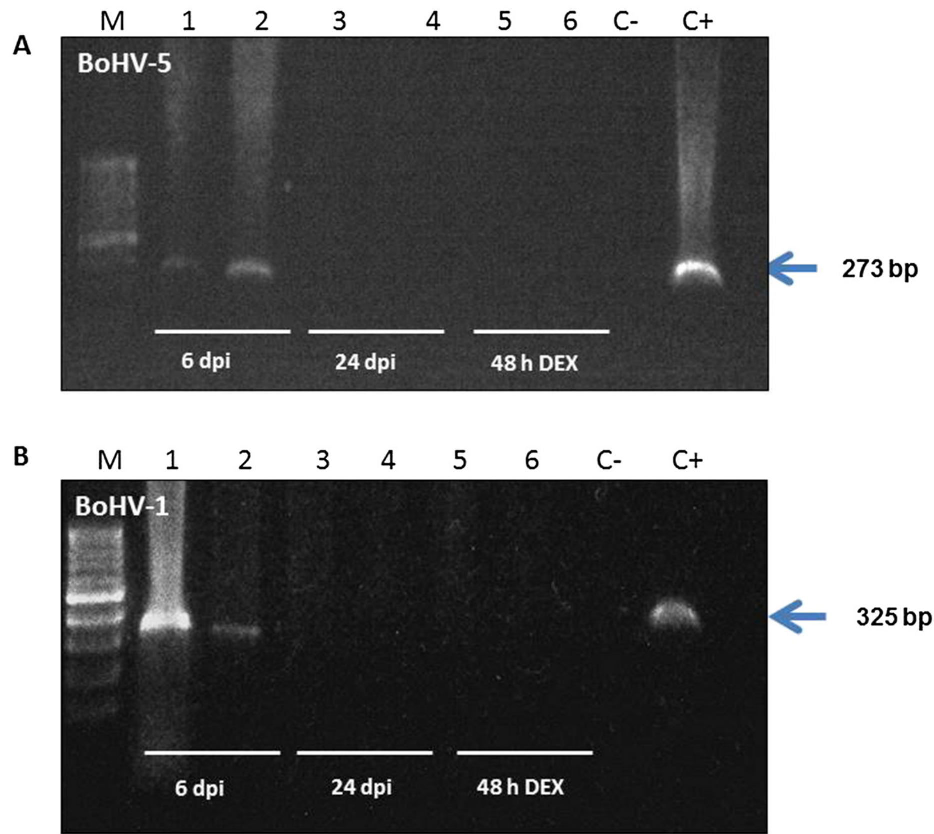

Fig. 4. Detection of BoHV-1 and BoHV-5 genome in tonsils of intranasally-inoculated calves. A: nested PCR for detection of the gB gene of BoHV-5 in tonsils obtained at 6 days post infection (dpi) (primary acute infection), 24 dpi (latency) and 48 h after finishing dexamethasone treatment (reactivation). Arrow indicates the 273 bp PCR product. B: nested PCR for detection of the gD gene of BoHV-1 in tonsils obtained at 6 dpi (primary acute infection), 24 dpi (latency) and 48 h after finishing dexamethasone treatment (reactivation). Arrow indicates the 325 bp PCR product.

Table 2
BoHV-1 and BoHV-5 genome detection in PBLs from calves inoculated with a high viral dose.

		Group 1($10^{6.3}$ $TCID_{50}$) – primary acute infection (dpi)						
Virus	Calf ID	0	1	2	3	4	5	6
BoHV-5	2	–	–	+	–	–	–	–
	3	–	+	–	+	–	+	–
BoHV-1	4	–	–	+	+	–	–	+
	5	–	–	–	+	+	+	–
Mock	1	–	–	–	–	–	–	–

Calves in group 1 were euthanased at 6 dpi. –/+ indicate negative or positive virus genome detection in PBLs, respectively.

Conflict of interest statement

Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) who 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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Table 3
BoHV-1 and BoHV-5 genome in PBLs of calves experimentally inoculated with a low virus dose.

		Groups 2 and 3 (10^3 $TCID_{50}$)										
		Primary infection (dpi)							Latency and reactivation (dpi)			
Virus	Calf ID	0	1	2	3	4	5	6	13	20	24	25
BoHV-5	7	–	–	+	–	+	+	–	–	–	+	
	8	–	–	+	–	–	–	–	+	+		+
	9	–	–	–	–	–	–	–	+	–		+
	10	–	–	+	–	–	–	–	–	+	+	
BoHV-1	11	–	–	+	–	–	+	+	–	+		–
	12	–	–	+	–	–	–	–	–	+	+	
	13	–	+	+	–	+	–	–	+	+	–	
	14	–	+	+	–	+	–	–	–	–		–
Mock	6	–	–	–	–	–	–	–	–	–	–	–

Dexamethasone administration for calves in group 3 was initiated on 21 days post infection (dpi). Calves in groups 2 (latency) and 3 (reactivation) were euthanased at 24 and 25 dpi, respectively. –/+ indicate negative or positive virus genome detection in PBLs, respectively.

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