Research in Veterinary Science 107 (2016) 1–7

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Involvement of toll-like receptors 3 and 7/8 in the neuropathogenesis of bovine herpesvirus types 1 and 5

Daniel Rensetti [a], Maia Marin [b,d], Silvina Quintana [c], Pedro Morán [a], Andrea Verna [b,d], Anselmo Odeón [d], Sandra Pérez [e,*]

[a] Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Paraje Arroyo Seco S/N, Tandil (7000), Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Rivadavia 1917, C1033AAJ Buenos Aires, Argentina
[c] Instituto de Análisis Fares Taie, Rivadavia 3331, (7600) Mar del Plata, Buenos Aires, Argentina
[d] Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria Balcarce, Ruta 226 Km 73.5 (7620), Balcarce, Buenos Aires, Argentina
[e] 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

ARTICLE INFO

Article history:
Received 15 January 2016
Received in revised form 8 April 2016
Accepted 29 April 2016
Available online xxxx

Keywords:
Bovine herpesvirus
Toll-like receptors
Neuropathology
Innate immunity

ABSTRACT

Bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) are closely related alpha-herpesviruses. BoHV-5 is the causal agent of non-suppurative meningoencephalitis in calves. BoHV-1 causes respiratory disease, abortions, genital disorders and, occasionally, encephalitis in cattle. Both viruses are neurotropic and they share similar biological properties. Nevertheless, they differ in their ability to cause neurological disease. Toll-like receptors (TLRs) are involved in the innate immune response to pathogens. In this study, the variations in the expression levels of TLRs were evaluated in different regions of the bovine central nervous system during the acute infection and reactivation of BoHV-1 and BoHV-5- infected cattle. With the exception of TLR9, significant up-regulation of all TLRs was detected following primary infection of neural tissues by both bovine alpha-herpesviruses. Furthermore, the stages of acute infection and reactivation were characterized by a distinguishable TLR expression pattern. Important differences in TLR expression upon infection of the central nervous system by BoHV-1 or BoHV-5 were not detected. The striking differences in TLR mRNA levels during acute infection and reactivation provide evidence that the innate immune response may be involved in the clinical outcomes observed at each stage. Further research is required to analyze the mechanisms that initiate TLR activation and the signaling cascade mediated by each TLR to elucidate the precise role these receptors play in bovine herpesvirus encephalitis.

1. Introduction

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. A closely related alpha-herpesvirus, bovine herpesvirus type 1 (BoHV-1), causes several syndromes in cattle, including respiratory disease, abortions and genital disorders (Tikoo et al., 1995). BoHV-1 is also responsible for some cases of encephalitis in cattle (Silva et al., 2007; Rissi et al., 2008). However, these particular cases are not as frequent as those associated with BoHV-5. Both viruses are neurotropic and they share similar biological properties. Nevertheless, it is unknown why these alpha-herpesviruses differ in their ability to cause neurological disease in cattle.

Herpesviruses' life cycle is characterized by stages of acute infection, latency and reactivation. Sensory neurons of the trigeminal ganglion are the main site of latency of alpha-herpesviruses. Sporadic virus reactivation from latency can occur under conditions of natural stress or it can be experimentally induced by glucocorticoids administration. These episodes of reactivation are the main source of virus dissemination (Rock et al., 1992). BoHV-1 reactivation is subclinical. However, reactivation of BoHV-5 may occur in the presence of mild neurological signs (Pérez et al., 2002; Vogel et al., 2003).

In many viral infections, an inappropriate or exacerbated immune response may be responsible for pathological alterations. Particularly, it has been described that the inflammatory response mediated by toll-like receptors (TLRs) during virus infection can result in tissue damage (Carty et al., 2014). It is unknown whether the innate immune response plays a role in the development of meningo-encephalitis by alpha-herpesviruses in cattle. Furthermore, it has not been reported whether differences in the immune response might be associated with differences in the pathogenesis of BoHV-1 and BoHV-5.

* Corresponding author.
E-mail address: seperez@vet.unicen.edu.ar (S. Pérez).

http://dx.doi.org/10.1016/j.rvsc.2016.04.009
0034-5288/

TLRs allow the recognition of pathogens by the innate immune system (Kawai and Akira, 2005; Akira et al., 2006). These receptors are transmembrane signaling proteins which mediate the recognition of pathogen associated molecular patterns (PAMPs). TLRs can also be activated by danger associated molecular patterns (DAMPs), which derive from the host after tissue injury (Lee et al., 2013). They are expressed by cells of the immune system or by those cells which are primary target for infectious agents (Zarember and Godowski, 2002). TLRs 3, 7, 8 and 9 are involved in the immune response against viral infections (Wang et al., 2006; Cargill and Womack, 2007) and they are located in the membranes of endosomic compartments (Latz et al., 2004). TLR3 recognizes double-stranded RNA, TLR7 and TLR8 are activated by single-stranded, GU-rich RNA and sequences of double-stranded DNA, rich in un-methylated CpG, are the stimulus for TLR9 activation (Borrow et al., 2010). TLRs stimulation induces a series of events that result in the production of pro-inflammatory cytokines and interferon and, finally, in the stimulation of the adaptive immune response (Smith et al., 2005; Rosenthal, 2006). For this reason, many TLR agonists may have therapeutic efficacy in the control of viral infections of the central nervous system (CNS). Nevertheless, research must be focused on determining whether the triggering of the innate immune response leads to the control of the infection or to the induction of pathology.

Previous studies by Marin et al. (2014a, 2014b) have shown that TLRs participate in the response to bovine herpesviruses infections *in vitro* and *in vivo*. The aim of this study was to evaluate whether TLR expression in the CNS of BoHV-1- and BoHV-5-experimentally infected calves was involved in the differences in the neuropathogenesis observed after infection of cattle with each alpha-herpesvirus.

2. Materials and methods

2.1. Cells

For viral stocks, virus isolation and *in vitro* tests, Madin-Darby bovine kidney (MDBK) cells grown in minimum essential medium (MEM) supplemented with 10% fetal calf serum and antibiotics were used. Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

2.2. Virus

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 tissue culture infectious dose $(TCID)_{50}$/ml (Reed and Müench, 1938).

2.3. 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). Ten BoHV-1- and BoHV-5-free and seronegative cross-bred, 1 year-old calves were used. Calves were randomly assigned to one of three 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). In Group 2 (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. With this low dose inoculum, virus latency is established without the presence of clinical signs during the acute stage of infection, as previously demonstrated by Pérez et al. (2002). At 20 dpi they received an intravenous dose of 0.1 mg/kg bodyweight dexamethasone (DEX) (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 DEX treatment. Establishment of latency in these animals was confirmed by serology and absence of virus excretion in nasal and ocular swabs after the period of primary infection (data not shown). Group 3 (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 DEX using the same regime as the calves in Group 2. This calf was euthanased at 25 dpi.

2.4. Tissue samples

At necropsy, once the brain was removed, it was transversally sliced into eight 1–2 cm-thick sections. Samples from anterior cerebral cortex, including the olfactory and frontal cortex; posterior cerebral cortex (ectomarginal groove area) and cervical medulla were collected aseptically and individually for virus isolation and Real Time-PCR. Samples from posterior cortex were not available from calves in Group 2. Neural tissues were also placed in 10% neutral buffered-formalin and processed for histopathology.

2.5. Virus isolation and histopathology from nervous tissue

Tissue homogenates were prepared as a 10% solution in MEM with antibiotics. Homogenates were centrifuged at 1000 $\times g$ for 30 min at 4 °C. One hundred microliters of supernatant were inoculated into MDBK cells in 24 well-plates in duplicate. Cultures were observed daily for cytopathic effect and the supernatants were passaged every three days, for a total of three passages. Samples were evaluated by direct immunofluorescence using an anti-BoHV polyclonal antibody (VMRD). For histopathology, neural tissue sections were cut at 5 μm and stained with hematoxylin-eosin.

2.6. Nucleic acids extraction

Total DNA from tissue samples of experimentally-infected animals was extracted using a commercial kit (Dneasy Blood and Tissue kit, Qiagen Inc., Valencia, CA, USA), as recommended by the manufacturer. The DNA concentration was measured by absorbance at 260 nm using an Epoch Microplate Spectrophotometer (BioTeK, Winooski, VT, USA). To test the efficiency of DNA extraction or the presence of inhibitors in the Real-Time PCR reaction, the detection of cytochrome B, a gene with constitutive expression in bovine tissues, was evaluated. The amplifications were carried out in a final volume of 20 μl using EvaGreen as intercalating fluorescent dye (KAPA HRM FAST Master Mix, Biosystems, Woburn, USA) and primers that amplify a 134 bp fragment of bovine cytochrome B DNA (Santaclara et al., 2007). The cycling program consisted of an initial denaturation of 2 min at 95 °C and 40 cycles of 10 s at 95 °C, 15 s at 56 °C and 20 s at 72 °C. Samples with bovine cytochrome B Cycle Threshold (Ct) values below 35 were considered suitable for further analysis. The melting temperature of bovine cytochrome B specific amplification fragment was 84 °C. All real-time PCR reactions were carried out in a Rotor Gene Q thermocycler and were performed in duplicate.

Total RNA from nervous tissue samples was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and digested with DNase I for 30 min at 37 °C to remove any contaminating genomic DNA (gDNA). The quality and quantity of the resulting RNA were determined using an Epoch Microplate Spectrophotometer (BioTeK, Winooski, VT, USA). Complementary DNA (cDNA) was synthesized using a reaction mixture containing 1 μg of total RNA, random hexamers (12 ng/μl) (Promega, Madison, WI, USA) and Moloney murine leukaemia virus reverse transcriptase (10 U/μl) (Promega, Madison, WI, USA), following the procedures suggested by the manufacturer. Negative controls, omitting the RNA or the reverse transcriptase, were included.

2.7. Real Time PCR with High Resolution Melting (HRM) for virus genome detection

Real-time PCR reactions to detect BoHV-1 and BoHV-5 DNA were performed using primers that amplify a 189 bp fragment from the DNA polymerase gene of BoHV-1 and BoHV-5 (Diallo et al., 2011). Real-Time PCR assays were carried out in a Rotor Gene Q thermocycler, in a final volume of 20 μl using EvaGreen as intercalating fluorescent dye (KAPA HRM FAST Master Mix, Biosystems, Woburn, USA). The cycling program consisted of an initial denaturation of 3 min at 95 °C and 50 cycles of 15 s at 95 °C, 15 s at 58 °C and 20 s at 72 °C. After amplification, a HRM curve analysis was performed. The genotype confidence cut off value of 93% was set up in the genotyping module of the software. The melting temperatures of BoHV-1 and BoHV-5 were 89.7 °C and 91.1 °C, respectively.

2.8. Real Time RT-PCR for TLRs

Real Time RT-PCR reactions for bovine TLR3, TLR7, TLR8 and TLR9 were carried out using primers and probes described by Marin et al. (2014a). Expression of the "housekeeping" gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control (McGuire et al., 2004). The PCR reactions contained 800 nM specific forward and reverse primers, 200 nM specific probe, 1× PCR Mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems, Branchburg, NJ, USA) and 1 μl of cDNA sample in a final volume of 25 μl. The amplification and detection of the specific products were carried out in an Applied Biosystems 7500 cycler, with the following amplification conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 20 s at 95 °C and 60 s at 60 °C. In all cases, the experiments were performed in duplicate. Negative controls for cDNA synthesis and PCR procedures were included in all cases. The amplification efficiency was determined for each gene using 10-fold dilutions of the cDNA. The results are reported as the mean fold change of TLR transcription levels in the regions of the nervous system of infected calves over levels detected in tissue sections of uninfected calves, which served as the control group.

2.9. Statistical analysis

The relative expression analysis of the target genes was performed using the Relative Expression Software Tool (REST, Qiagen Inc., Valencia, CA, USA), which compares the expression of the target gene in a sample group relative to the control group with 2 to 16 data points and evaluates the group differences for significance with a pair-wise fixed reallocation randomization test (Pfaffl et al., 2002). The real-time RT-PCR efficiency for each gene was determined by a linear regression model according to the equation: $E = 10[-1/\text{slope}]$. For comparison of data between BoHV-1 and BoHV-5-infected calves, data for each TLR gene were normalized with respect to the data obtained from the uninfected calves and GAPDH. Data were then analyzed by ANOVA and Student's *t*-test. A 5% significance level was used.

3. Results

3.1. Microscopic lesions and detection of BoHV-1 and BoHV-5 in nervous tissue by virus isolation and Real Time PCR-HRM

As shown in Table 1, during acute infection, BoHV-5 was not isolated from any of the nervous tissue samples analyzed. However, BoHV-1 was isolated from olfactory (2 out of 2 calves), frontal (1/2) posterior brain cortex (1/2) and cervical medulla (1/2). BoHV-1 titers in brain samples ranged from $10^{6.3}$ to $10^{7.3}$ $TCID_{50}$/ml. In cervical medulla the virus titer was $10^{7.97}$ $TCID_{50}$/ml. BoHV-1 was not isolated from brain sections or cervical medulla of calves that received DEX to induce reactivation. On the contrary, during BoHV-5 reactivation, the virus was isolated from olfactory (1/2) and posterior cortex (1/2), with virus titers of $10^{7.07}$ and $10^{6.07}$ $TCID_{50}$/ml, respectively. As expected, BoHV-1 and BoHV-5 were not isolated from any of the tissue samples from mock-infected calves. Microscopic lesions in BoHV-1- and BoHV-5-acutely-infected calves consisted of cortical and sub-meningeal hemorrhages in frontal brain cortex and edema and congestion in posterior cortex. During reactivation, mild multifocal hemorrhages in anterior brain cortex of BoHV-1- and BoHV-5-infected calves were observed. Small areas of gliosis and satellitosis were present in medulla of BoHV-1-infected calves at this stage. As expected, microscopic lesions were not observed in mock-infected cattle (Fig. 1).

Table 1
Virus isolation and Real-Time PCR with HRM analysis in nervous tissue samples from BoHV-1- and BoHV-5-infected calves. I: virus isolation, HRM: Real Time-PCR with High Resolution Melting (HRM) analysis.

Infection	Virus	Central nervous system section							
		Olfactory cortex		Frontal cortex		Posterior cortex		Cervical medulla	
Acute		I	HRM	I	HRM	I	HRM	I	HRM
	BoHV-5# 2	−	+	−	+	−	−	−	+
	BoHV-5# 3	−	+	−	−	−	−	−	+
	BoHV-1# 4	+	−	−	+	−	+	+	+
	BoHV-1# 5	+	+	+	+	+	+	−	+
Reactivation	BoHV-5# 8	−	+	−	−	−	−	−	−
	BoHV-5# 9	+	+	−	+	+	+	−	+
	BoHV-1# 11	−	−	−	−	−	−	−	−
	BoHV-1# 14	−	−	−	−	−	−	−	−
Mock	Calf # 1	−	−	−	−	−	−	−	−
	Calf# 6	−	−	−	−	−	−	−	−

By using Real Time-HRM analysis, it was demonstrated that during acute BoHV-5 infection, the viral genome was present in olfactory (2/2) and frontal (1/2) cortex and cervical medulla (2/2). At the same stage of infection, BoHV-1 genome was consistently detected in olfactory (1/2), frontal (2/2), posterior cortex (2/2) and cervical medulla (2/2). Olfactory (2/2), frontal (1/2), posterior cortex (1/2) and cervical medulla (1/2) harboured BoHV-5 DNA 48 h after DEX-induced reactivation. However, BoHV-1 DNA was not detected at this time-point (Table 1).

3.2. Expression of TLRs in the CNS of BoHV-1- and BoHV-5-acutely-infected calves

TLR mRNA levels were evaluated in brain sections and cervical medulla during the acute, primary infection of cattle intranasally challenged with BoHV-1 and BoHV-5. Samples from experimentally-inoculated cattle were compared with TLR expression levels in neural tissue from mock-infected calves. Then, data were normalized and TLR expression was compared between BoHV-1- and BoHV-5-inoculated animals.

At 6 dpi, TLR3 levels were significantly up-regulated ($p \leq 0.05$) in frontal, posterior cortex and cervical medulla of BoHV-1- and BoHV-5-infected calves when compared to the samples from uninfected calves. In the case of BoHV-1, TLR3 was also up-regulated in the olfactory cortex ($p \leq 0.05$). Interestingly, the highest increase in TLR3 expression was detected in cervical medulla of both groups of infected animals (4.6-fold for BoHV-5 and 8.8-fold for BoHV-1 over uninfected calves). For TLR7, significant increases ($p \leq 0.05$) in mRNA levels were detected in all brain sections and cervical medulla of BoHV-5- infected calves. Similar findings were obtained for TLR7 expression in BoHV-1-acutely-infected calves, with the exception of posterior cortex, in which receptor levels did not differ ($p \geq 0.05$) from the uninfected control. TLR8 expression was up-regulated ($p \leq 0.05$) in posterior cortex and cervical

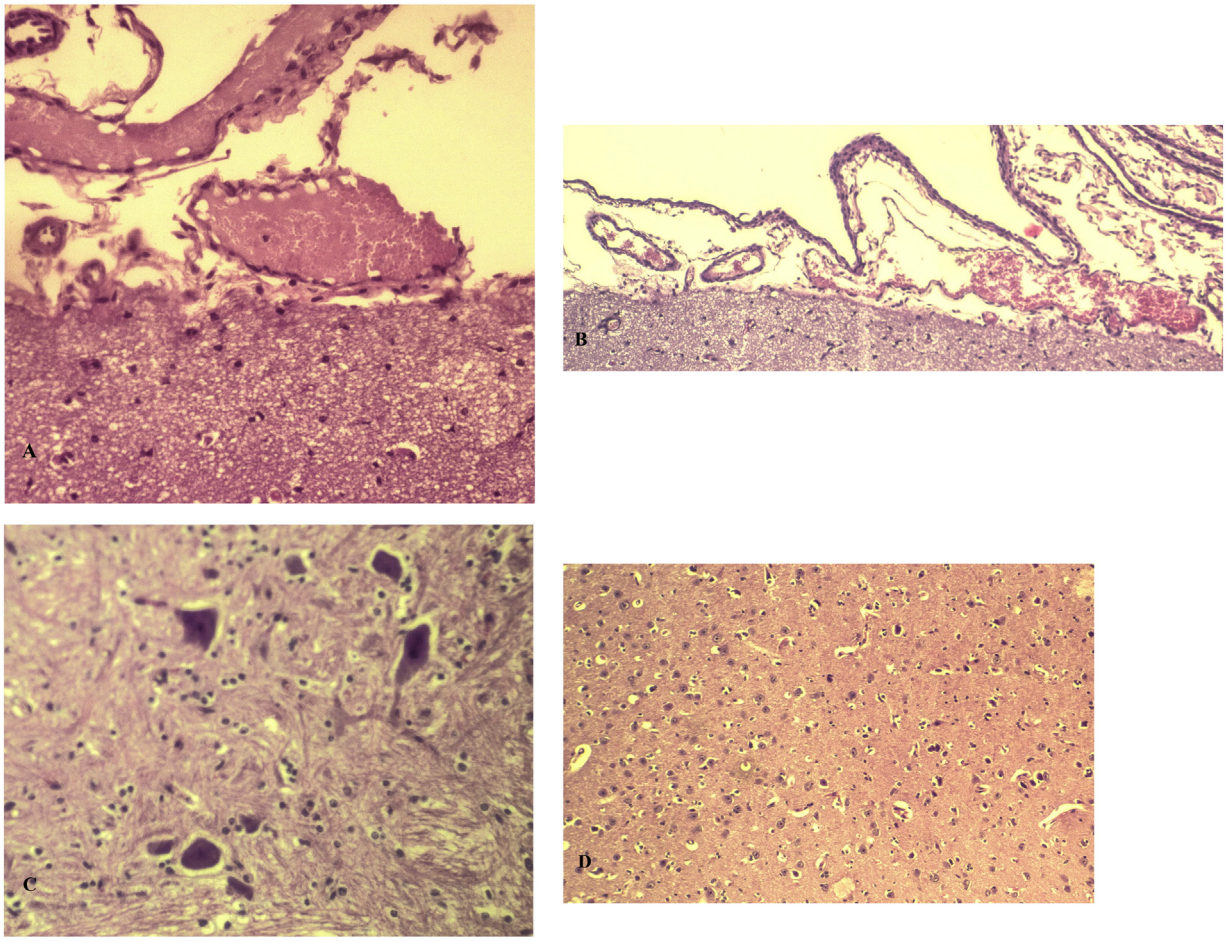

Fig. 1. Microscopic lesions in neural tissues of BoHV-1and BoHV-5-infected calves. (A). Sub-meningeal edema in posterior cortex of a BoHV-5-infected calf (6 dpi) (20×), (B). Sub-meningeal hemorraghes in posterior cortex of a BoHV-1-infected calf (20×), (C). Areas of gliosis and satellitosis in cervical medulla of a BoHV-1-infected calf after dexamethasone-induced reactivation (40×), (D). Posterior brain cortex of a mock-infected calf (20×).

medulla of BoHV-5-acutely-infected calves and in the olfactory and cervical medulla of BoHV-1- infected animals. TLR9 mRNA level was down-regulated in the frontal cortex of BoHV-1-infected calves. Nevertheless, TLR9 expression was not significantly affected ($p \geq 0.05$) in any other area of the CNS by BoHV-1 or BoHV-5-infection (Table 2).

After normalizing by the TLR levels in uninfected tissues, comparisons were established between TLR mRNA expression in BoHV-1- and BoHV-5-infected animals. As shown in Fig. 2, at 6 dpi, only TLR3 expression was significantly higher ($p \leq 0.05$) in the olfactory cortex of BoHV-1-infected calves when compared with BoHV-5-infection. Differences in the expression levels of the other TLRs were not recorded ($p \geq 0.05$).

3.3. Expression of TLRs in the CNS of calves experimentally-inoculated with BoHV-1 or BoHV-5 and that received DEX to induce reactivation

The expression of TLRs mRNA was evaluated in the brain and cervical medulla 48 h after the administration of DEX to induce BoHV-1 and BoHV-5 reactivation. In BoHV-1 and BoHV-5-infected calves, a significant down-regulation ($p \leq 0.05$) of TLR3 and TLR8 mRNA levels was detected in frontal cortex when compared with samples from the uninfected calf. Furthermore, in BoHV-5-infected calves TLR3 levels were up-regulated ($p \leq 0.05$) in cervical medulla. In the case of BoHV-1, TLR3 levels significantly decreased in olfactory cortex at 48 h after DEX administration ($p \leq 0.05$). Expression levels of all other TLRs were

Table 2
TLR mRNA expression levels in neural tissue sections of BoHV-1 and BoHV-5 acutely-infected animals and uninfected calves. * in the same row (*i.e*, for each TLR) denotes statistically significant differences with respect to mock-infected calves ($p \leq 0.05$).

	Neural tissue section											
	Olfactory cortex			Frontal cortex			Posterior cortex			Cervical medulla		
TLR	BoHV-1	BoHV-5	M	BoHV-1	BoHV-5	M	BoHV-1	BoHV-5	M	BoHV-1	BoHV-5	M
3	3.85*	1.36	1	3.08*	1.28*	1	3.14*	2.70*	1	9.85*	5.63*	1
7	4.66*	3.50*	1	2.82*	2.30*	1	1.65	2.56*	1	2.21*	2.58*	1
8	3.24*	1.60	1	3.14	2.59	1	1.48	2.44*	1	3.09*	3.89*	1
9	1.88	2.89	1	0.74*	2.63	1	0.61	1.77	1	1.19	0.79	1

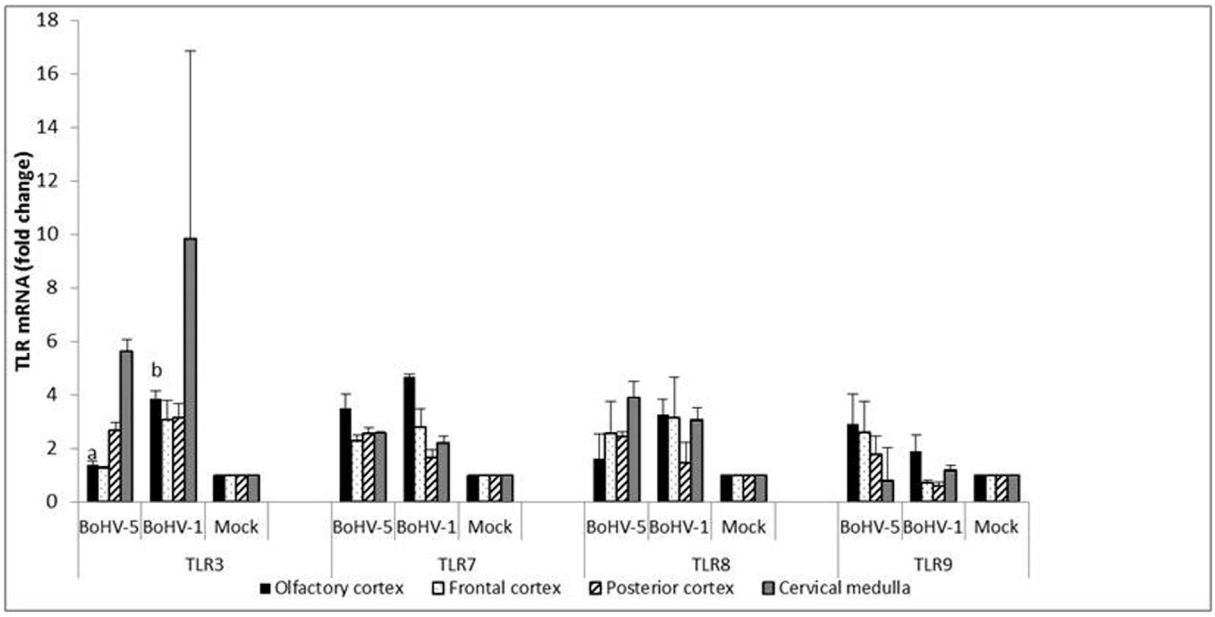

Fig. 2. Expression of TLR3, TLR7, TLR8 and TLR9 mRNA in nervous tissue of BoHV-5- and BoHV-1-actuely infected calves. Different letters indicate statistically significant differences ($p \leq 0.05$) between infected groups.

not significantly affected ($p \geq 0.05$) during reactivation of BoHV-1 or BoHV-5 (Table 3).

After determining the variations in TLR expression levels upon infection with respect to mock-infected animals, it was important to establish whether variations were recorded between BoHV-1- and BoHV-5-infected calves once virus reactivation from latency was induced. As shown in Fig. 3, after DEX-induced reactivation, a significant decrease ($p \leq 0.05$) in TLR3 expression was detected in the frontal cortex of BoHV-1-infected calves, when compared with BoHV-5-infection. Similar findings were observed in the expression of TLR8 in the frontal cortex during reactivation of BoHV-1. Differences in the expression levels of other TLR and in the different tissue sections evaluated were not recorded ($p \geq 0.05$).

4. Discussion

The role of TLRs in the recognition of pathogens has been widely recognized. However, the immune mechanisms involved in the control of these pathogens after TLR stimulation have been partially characterized, particularly in the case of animal diseases. In this study, the changes in the expression levels of TLRs in the CNS after BoHV-1 and BoHV-5 infection in relation to uninfected animals have been analyzed. Then, comparisons in TLR mRNA levels were established between BoHV-1 and BoHV-5-infected cattle to determine whether it is possible to associate a distinct TLR expression arrangement with the pathogenesis of each virus in neural tissue. With the exception of TLR9, significant up-regulation of all TLRs was detected following primary infection of neural tissues by both bovine alpha-herpesviruses. Furthermore, the stages of acute infection and reactivation were characterized by a distinguishable TLR expression pattern. The magnitude in the variation of TLR expression is in agreement with the clinical manifestations that can be observed at each stage of the viruses' life cycle in cattle.

Herpes simplex virus 1 (HSV-1), which shares biological properties with BoHV-1 and BoHV-5, causes severe encephalitis in humans. Recently, Zhang et al. (2007) demonstrated that a TLR3 deficiency in the CNS correlates with the development of neurological disease caused by HSV-1. It was speculated that TLR3 deficiency is associated with inefficient virus control in the brain, leading to persistence of the virus in this tissue and to a high rate of encephalitis due to virus reactivation. Contrary to these studies with HSV-1, it has been previously shown that TLR3 mRNA levels are up-regulated in dorso-lateral brain cortex after infection of calves with BoHV-5 (Marin et al., 2014b). Furthermore, in this study, the up-regulation of TLR3 was detected in all CNS regions evaluated. Surprisingly, the highest increase in TLR3 levels was detected in cervical medulla of BoHV-1-infected calves and to a lesser extent in the same sample of BoHV-5-infected animals. Thus, the findings of this study consistently demonstrate that there is an involvement of TLR3 in the innate immune response to the infection of the CNS by both alpha-herpesviruses. Significant differences in TLR3 mRNA levels between BoHV-1 and BoHV-5-infected calves were only evident in olfactory cortex, the site where virus invasion of the brain is initiated during primary acute infection (Pérez et al., 2002). However, it is apparent that TLR3 stimulation was not involved in halting BoHV-1 or BoHV-5 progression into the brain since virus replication and/or genomes were detected within the different neural areas analyzed. On the contrary, it has been shown that HSV-1 replicates more efficiently in TLR3 deficient neurons where type I IFN production is impaired (Lim et al., 2014). Thus, the findings from this study also demonstrate that TLR modulation by herpesvirus infection, at least in neural tissue, may lead to different outcomes of infection. In agreement with our findings, it

Table 3
TLR mRNA expression levels in neural tissue sections of BoHV-1 and BoHV-5 latently-infected animals after dexamethasone-induced reactivation and uninfected calves. * in the same row (*i.e.*, for each TLR) denotes statistically significant differences with respect to mock-infected calves ($p \leq 0.05$).

	Neural tissue section								
	Olfactory cortex			Frontal cortex			Cervical medulla		
TLR	BoHV-1	BoHV-5	M	BoHV-1	BoHV-5	M	BoHV-1	BoHV-5	M
3	0.60*	1.22	1	0.49	0.76*	1	0.88	1.17*	1
7	0.78	1.73	1	0.77	1.08	1	1.13	0.79	1
8	0.42	1.34	1	0.26*	0.66*	1	0.83	0.44	1
9	0.87	0.46	1	0.52	0.46	1	1.06	0.42	1

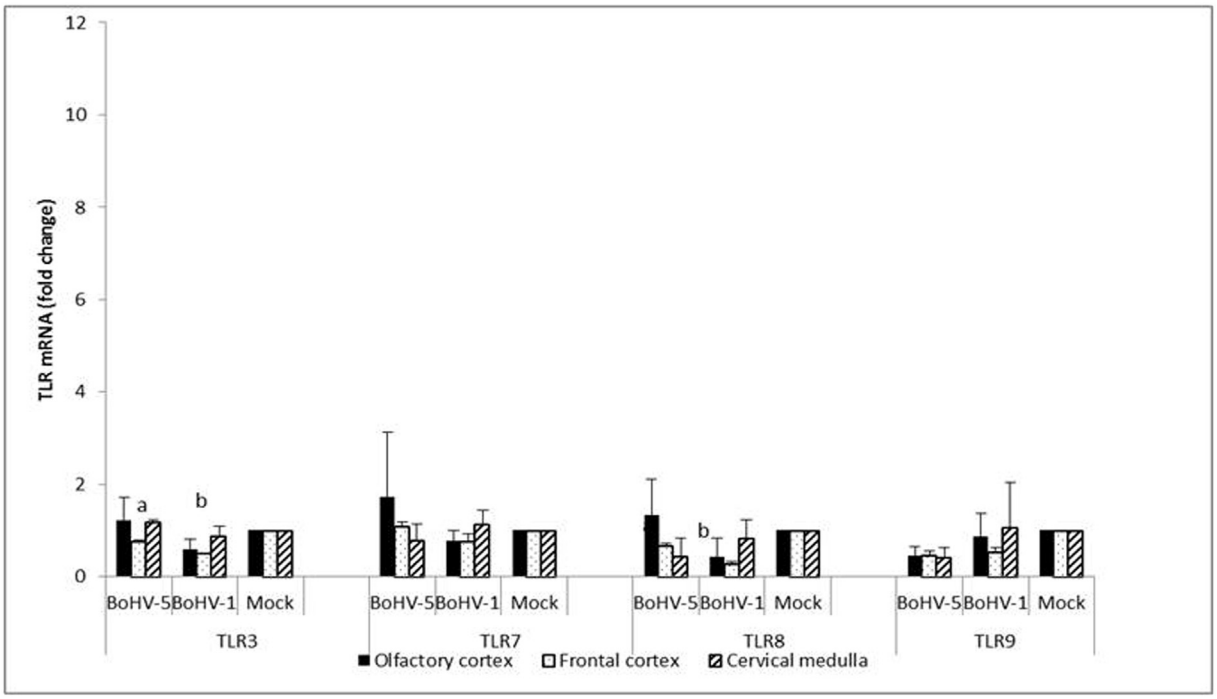

Fig. 3. Expression of TLR3, TLR7, TLR8 and TLR9 mRNA in nervous tissue of BoHV-5- and BoHV-1- infected calves after dexamethasone-induced reactivation. Different letters indicate statistically significant differences ($p \leq 0.05$) between infected groups.

has been shown that for West Nile Virus- (WNV) (Wang et al., 2004) and simian immunodeficiency virus (SIV) and human immunodeficiency virus- (HIV) induced encephalitis (Suh et al., 2009) there is an up-regulation of TLR3 levels, which is responsible for the inflammatory response in CNS. However, Daffis et al. (2008) have also shown that in experimentally-infected mice, TLR3 might favor WNV pathogenesis in some cases or protects the host from virus dissemination and encephalitis in others. Nevertheless, it can be inferred according to the results obtained here that the role of TLR3 in bovine alpha-herpesvirus infection of the CNS is different from that observed in the encephalitis by HSV-1 in humans. Furthermore, it is important to consider that TLR3 may have functions in the CNS involving mechanisms which are unrelated to the innate immune response (Cameron et al., 2007).

The results from this study also provide evidence of a role for TLR7 and TLR8 in the neurological disease caused by bovine alpha-herpesviruses. During acute BoHV-5- and BoHV-1-infection, TLR7 levels remained above the values found in uninfected animals in all brain sections, except in the posterior cortex of BoHV-1-infected calves. In previous studies it was demonstrated that the levels of TLR7 mRNA significantly increase after the infection of bovine peripheral white blood cells with BoHV-1 and BoHV-5 and that the treatment with Imiquimod, a TLR7/8 agonist, reduces viral replication (Marin et al., 2014a).

In general, in this study it was determined that TLR3 and TLR7/8 are significantly higher in the olfactory cortex of BoHV-1-acutely-infected calves but only TLR7 increases in this region of the brain of BoHV-5-infected cattle. Difficultness in the isolation of BoHV-5 from neural tissue samples has been previously reported (Belknap et al., 1994; Pérez et al., 2002) and this is likely due to the low viral titers reached in this tissue (Belknap et al., 1994). According to the results of this work it can also be speculated that virus stimulation of TLR7 during acute infection is leading to a negative feedback in virus replication within the CNS, as it has been shown by Baker et al. (2013) in studies with Langat virus, a member of the tick-borne encephalitis virus sero-complex. The authors suggested that TLR7 may not have influence in neurovirulence but may play a direct role in reducing the viral load within the brain.

In contrast to the findings during acute infection, TLR3 and TLR8 levels were significantly down-regulated during virus reactivation. These findings are expected since virus replication during reactivation episodes should be compatible with neuronal survival and, therefore, TLR stimulation should not be greatly affected at this stage. Downregulation of TLR7/8 during virus reactivation might favor virus replication, particularly in the case of BoHV-5, allowing the virus to spread to further regions. It is known that reactivation of BoHV-5 results in the establishment of latent infection in additional sites of the brain (Vogel et al., 2003).

A role for TLR9 in herpesvirus encephalitis could not be demonstrated in this work. It has been reported that TLR9 mRNA levels are increased within the trigeminal ganglion during BoHV-5 infection (Marin et al., 2014b). However, according to the results from this study, this receptor is not particularly affected by virus infection of the CNS.

At this time, important conclusions on TLR expression in cervical medulla cannot be reached. However, it is evident that virus stimulation of TLR occurs in this tissue.

Overall, the results from this study demonstrate that expression of at least TLR3, TLR7 and TLR8 is stimulated upon bovine alpha-herpesvirus infection of the brain. Important differences in the patterns of TLR expression upon infection of the CNS by BoHV-1 or BoHV-5 were not detected. The striking differences observed with TLR mRNA levels during acute infection and reactivation provide evidence that the innate immune response may be involved in the clinical outcomes observed at each stage. However, further research will be required to analyze the mechanisms that initiate TLR activation and the signaling cascade mediated by each TLR to elucidate the precise role these receptors play in bovine herpesvirus encephalitis.

Conflict of interest statement

The authors declare no potential conflicts of interest with respect to the research, authorship, publication of this article and/or financial or personal relationships that could inappropriately influence this work.

Acknowledgements

The authors gratefully acknowledge the assistance given by DVM Mariano Grosso in animal handling, necropsies and samples collection. This research was supported by grant PICT 2008-1421 (ANPCyT) and PIP 098 (CONICET).

References

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801.

Baker, D., Woods, T., Butchi, N., Morgan, T., Taylor, R., Sunyakumthorn, P., Mukherjee, P., Lubick, K., Best, S., Peterson, K., 2013. Toll-like receptor 7 suppresses virus replication in neurons but does not affect viral pathogenesis in a mouse model of Langat virus infection. J. Gen. Virol. 94, 336–347.

Belknap, E., Collins, J., Ayers, V., Schultheiss, P., 1994. Experimental infection of neonatal calves with neurovirulent bovine herpesvirus type 1.3. Vet. Pathol. 31, 358–365.

Borrow, B., Martínez-Sobrido, L., De la Torre, J., 2010. Inhibition of the type I interferon antiviral response during arenavirus infection. Virology 2, 2443–2480.

Cameron, J., Alexopoulou, L., Sloane, J., DiBernardo, A., Ma, Y., Kosaras, B., Flavell, R., Strittmatter, S., Volpe, J., Sidman, R., Vartanian, T., 2007. Toll-like receptor 3 is a potent negative regulator of axonal growth in mammals. J. Neurosci. 27, 13033–13041.

Cargill, E., Womack, J., 2007. Detection of polymorphisms in bovine toll-like receptors 3, 7, 8, and 9. Genomics 89, 745–755.

Carty, M., Reinert, L., Paludan, S., Bowie, A., 2014. Innate antiviral signalling in the central nervous system. Trends Immunol. 35, 79–87.

Daffis, S., Samuel, M., Suthar, M., Gale Jr., M., Diamond, M., 2008. Toll-like receptor 3 has a protective role against West Nile virus infection. J. Virol. 82, 10349–51038.

Diallo, I., Corney, B., Rodwell, B., 2011. Detection and differentiation of bovine herpesvirus 1 and 5 using a multiplex real-time polymerase chain reaction. J. Virol. Methods 175, 46–52.

Inman, M., Lovato, L., Doster, A., Jones, C., 2002. A mutation in the latency-related gene of bovine herpesvirus 1 disrupts the latency reactivation cycle in calves. J. Virol. 76, 6771–6779.

Kawai, T., Akira, S., 2005. Pathogen recognition with Toll-like receptors. Curr. Opin. Immunol. 17, 338–344.

Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K., Monks, B., Knetter, C., Lien, E., Nilsen, N., Espevik, T., Golenbock, D., 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat. Immunol. 5, 190–198.

Lee, H., Lee, S., Cho, I., Lee, S., 2013. Toll-like receptors: sensor molecules for detecting damage to the nervous system. Curr. Protein Pept. Sci. 14, 33–42.

Lim, H., Seppänen, M., Hautala, T., Ciancanelli, M., Itan, Y., Lafaille, F., Dell, W., Lorenzo, L., Byun, M., Pauwels, E., Rönnelid, Y., Cai, X., Boucherit, S., Jouanguy, E., Paetau, A., Lebon, P., Rozenberg, F., Tardieu, M., Abel, L., Yildiran, A., Vergison, A., Roivainen, R., Etzioni, A., Tienari, P., Casanova, J., Zhang, S., 2014. TLR3 deficiency in herpes simplex encephalitis: high allelic heterogeneity and recurrence risk. Neurology 83, 1888–1897.

Marin, M., Quintana, S., Faverín, C., Leunda, M., Odeón, A., Pérez, S., 2014a. Toll-like receptor activation and expression in bovine alpha-herpesvirus infections. Res. Vet. Sci. 96, 196–203.

Marin, M., Quintana, S., Faverín, C., Leunda, M., Odeón, A., Pérez, S., 2014b. Toll-like receptor expression in the nervous system of bovine alpha-herpesvirus-infected calves. Res. Vet. Sci. 97, 422–429.

McGuire, K., Manuja, A., Russell, G., Springbett, A., Craigmile, S., Nichani, A., Malhotra, D., Glass, E., 2004. Quantitative analysis of pro-inflammatory cytokine mRNA expression in Theileria annulata-infected cell lines derived from resistant and susceptible cattle. Vet. Immunol. Immunopathol. 99, 87–98.

Pérez, S., Bretschneider, G., Leunda, M., Osorio, F., Flores, E., Odeón, A., 2002. Primary infection, latency, and reactivation of bovine herpesvirus type 5 in the bovine nervous system. Vet. Pathol. 39, 437–444.

Pfaffl, M., Horgan, G., Demplfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.

Reed, L., Müench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.

Rissi, D., Pierezan, F., Silva, M., Flores, E., de Barros, C., 2008. Neurological disease in cattle in southern Brazil associated with bovine herpesvirus infection. J. Vet. Diagn. Investig. 20, 346–349.

Rock, D., Lokensgard, J., Lewis, T., Kutish, G., 1992. Characterization of dexamethasone induced reactivation of latent bovine herpesvirus 1. J. Virol. 66, 2484–2490.

Rosenthal, K., 2006. Teaking innate immunity: the promise of innate immunologicals as anti-infectives. Canadian Journal of Infectious Diseases and Medical Microbiology 17, 307–314.

Santaclara, F., Espiñeira, M., Cabado, A., Vieites, J., 2007. Detection of land animal remains in fish meals by the polymerase chain reaction-restriction fragment length polymorphism technique. J. Agric. Food Chem. 55, 305–310.

Silva, M., Brum, M., Loreto, E., Weiblen, R., Flores, E., 2007. Molecular and antigenic characterization of Brazilian bovine herpesvirus type 1 isolates recovered from the brain of cattle with neurological disease. Virus Res. 129, 191–199.

Smith, P., Lombarda, G., Foster, G., 2005. Type I interferons and the innate immune response more than just antiviral cytokines. Mol. Immunol. 42, 869–877.

Suh, H., Brosnan, C., Lee, S., 2009. Toll-like receptors in CNS viral infections. Curr. Top. Microbiol. Immunol. 336, 63–81.

Tikoo, S., Campos, M., Babiuk, L., 1995. Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control. Adv. Virus Res. 45, 191–223.

Vogel, F., Caron, L., Flores, E., Weiblen, R., Winkelmann, E., Meyer, S., Bastos, R., 2003. Distribution of bovine herpesvirus type 5 DNA in the central nervous systems of latently, experimentally infected calves. J. Clin. Microbiol. 41, 4512–4520.

Wang, T., Town, T., Alexopoulou, L., Anderson, J., Fikrig, E., Flavell, R., 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat. Med. 10, 1366–1373.

Wang, J., Shao, Y., Bennett, T., Shankar, R., Wightman, P., Reddy, L., 2006. The functional effects of physical interactions among Toll-like receptors 7, 8, and 9. J. Biol. Chem. 281, 37427–37434.

Zarember, K., Godowski, P., 2002. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J. Immunol. 168, 554–561.

Zhang, S., Jouanguy, E., Ugolini, S., Smahi, A., Elain, G., Romero, P., Segal, D., Sancho-Shimizu, V., Lorenzo, L., Puel, A., Picard, C., Chapgier, A., Plancoulaine, S., Titeux, M., Cognet, C., von Bernuth, H., Ku, C., Casrouge, A., Zhang, X., Barreiro, L., Leonard, J., Hamilton, C., Lebon, P., Héron, B., Vallée, L., Quintana-Murci, L., Hovnanian, A., Rozenberg, F., Vivier, E., Geissmann, F., Tardieu, M., Abel, L., Casanova, J., 2007. TLR3 deficiency in patients with herpes simplex encephalitis. Science 317, 1522–15227.