



Toll-like receptor expression in the nervous system of bovine alpha-herpesvirus-infected calves

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ABSTRACT

In this study, the expression levels of viral Toll-like receptors (TLRs) in the nervous system of bovine herpesvirus type 5 (BoHV-5)-infected calves were investigated. A significant increase in the expression of TLRs 3 and 7–9 was found in the anterior cerebral cortex during acute infection and viral reactivation. In the trigeminal ganglia, only TLR9 expression was significantly affected. The magnitude of the increase was lower in BoHV-1-infected calves, suggesting that a restricted immune response might protect against exacerbated inflammatory responses in the brain. This work describes, for the first time, the involvement of TLRs 3 and 7–9 in the recognition of BoHV in the bovine nervous system, indicating that the expression of these receptors might be associated with the development of neurological disease. Modulation of the signalling pathways mediated by TLRs might provide an effective approach to control the neuro-immune response to BoHV-5, which may be responsible for neurological lesions.

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1. Introduction

Alpha-herpesviruses are capable of causing neurologic disease in cattle. Bovine herpesvirus type 1 (BoHV-1) and type 5 (BoHV-5) are neuroinvasive. Whereas encephalitis caused by BoHV-1 has been reported occasionally (Roels et al., 2000; Silva et al., 2007), BoHV-5 has a marked neurotropism and is the causative agent of necrotising meningoencephalitis in young cattle (Pérez et al., 2002). Both BoHV-1 and BoHV-5 are genetically and antigenically related. BoHV-1 has a worldwide distribution, whereas BoHV-5 has a more limited geographic distribution, as it is most frequently reported in southern Brazil and Argentina (Carrillo et al., 1983; Salvador et al., 1998; Weiblen et al., 1989). Like other alpha-herpesviruses, BoHV-1 and BoHV-5 establish a lifelong latent infection primarily in the neural ganglia of animals that survive acute infection (Meyer et al., 2001; Pérez et al., 2002). The reactivation of a latent infection may occur under certain natural or induced stimuli, and this phenomenon provides adequate means for virus transmission and spread (Del Médico Zajac et al., 2010). Unlike BoHV-1 reactivation, natural or pharmacologically induced reactivation of BoHV-5 is frequently accompanied by the recrudescence of neurological disease (Pérez et al., 2002; Vogel et al., 2003). The mechanisms underlying the clinical manifestations after an infection caused by BoHV, especially

encephalitis, have not been well-defined. However, it is likely that the immune system plays an important role in the development of this neurological condition (Abril et al., 2004).

Toll-like receptors (TLRs) are a broad family of evolutionarily conserved innate immune receptors that recognise pathogen-associated molecular patterns (PAMPs) from diverse organisms (Mogensen, 2009). Among the TLRs, TLRs 3, 7, 8 and 9, which are expressed in intracellular vesicles, recognise microbial nucleic acids, particularly of viral origin, and constitute a powerful sensor system that detects viral invasion. Double-stranded RNA (dsRNA) is recognised by TLR3, single-stranded RNA (ssRNA) is detected by TLR7/8, and TLR9 recognises the unmethylated CpG dinucleotides in DNA molecules. These components are often present in the viral genome, and they are generated during the replication of many viruses (Borrow et al., 2010). The activation of TLRs by pathogens and pathogen-derived products induces the expression of pro-inflammatory mediators and anti-microbial effector molecules (Mogensen, 2009). Efficient inflammatory responses mediated by TLRs are required for the host defence against invading pathogens and tissue repair. However, exaggerated or chronic inflammation induced by the activation of these receptors may also lead to tissue damage and the development of disease. In the central nervous system (CNS), TLRs are expressed in various cell types, including microglia, astrocytes, neurons and cerebral vascular cells (Kong and Le, 2011). Accumulating evidence supports a model in which TLRs play a major role in brain infection and injury. Presently, investigation is aimed at demonstrating how the outcome of TLR

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involvement can lead to the resolution of infection, neurodegeneration or neural repair, depending on the context (Konat et al., 2006; Ransohoff and Brown, 2012).

Previous research on the neuropathogenesis of BoHV-5 has focused on the role of envelope glycoproteins (Chowdhury et al., 2000, 2002; Kaashoek et al., 1998), and investigations addressing the involvement of other viral products or host factors are lacking. Mechanisms mediated by TLRs have been recognised as key factors in many human infectious diseases. However, no information about the participation of these receptors in viral diseases of cattle is available. Thus, the aim of this work was to determine whether variations in the expression levels of viral TLRs might be detected at different stages of the infectious cycle of BoHV-5 in the nervous system of its natural host. Understanding the mechanisms that govern TLR signalling during a BoHV-5 infection in the nervous system will undoubtedly facilitate the design of effective therapeutic or preventive measures for the control of BoHV infection.

2. Materials and methods

2.1. Animals

Thirty-five crossbred calves, 6–8 months old, were used in this experiment. All of the animals were free of detectable antibodies to BoHV-1, BoHV-5 and bovine viral diarrhoea virus.

2.2. Cells and virus

Madin–Darby Bovine Kidney (MDBK) cells from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used for this study. MDBK cells were propagated in Minimum Essential Medium (Eagle) with Earle salts (E-MEM) (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% foetal bovine serum (Bioser, Buenos Aires, Argentina), certified free from adventitious viruses and antibodies, and with antibiotic–antimycotics (Gibco, Langley, OK, USA) at a concentration of 100 U/ml penicillin G, 100 µg/ml streptomycin sulphate and 0.025 µg/ml amphotericin B. Cells were incubated at 37 °C in a 5% CO₂ atmosphere.

The reference strain Los Angeles 38 (LA38) (BoHV-1.1) and the Argentinean BoHV-5 field strain (identified as 97/613), both in the eighth passage, characterised and provided by the Specialised Veterinary Diagnostic Service, INTA Balcarce (Argentina), were used for animal challenges. The isolate 97/613 was recovered from the brain of a 2-year-old cow with necrotising encephalitis. The virus identification was confirmed by isolation in cell culture followed by direct immunofluorescence using a polyclonal antibody against BoHV (American BioResearch, Sevierville, TN, USA) and nested-PCR (Campos et al., 2009). Viral inocula were propagated in MDBK cells in T-175 flasks (Greiner Bio-one, Frickenhausen, Germany) for 24 h. Supernatants were harvested and stored at –80 °C until use. Virus titres were determined by the endpoint titration method and expressed as tissue culture infective doses (TCID₅₀) according to Reed and Muench (1938).

2.3. Experimental design and BoHV challenge

A total of 35 BoHV-1- and BoHV-5-free and seronegative British cross-bred, 1-year-old calves were used in this study. To evaluate the expression levels of viral TLRs at different stages of the BoHV-5 infectious cycle, 23 bovines were randomly assigned to two main experimental groups: **Group 1** (12 calves) for the study of BoHV-5 acute primary infection, and **Group 2** (11 calves) for the study of BoHV-5 reactivation. An additional group was designed to evaluate the expression levels of TLRs during BoHV-5 latency: **Group 3** (two calves). Furthermore, to establish comparisons of TLR expression levels in neural tissues from BoHV-1-infected calves, animals

were also assigned to the following groups: **Group 4** (two calves) for BoHV-1 acute primary infection, and **Group 5** (two calves) for BoHV-1 latency and reactivation. Additionally, **Group 6**, consisting of six mock-infected calves, was also included.

Calves were challenged intranasally by aerosolisation with 25 ml of inoculum, evenly distributed in both nostrils. The virus inoculum for calves in Group 1 and Group 4 contained 10^{6.3} TCID₅₀ of the respective virus to induce acute infection. The virus inoculum for calves in Groups 2, 3 and 5 contained 10³ TCID₅₀ to induce a latent infection. Mock-infected calves were intranasally inoculated with 25 ml of E-MEM.

Calves from each treatment group were maintained in isolated pens and fed on grass hay, commercial concentrate foodstuff and water *ad libitum*.

To induce reactivation from latency, at 3 months after the primary inoculation (PI), all calves in Groups 2 and 5 and three calves in Group 6 were given dexamethasone (DXM; Dexametasona Vet, Schering Plough, Sanidad Animal, Argentina) at a dose of 0.1 mg/kg/day intravenously for 5 consecutive days. Animals in Groups 1 and 2 were sequentially killed, one calf per day, on days 6–17 PI and on days 6–16 after DXM treatment (day post-reactivation [PR]), respectively. Calves in Group 3 were killed on day 54 PI. The euthanasia of calves in Group 4 was performed on day 7 PI and for calves in Group 5 on day 6 PR. Mock-infected calves were killed on days 6, 10, and 16 PI (three control calves for the study of BoHV-1 and BoHV-5 acute infection) and on the same day PR (three control calves for the study of BoHV-1 and BoHV-5 latency and reactivation). To minimise the use of animals according to the Institutional Committee for Care and Use of Experimental Animals (CICUAE) of INTA, which is where the experiments were held, and because the only objective of this group is to obtain uninfected tissues, the organs of mock-infected calves killed during the acute infection are used as controls for the study of BoHV-5 latency. Calves were deeply anaesthetised and killed according to the regulations of the CICUAE of INTA, Argentina.

2.4. Sample collection

After euthanasia, the brain was removed and transversally sliced into eight 1- to 2-cm-thick sections. Different sections of the CNS were collected aseptically and individually for viral isolation and viral DNA detection. The following areas were evaluated: anterior cerebral cortex (three samples: olfactory cortex, frontal cortex and dorsolateral cortex), posterior cerebral cortex (two samples: marginal groove area and ectomarginal groove area), cerebellum (one sample), medulla and pons (three samples: cervical medulla, medulla oblongata and pons) and diencephalon (one sample). Trigeminal ganglia (TG) were also collected. Samples from the anterior cerebral cortex, where microscopic lesions of nonsuppurative meningoencephalitis are usually observed (Pérez et al., 2002), and the TG were selected for TLR expression studies by real-time RT-PCR.

2.5. Virus isolation and identification

Tissue samples were homogenised in 1X Hank's solution (10% w/v), and the suspensions were centrifuged at 1000 × g for 15 min at 4 °C. Fifty microlitres of supernatant were inoculated in duplicate into monolayers of MDBK cells in 96-well plates (Greiner Bio-one) and incubated at 37 °C. Samples were passaged every 3 days, for a total of four to six times, and monitored daily for the presence of cytopathic effects. At the end of each passage, the samples were tested by direct immunofluorescence using a polyclonal antibody against BoHV conjugated with fluorescein isothiocyanate (American BioResearch).

2.6. DNA extraction and PCR

Homogenates of tissue samples in 1X Hank's solution (10% w/v) were centrifuged for 10 min at 1500 × g at 4 °C. Five hundred microlitres of supernatants of homogenised tissues was placed in 1 ml lysis buffer (10 mM Tris–HCl pH 7.4, 25 mM EDTA, pH 8, 100 mM ClNa, 0.5% SDS and 100 mg proteinase K [Promega, Madison, WI, USA]) and digested overnight at 37 °C. Total DNA was extracted with phenol–chloroform–isoamyl alcohol (25:24:1) and precipitated with 3 M sodium acetate and cold 100% ethanol. After incubation for 1 h at –80 °C, the nucleic acid solution was centrifuged at 12,000 × g for 30 min. The supernatant was discarded, and the pellet was rinsed with cold 70% ethanol. The pellet was vacuum dried for 10 min and resuspended in 50 µl sterile water. The DNA concentration was measured by absorbance at 260 nm using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA). A *nested*-PCR technique, according to Campos et al. (2009), was performed to detect BoHV-1 and BoHV-5 DNA in nervous tissue samples. Details of the primers are provided in Table 1. To test the efficiency of DNA extraction or the presence of inhibitors in the PCR reaction in those samples in which BoHV-1 and BoHV-5 were not amplified, the detection of cytochrome B, a gene with constitutive expression in bovine tissues, was evaluated using primers described by Santaclara et al. (2007). The PCR reactions contained 800 nM specific forward and reverse primers, 1X PCR Mastermix (Eva Green PCR Mastermix, Biodynamics, Buenos Aires, Argentina) and 1 µl of the DNA sample in a final volume of 10 µl. The amplification and detection of the specific products were carried out using a Rotor Gene 6000 cycler, with the following amplification conditions: 3 min at 95 °C and 35 cycles of 20 s at 94 °C, 40 s at 56 °C and 40 s at 72 °C.

2.7. RNA extraction, DNase treatment and reverse transcription

The total RNA from the nervous system was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and digested with DNase I Amplification Grade (Invitrogen) 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). Complementary DNA (cDNA) was synthesised using a reaction mixture containing 1 µg of total RNA, random hexamers

(12 ng/µl) (Promega) and Moloney murine leukaemia virus reverse transcriptase (10 U/µl) (Promega), following the procedures suggested by the manufacturer. Negative controls, omitting the RNA or the reverse transcriptase, were included.

2.8. Primers and probes

The primers and probes were designed for bovine TLRs, based on sequences available in the GenBank database (<http://www.ncbi.nlm.nih.gov/>), using the Primer Premier software (PREMIER Biosoft International, Palo Alto, CA, USA). The sense and antisense primers for TLR3 and TLR9 were placed in two consecutive exons of the respective gene. The probe spanned the junction of the two exons, which were covered by the forward and reverse primers to ensure proper discrimination between cDNA and gDNA. Primers and probes for TLR7 and TLR8 were designed for the only exon that comprises these gene coding regions. Expression of the "housekeeping" gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control (McGuire et al., 2004). Each probe was labelled at the 5'-end with the reporter dye FAM (6-carboxyfluorescein) and at the 3'-end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine). All primers and probes were commercially synthesised (Eurofins MWG Operon, Huntsville, AL, USA). Details of the primers and probes are provided in Table 1.

2.9. Real-time RT-PCR

Real-time RT-PCR reactions for bovine GAPDH and TLR3, TLR7, TLR8 and TLR9 were run in separate wells. The PCR reactions contained 800 nM specific forward and reverse primers, 200 nM specific probe, 1X 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

Table 1

Sequences of primers and probes for viral DNA detection by *nested*-PCR according to Campos et al. (2009) and for TLR mRNA detection by real-time RT-PCR.

Primer	Primer sense or probe	Amplicon size (base pairs)	5'-3' sequences
BoHV-1/BoHV-5 (First round PCR)	F ^a	575/572	CGGCCACGACGCTGACGA CGCCGCCGAGTACTACCC
	R ^b		
BoHV-1 (Second round PCR)	F	161	CTAACATGGAGCGCGCTT CGGGCGCATGCCGTC
	R		
BoHV-5 (Second round PCR)	F	236	GTGGAGCGCCGCTTCGC TATCGCGGAGAGCAGGCCG
	R		
GAPDH	F	112	TTCTGGCAAAGTGGACATCGT CTTGACTGTGCCGTTGAACCTG
	R		
TLR3	Probe	143	ACATGGTCTACATGTTCCAGTATGATTCCACCC CAGGTCAACAGTCCCGAA
	F		
TLR7	R	144	GCAGCACATTCACCAT ACAGTAAGTGAAGGAAAACCATGAGCAGA
	Probe		
TLR8	F	117	TAAAACTCTGCCCTGTGATG CTTGCTATGTGGTTAATGGT
	R		
TLR9	Probe	113	ATTCTGCAATGCCACCAAC TTATTGAGAATGTAATGGTCCG
	F		
TLR9	R	113	GAAAGGATTCATTCTTACCC ACCCAAAACAGTGGACAAAAGATGTGACC
	Probe		
			AACCTGCCGCCAGACCTT GCCAGGGCCACTGCCAGTG ATTCCTGTCTATGGCCCTACTGTGCCCC

^a Forward primer.

^b Reverse primer.

nervous system of infected calves over levels detected in tissue sections of uninfected calves, which served as the control group.

2.10. 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 2–16 data points and evaluates the group differences for significance with a pair-wise fixed reallocation randomisation 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: $E = 10[-1/\text{slope}]$.

3. Results

3.1. Viral isolation and detection of viral DNA in nervous tissues

In calves acutely infected with BoHV-1 (Group 4), viral isolation was successful from all CNS sections evaluated (anterior and posterior cerebral cortex, cerebellum, medulla, pons and diencephalon) and from the TG. BoHV-1 and BoHV-5 were not isolated from tissue samples from the other treatment groups.

The distribution of viral DNA in different sections of the CNS and TG of calves in all experimental groups was investigated by nested-PCR. The frequency of the detection of viral DNA by this technique in neural tissues is presented in Table 2. In general, viral DNA was detected in the different areas of the anterior cortex and in the TG. Nevertheless, the frequency of positive animals for each neural region varied depending on the alpha-herpesvirus and the infection stage studied. BoHV-5 DNA was frequently detected in the frontal cortex (8/12), the olfactory cortex (6/12), the dorsolateral cortex (6/12) and the TG (6/12) of calves in Group 1 (acute infection). The distribution of BoHV-5 DNA in the brains of calves in Group 2 upon viral reactivation was similar to that of calves in Group 1 during acute infection, but the frequency of positivity decreased to 4/11 animals in the olfactory cortex and frontal cortex and to 3/11 animals in the dorsolateral cortex and TG. In latently infected calves (Group 3), BoHV-5 DNA was consistently detected in the TG (2/2) and in the anterior and dorsolateral cortex in 1/2 animals. Interestingly, in calves acutely infected with BoHV-1 (Group 4), the presence of viral DNA exhibited a wide distribution in the bovine nervous system. In this case, the virus genome was detected in the olfactory cortex (2/2), the frontal cortex (2/2), the dorsolateral cortex (1/2), the marginal groove area (1/2) and the ectomarginal groove area (1/2) of the posterior cerebral cortex, the cerebellum (2/2), the cervical medulla (2/2) and the TG (2/2). Similar to the detection of BoHV-5 DNA during acute infection and reactivation, in calves in Group 5 (BoHV-1 reactivation), viral DNA was detected in samples from the anterior cortex and the TG. The frequency of positive animals at this stage of the infection was 1/2 for the olfactory cortex and 2/2 for the frontal and dorsolateral cortex and TG. As expected, viral DNA was not detected in any of the samples from uninfected control animals.

In all samples in which viral DNA was not detected, the bovine cytochrome B gene, an internal control for the PCR assay, was amplified (data not shown).

3.2. TLR expression in nervous tissues

Messenger RNA expression levels of the major antiviral TLRs were analysed in the nervous tissue of infected calves at different stages of BoHV-5 and BoHV-1 infection and compared with the levels in non-infected control calves. The nervous system areas evaluated in this study were selected based on the presence of viral DNA, which was primarily detected in the anterior cortex and TG, as

Table 2
Detection of viral DNA by PCR in brain sections of calves inoculated with BoHV-5 or BoHV-1.

Group and treatment	Anterior cerebral cortex			Posterior cerebral cortex		Cerebellum	Medulla and pons		Diencephalon	Trigeminal Ganglia
	Olfactory	Frontal	Dorso-lateral	Marginal groove area	Ectomarginal groove area		Cervical	Oblongata		
Group 1 BoHV-5 Acute infection	6/12*	8/12	6/12	-	-	-	-	-	-	6/12
Group 2 BoHV-5 Latency	-	1/2	1/2	-	-	-	-	-	-	2/2
Group 3 BoHV-5 Latency and reactivation	4/11	4/11	3/11	-	-	-	-	-	-	3/11
Group 4 BoHV-1 Acute infection	2/2	2/2	1/2	1/2	1/2	2/2	2/2	-	-	2/2
Group 5 BoHV-1 Latency and reactivation	1/2	2/2	2/2	-	-	-	-	-	-	2/2
Group 6 Controls	-	-	-	-	-	-	-	-	-	-

* Ratio of calves in which viral DNA was detected in different areas of the nervous system.

-, Viral DNA was not detected in the area of the nervous system analysed.

previously described. These neural regions are frequent sites for the replication and latency of alpha-herpesviruses of cattle (Pérez et al., 2002; Vogel et al., 2003).

GAPDH expression levels remained constant in samples from calves in different experimental groups, and a linear relationship between the amount of the template and CT values was observed when the amplification efficiency for each gene was determined (data not shown). The relative levels of TLR mRNA and the statistical significance ($P < 0.05$) of the differences in TLR mRNA expression levels obtained for each receptor studied are detailed below.

3.2.1. TLR3

The relative expression of TLR3 in the CNS and TG of infected-calves is presented in Fig. 1A. Calves in Group 1, corresponding to BoHV-5 acute infection, showed an increase in the relative levels of TLR3 mRNA in the different areas of the CNS. TLR3 was strongly up-regulated in the dorsolateral cortex, with a statistically significant increase ($P < 0.05$) of 44-fold with respect to the control group. TLR3 levels in the olfactory and anterior cortex were 4- and 4.9-fold higher, respectively. However, statistically significant differences were not observed ($P > 0.05$). At this stage of infection, the relative levels of this receptor mRNA did not change in TG. During BoHV-5 reactivation (Group 2), TLR3 relative expression was 33-fold higher in the anterior cerebral cortex. TLR3 levels did not show significant differences ($P > 0.05$) in the other analysed areas of nervous tissue. However, TLR3 expression in the dorsolateral cortex and the TG was three- and twofold higher than in uninfected animals. In calves latently infected with BoHV-5 (Group 3), the relative levels of TLR3 mRNA did not change significantly ($P > 0.05$). Similar to the animals acutely infected with BoHV-5 (Group 1), during BoHV-1 acute infection (Group 4), an increase in the levels of TLR3 mRNA

in the cerebral cortex was observed. However, the magnitude of the increase was lower, only 11- and 13-fold in the olfactory and anterior cortex, respectively. Nevertheless, as in BoHV-5 infection, acute infection with BoHV-1 did not elicit a significant variation in TLR3 levels in TG ($P > 0.05$). During BoHV-1 reactivation, the relative TLR3 mRNA levels did not differ significantly ($P > 0.05$) in any area of the nervous system of infected animals (Group 5) when compared to uninfected calves.

3.2.2. TLR7

The study of the TLR7 expression in the different areas of the nervous system (Fig. 1B) revealed that there was an increase in the range of 3.5- to 9.5-fold in the relative mRNA levels in Group 1, corresponding to an acute BoHV-5 infection. These differences were statistically significant ($P < 0.05$) in the anterior and dorsolateral cortex. During BoHV-5 and BoHV-1 reactivation (Groups 2 and 5), the TLR7 expression was markedly up-regulated in the anterior cortex of the brain at a magnitude of 83- and 63-fold, respectively. The TLR7 expression was not affected in calves latently infected with BoHV-5 (Group 3) or acutely infected with BoHV-1 (Group 4). During acute BoHV-1 infection, TLR7 mRNA was not detectable in TG.

3.2.3. TLR8

The relative expression levels of TLR8 mRNA in the CNS and TG of infected calves are presented in Fig. 1C. The study of acute infection with BoHV-5 (Group 1) also revealed the up-regulation of TLR8 in the different evaluated areas of the CNS, with variations in the range of 9.9- to 12-fold higher in tissues from infected animals when compared with the levels in mock-infected calves. In Group 2, the relative levels of TLR8 mRNA increased by a factor of 43.8 in

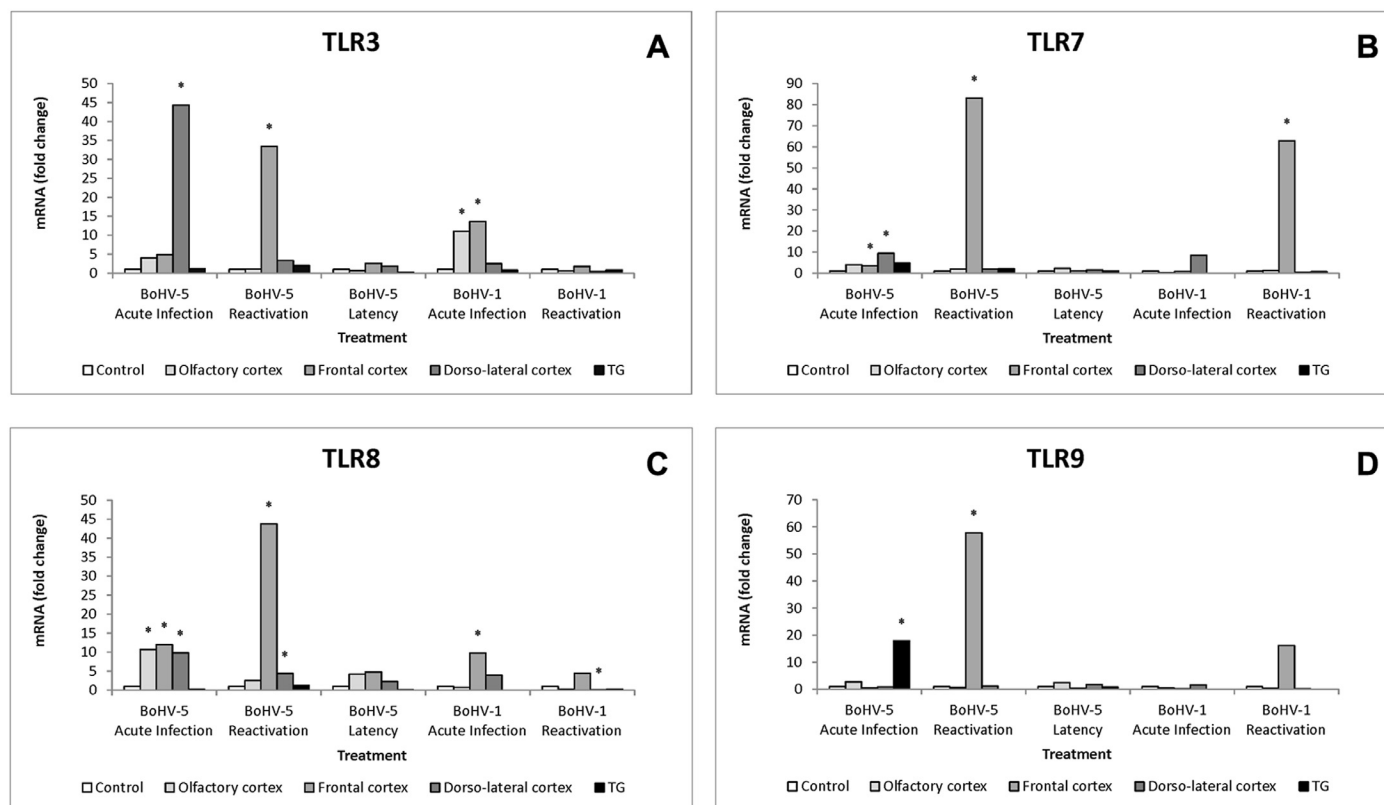


Fig. 1. Relative expression of TLR3 (A) and TLR7–9 (B–D) in the nervous system of BoHV-1- and BoHV-5-infected calves. The results represent the mean fold change of TLR transcription levels in the areas of the nervous system of infected calves over levels detected in tissue sections of uninfected calves, which served as the control group. TG, trigeminal ganglia; *statistically significant differences ($P < 0.05$) with respect to the uninfected control.

the anterior cortex area. Furthermore, a significant increase ($P < 0.05$) in the relative expression of TLR8 was observed in the dorsolateral cortex. However, this increase was only 10% higher with respect to the anterior cortex, by a factor of 4.4. Significant levels of TLR8 expression in the nervous tissues of calves in Group 3 (BoHV-5 latency) were not detected ($P > 0.05$). During acute infection with BoHV-1 (Group 4), a significant increase (9.8-fold) ($P < 0.05$) in the anterior cortex was determined. In this group of calves, TLR8 mRNA was not detected in TG ($P > 0.05$). During BoHV-1 reactivation (Group 5), a significant decrease in the relative expression levels in the dorsolateral cortex was observed.

3.2.4. TLR9

Relative TLR9 mRNA levels were significantly higher (18-fold) ($P < 0.05$) in the TG of BoHV-5-infected animals during acute infection compared to the levels detected in control animals (Fig. 1D). However, during BoHV-1 acute infection, the relative levels of TLR9 were under the limit of detection in the TG of infected calves. Similar to the findings during BoHV-5 acute infection, it was also possible to detect TLR9 transcripts in the TG of calves in Groups 2 and 5, which corresponds to the studies of viral reactivation in calves infected with BoHV-5 and BoHV-1, respectively. These results demonstrate that the receptor levels increase during reactivation. However, the relative levels of TLR9 could not be estimated because the receptor expression was undetectable in the TG of uninfected calves. Interestingly, TLR9 was the only receptor for which the expression was significantly affected after infection of the TG by alpha-herpesviruses. With respect to the areas of the cerebral cortex evaluated in this study, only the anterior cortex showed an increase (58-fold) in the relative expression of TLR9 during reactivation of BoHV-5. During BoHV-1 reactivation, a 16-fold increase in the relative levels of the transcripts was determined. Nevertheless, the differences observed were not statistically significant ($P > 0.05$).

4. Discussion

The neuropathogenesis of BoHV-5 is not completely understood. However, multiple factors, either host, viral or environmental, likely influence the development of clinical signs and neuropathology (Brum et al., 2010). Previous studies were primarily focused on the role of envelope glycoproteins in neurovirulence (Chowdhury et al., 2000, 2002; Kaashoek et al., 1998). Nevertheless, very little has been done to determine the role of other viral products or host factors. Although the participation of innate immunity in several infectious diseases is now widely recognised, the link between TLRs and viral animal diseases has not been extensively analysed. The focus of this work was to analyse the expression levels of TLR mRNA in cattle experimentally inoculated with BoHV-1 and BoHV-5 as an initial step to find a relationship between TLRs and the development of neurologic disease. The most relevant finding of this study was the significant variation in the expression levels of TLRs involved in viral nucleic acid recognition in the CNS and TG from BoHV-5- and BoHV-1-infected calves during acute infection and viral reactivation. To date, only a few studies have been performed to address the expression of bovine TLRs that detect viral nucleic acids (Griebel et al., 2005; Marin et al., 2014; Schneberger et al., 2011). Furthermore, the association of these receptors with BoHV infection has not been analysed yet.

In this study, an increase was found in the relative expression of TLR3, TLR7 and TLR8 in the anterior cortex of BoHV-5-infected calves, both during acute infection and viral reactivation. The relative levels of TLR9 mRNA were higher only during BoHV-5 reactivation. For calves infected with BoHV-1, an increase in the expression of these receptors in the anterior cortex was also observed. Nevertheless, the magnitude of this change was lower when compared to the changes observed in BoHV-5 infections. During

acute BoHV-1 infection, an increase in the relative levels of TLR3 and TLR8 was detected. However, during reactivation of BoHV-1, only increased mRNA levels of TLR7 were observed. The TG is the major site of latency of alpha-herpesviruses (Meyer et al., 2001; Pérez et al., 2002). In this area of the peripheral nervous system, TLR9 was the only receptor that was significantly affected by viral infection. The relative mRNA levels of TLR9 transcripts increased in animals acutely infected with BoHV-5. TLR9 transcripts were also detected in TG during dexamethasone-induced reactivation of both alpha-herpesviruses, suggesting that this receptor might play a role during viral reactivation.

In a previous study, Pérez et al. (2002) observed that the nature and location of CNS lesions caused by BoHV-5 were similar during acute infection and viral reactivation. At both stages of infection, lesions were more severe in the anterior cerebral cortex. The findings of microscopic changes, along with the detection of viral DNA in this area, indicate that this region of the nervous system is relevant for studies of neuro-immunological responses in herpesvirus encephalitis.

Alpha-herpesviruses replicate rapidly and produce a cytopathic effect in cell cultures. However, in this work, BoHV-5 was not isolated from any neural tissue samples. The failure to isolate BoHV-5 from this type of sample has also been reported by Cascio et al. (1999) and Pérez et al. (2002). It is possible that the low to moderate viral titres in neural tissue (Belknap et al., 1994) are responsible for the difficulty in isolating BoHV-5. In this study, the possibility of detecting viral DNA by PCR in nervous tissues confirmed the importance of applying molecular techniques to improve the sensitivity of the tests used for the identification of these pathogens.

Interestingly, the neuroinvasion after experimental acute infection with BoHV-1, which was achieved after the inoculation of a high dose of viral inoculum, was truly extensive. The wide distribution of BoHV-1 DNA in the CNS a few days after infection correlates with the recovery of infectious virus from all tissue samples. During latent infection and the subsequent reactivation of BoHV-1, the viral distribution in the CNS was restricted, showing that the host was able to contain the spread during reactivation episodes. Usually, only the reactivation of BoHV-1 is subclinical (Muyilkens et al., 2007). The differences between BoHV-1 and BoHV-5 clinical profiles might be related to the efficiency of the components of the immune system in the containment of virus reactivation. In a natural infection by the respiratory route, both BoHV-1 and BoHV-5 replicate at similar levels in the nasal mucosa. However, striking differences may be observed in their neuroinvasiveness (Del Médico Zajac et al., 2010; Meyer et al., 2001; Pérez et al., 2002). Unlike the findings in this study, it has been previously reported that BoHV-1 only reaches the first-order neurons located in the TG where latency is established (Meyer et al., 2001). Consequently, viral encephalitis caused by BoHV-1 is not frequently observed.

The results presented here demonstrate, for the first time, the involvement of TLRs 3, 7, 8 and 9 in the recognition of BoHV-1 and BoHV-5 in bovine nervous tissues. The involvement of these receptors in viral encephalitis caused by Herpes Simplex Virus (HSV) in humans has been previously documented. It has been demonstrated that a deficiency of TLR3 or some of the key molecules in the TLR3 signalling pathway would be associated with the development of the neurological disease caused by HSV-1 (Pérez de Diego et al., 2010; Sancho-Shimizu et al., 2011). Protective immunity against HSV-1 has also been attributed to TLR7, TLR8 and TLR9 because mutations in key molecules of their signalling pathways predispose hosts to encephalitis (Casrouge et al., 2006). Paradoxically, the expression of TLR3 was associated with the development of neurological disease caused by West Nile virus in humans. TLR3 leads to the leakiness of the blood–brain barrier as a result of the inflammatory response to the virus, allowing the infection to progress to the brain

(Wang et al., 2004). Thus, the importance of the TLR-dependent immune response might be associated with the type of pathogen involved and its ability to subvert or exploit these pathways for its own benefit.

In comparison to BoHV-5, in this study, a reduced number of calves were experimentally inoculated with BoHV-1. However, the results obtained from this group of animals indicate that the magnitude of the increase in mRNA expression levels of the four viral TLRs analysed was always lower in BoHV-1-infected calves. This was particularly evident during acute infection and reactivation from latency. Therefore, the results from this study suggest that the increased expression of these receptors in the nervous system might be associated with the development of neurological disease with BoHV-5. It can be speculated that a restricted and more controlled immune response in BoHV-1 infection might protect against exacerbated responses that would contribute to the development of neurological lesions and clinical signs. This hypothesis is also supported by studies performed with the prototype of alpha-herpesviruses, HSV. It has been suggested that the timing of the activation of the TLR9 response following HSV infection and its subsequent modulation are crucial for a well-controlled immune response during encephalitis. Recently, Boivin et al. (2012) reported that the neutralisation of TLR9 activity after an infection in mice decreased mortality, likely by controlling the exaggerated inflammatory response in the brain. Interestingly, in the present study, the expression of TLR9 significantly increased in the TG during acute infection and in the CNS during reactivation only in BoHV-5-infected calves. Multiple TLRs are up-regulated in the CNS in response to pathogen infections (Aravalli et al., 2007; McKimmie et al., 2005). Of these receptors, several have been shown to contribute to neuroinflammatory responses and pathogenesis, including TLR2, TLR3, TLR4, and TLR9 (Aravalli et al., 2005; Kurt-Jones et al., 2004; Pedras-Vasconcelos et al., 2006; Wang et al., 2004). TLR7 and TLR8 are also important in initiating the innate immune response in the CNS, as determined in this study. However, there is still a lack of basic understanding of the neuroinflammatory properties of these receptors (Butchi et al., 2008). Thus, in the future, modulation of the signalling pathways mediated by TLRs might provide an effective approach to control the neuro-immune response that might occur during the encephalitis induced by BoHV-5 and which could be, in part, responsible for the development of the neurological disease. The complex interrelation of the components involved in the innate and adaptive immune defences in mammals necessitates further study to evaluate the way in which the activation of the different TLRs contributes to the restriction of herpesvirus infections or their participation in the development of disease.

This study provides an initial analysis of viral TLRs that might be involved in herpesvirus encephalitis in cattle. An increase in the expression of TLR3 and TLRs 7–9 during infection by BoHV-1 and BoHV-5 in the bovine nervous system has been demonstrated. Nevertheless, it is unknown whether this increase is due to a higher expression level of the receptors as a consequence of the infection in neural cells, in infiltrating immune cells, or in both cell types. In this case, additional studies are required to detect the variation in the expression levels and cell co-localisation. Overall, the results presented here elucidate immune factors triggered in the host that determine the different outcomes of infection by two closely related alpha-herpesviruses of cattle.

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References

- Abril, C., Engels, M., Liman, A., Hilbe, M., Albin, S., Franchini, M., et al., 2004. Both viral and host factors contribute to neurovirulence of bovine herpesviruses 1 and 5 in interferon receptor-deficient mice. *Journal of Virology* 78, 3644–3653.
- Aravalli, R., Hu, S., Rowen, T., Palmquist, J., Lokensgard, J., 2005. Cutting edge: TLR2-mediated proinflammatory cytokine and chemokine production by microglial cells in response to herpes simplex virus. *The Journal of Immunology* 175, 4189–4193.
- Aravalli, R., Peterson, P., Lokensgard, J., 2007. Toll-like receptors in defense and damage of the central nervous system. *Journal of Neuroimmune Pharmacology* 2, 297–312.
- Belknap, E., Collins, J., Ayers, V., Schultheiss, P., 1994. Experimental infection of neonatal calves with neurovirulent bovine herpesvirus type 1.3. *Veterinary Pathology* 31, 358–365.
- Boivin, N., Menasria, R., Piret, J., Boivin, G., 2012. Modulation of TLR9 response in a mouse model of herpes simplex virus encephalitis. *Antiviral Research* 96, 414–421.
- Borrow, B., Martínez-Sobrido, L., De la Torre, J., 2010. Inhibition of the type I interferon antiviral response during arenavirus infection. *Viruses* 2, 2443–2480.
- Brum, M., Bezerra dos Santos, C., Weiblen, R., Flores, E., 2010. Selection and characterization of brivudin resistant bovine herpesvirus type 5. *Brazilian Journal of Microbiology* 41, 124–132.
- Butchi, N., Pourciau, S., Du, M., Morgan, T., Peterson, K., 2008. Analysis of the neuroinflammatory response to TLR7 stimulation in the brain: comparison of multiple TLR7 and/or TLR8 agonists. *The Journal of Immunology* 180, 7604–7612.
- Campos, F., Franco, A., Hübner, S., Oliveira, M., Silva, A., Esteves, P., et al., 2009. High prevalence of co-infections with bovine herpesvirus 1 and 5 found in cattle in southern Brazil. *Veterinary Microbiology* 139, 67–73.
- Carrillo, B., Ambrogio, A., Schudel, A., Vasquez, M., Dahme, E., Pospischil, A., 1983. Meningoencephalitis caused by IBR virus in calves in Argentina. *Zentralblatt für Veterinärmedizin. Reihe B. Journal of Veterinary Medicine. Series B* 30, 327–332.
- Cascio, K., Belknap, E., Schultheiss, P., Ames, A., Collins, J., 1999. Encephalitis induced by bovine herpesvirus 5 and protection by prior vaccination or infection with bovine herpesvirus 1. *Journal of Veterinary Diagnostic Investigation* 11, 134–139.
- Casrouge, A., Zhang, S., Eidenschenk, C., Jouanguy, E., Puel, A., Yang, K., et al., 2006. Herpes simplex virus encephalitis in human UNC-93B deficiency. *Science* 314, 308–312.
- Chowdhury, S., Lee, B., Ozkul, A., Weiss, M., 2000. Bovine herpesvirus 5 glycoprotein E is important for neuroinvasiveness and neurovirulence in the olfactory pathway of the rabbit. *Journal of Virology* 74, 2094–2106.
- Chowdhury, S., Onderci, M., Bhattacharjee, P., Al-Mubarak, A., Weiss, M., Zhou, Y., 2002. Bovine herpesvirus 5 (BHV-5) Us9 is essential for BHV-5 neuropathogenesis. *Journal of Virology* 76, 3839–3851.
- Del Médico Zajac, M., Ladelfa, M., Kotsias, F., Muylkens, B., Thiry, J., Thiry, E., et al., 2010. Biology of bovine herpesvirus 5. *The Veterinary Journal* 184, 138–145.
- Griebel, P., Brownlie, R., Manuja, A., Nichani, A., Mookherjee, N., Popowych, Y., et al., 2005. Bovine toll-like receptor 9: a comparative analysis of molecular structure, function, and expression. *Veterinary Immunology and Immunopathology* 108, 11–16.
- Kaashoek, M., Rijsewijk, F., Ruuls, R., Keil, G., Thiry, E., Pastoret, P., et al., 1998. Virulence, immunogenicity and reactivation of bovine herpesvirus 1 mutants with a deletion in the gC, gG, gI, gE, or in both the gI and gE gene. *Vaccine* 16, 802–809.
- Konat, G., Kielian, T., Marriott, L., 2006. The role of Toll-like receptors in CNS response to microbial challenge. *Journal of Neurochemistry* 99, 1–12.
- Kong, Y., Le, Y., 2011. Toll-like receptors in inflammation of the central nervous system. *International Immunopharmacology* 11, 1407–1414.
- Kurt-Jones, E., Chan, M., Zhou, S., Wang, J., Reed, G., Bronson, R., et al., 2004. Herpes simplex virus 1 interaction with toll-like receptor 2 contributes to lethal encephalitis. *Proceedings of the National Academy of Sciences of the United States of America* 101, 1315–1320.
- Marin, M., Quintana, S., Faverin, C., Leunda, M., Odeón, A., Pérez, S., 2014. Toll-like receptor activation and expression in bovine alpha-herpesvirus infections. *Research in Veterinary Science* 96, 196–203.
- McGuire, K., Manuja, A., Russell, G., Springbett, A., Craigmile, S., Nichani, A., et al., 2004. Quantitative analysis of pro-inflammatory cytokine mRNA expression in *Theileria annulata*-infected cell lines derived from resistant and susceptible cattle. *Veterinary Immunology and Immunopathology* 99, 87–98.
- McKimmie, C., Johnson, N., Fooks, A., Fazakerley, J., 2005. Viruses selectively upregulate Toll-like receptors in the central nervous system. *Biochemical and Biophysical Research Communications* 336, 925–933.
- Meyer, G., Lemaire, M., Ros, C., Belak, K., Gabriel, A., Cassart, D., et al., 2001. Comparative pathogenesis of acute and latent infections of calves with bovine herpesvirus types 1 and 5. *Archives of Virology* 146, 633–652.
- Mogensen, T., 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews* 22, 240–273.
- Muylkens, B., Thiry, J., Kirten, P., Schyns, F., Thiry, E., 2007. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. *Veterinary Research* 38, 181–209.
- Pérez de Diego, R., Sancho-Shimizu, V., Lorenzo, L., Puel, A., Plancoulaine, S., Picard, C., et al., 2010. Human TRAF3 adaptor molecule deficiency leads to impaired Toll-like receptor 3 response and susceptibility to herpes simplex encephalitis. *Immunity* 33, 400–411.
- Pérez, S., Bretschneider, G., Leunda, M., Osorio, F., Flores, E., Odeón, A., 2002. Primary infection, latency and reactivation of bovine herpesvirus type 5 (BHV-5) in the bovine nervous system. *Veterinary Pathology* 39, 437–444.

- Pedras-Vasconcelos, J., Goucher, D., Puig, M., Tonelli, L., Wang, V., Ito, S., et al., 2006. CpG oligodeoxynucleotides protect newborn mice from a lethal challenge with the neurotropic Tacaribe arenavirus. *The Journal of Immunology* 176, 4940–4949.
- Pfaffl, M., Horgan, G., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* 30, e36.
- Ransohoff, R., Brown, M., 2012. Innate immunity in the central nervous system. *The Journal of Clinical Investigation* 122, 1164–1171.
- Reed, L., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. *American Journal of Hygiene* 27, 493–497.
- Roels, S., Charlier, G., Letellier, C., Meyer, G., Schynts, F., Kerkhofs, P., et al., 2000. Natural case of bovine herpesvirus 1 meningoencephalitis in an adult cow. *The Veterinary Record* 146, 586–588.
- Salvador, S., Lemos, R., Riet-Correa, F., Roehe, P., Osório, A., 1998. Meningoencefalite em bovinos causada por herpesvírus no Mato Grosso do Sul e São Paulo. *Pesquisa Veterinária Brasileira* 18, 76–83.
- Sancho-Shimizu, V., Pérez de Diego, R., Lorenzo, L., Halwani, R., Alangari, A., Israelsson, E., et al., 2011. Herpes simplex encephalitis in children with autosomal recessive and dominant TRIF deficiency. *The Journal of Clinical Investigation* 12, 4889–4902.
- 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. *Journal of Agricultural and Food Chemistry* 55, 305–310.
- Schneberger, D., Lewis, D., Caldwell, S., Singh, B., 2011. Expression of toll-like receptor 9 in lungs of pigs, dogs and cattle. *International Journal of Experimental Pathology* 92, 1–7.
- 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 Research* 129, 191–199.
- Vogel, F., Caron, L., Flores, E., Weiblen, R., Winkelmann, E., Mayer, S., et al., 2003. Distribution of bovine herpesvirus type 5 DNA in the central nervous systems of latently, experimentally infected calves. *Journal of Clinical Microbiology* 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. *Nature Medicine* 10, 1366–1373.
- Weiblen, R., Lombardo de Barros, C., Canabarro, T., Flores, I., 1989. Bovine meningoencephalitis from IBR virus. *The Veterinary Record* 124, 666–667.