Tissular Distribution of Argentinean Strains of Bovine Herpesvirus Type 4 (BoHV-4) in Experimentally-Infected Calves

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Although bovine herpesvirus type 4 (BoHV-4) is primarily associated with reproductive disorders of cattle, it can produce a variety of clinical signs. To determine the distribution, the presence and type of microscopic lesions caused by BoHV-4 strains of different genotypes, calves were infected with three phylogenetically different Argentinean BoHV-4 strains. Samples from nasal and ocular secretions, peripheral blood leukocytes, tissues and serum were analyzed. BoHV-4 was isolated from nasal and ocular secretions at 7 and 14 days post-inoculation (dpi). Viral DNA was detected by nested PCR in peripheral blood leukocytes at 14 and 21 dpi for two out of three strains and in tissues, such as nervous system, trachea, pulmonary and retropharyngeal lymph nodes, spleen and kidney, at 21 dpi. Antibody levels detected by viral seroneutralization test were mostly low and varied widely for the different strains. The tissue distribution of the BoHV-4 strains and the variations observed in the levels of neutralizing antibodies indicate that certain differences can be established among the patterns of biological behavior of each strain. This is an initial step to get insight into the biological characteristics of Argentinean BoHV-4 isolates. However, further evaluation involving a higher number of inoculated animals will be required to be conclusive on this aspect.

Key words: BoHV-4; Argentinean strains; Tissue Distribution; Cattle

INTRODUCTION

Bovine herpesvirus type 4 (BoHV-4) is a member of the family Herpesviridae (order Herpesvirales), subfamily Gammaherpesvirinae (Lomonte et al. 1996). BoHV-4 has been isolated from cattle with respiratory infections, vulvovaginitis, mastitis, abortions, endometritis and apparently healthy animals throughout the world (Wellemans et al. 1986, Miyano et al. 2004, Izumi et al. 2006, Bilge-Dagalt et al. 2010, 2012).

This virus has not yet been established as the causal agent of a particular disease entity; however, it is primarily associated with reproductive disorders of cattle, particularly in the postpartum period (Chastant-Maillard 2013).

Unlike other gammaherpesviruses, BoHV-4 can infect a wide range of domestic and wild ruminant species, such as American bison (Bison bison), buffaloes, goats, sheep, deer, and non-ruminant species like owl monkeys (Aotus trivirgatus), lions, domestic cats and rabbits (Kruger et al. 2000, Lin et al. 2000, Kálmán & Egyed 2005, Dewals et al. 2006). Previous works carried on natural- and experimentally-infected animals have
shown that BoHV-4 shows an irregular pattern of dissemination in bovine tissues (Naem et al. 1991, Eyged et al. 1996, Asano et al. 2003, Fabian et al. 2005, Campos et al. 2014). It has also been reported that there are genomic variations among BoHV-4 isolates from different regions. Emerging strains, whose restriction patterns do not fully match to the American (DN599 and 66-p-347) and European (V test, and Movar LVR140) reference strains groups have also been described (Bublot et al. 1990, Peleira et al. 2011). This variability is attributed to recombination events among strains and within the same strain, mainly in polyrepetitive DNA (prDNA) sequences located on both sides of the long unique region (LUR) of the genome, during viral replication (Bublot et al. 1991b, Donofrio et al. 2000a, Nikolin et al. 2007). In Argentina, this viral agent was virtually unknown until its detection and identification in 2007 in samples of cervical-vaginal mucus (CVM) from aborted cows (Verna et al. 2008), by the Specialized Veterinary Diagnostic Service of INTE Balcarce. Thereafter, the virus was isolated from nasal swabs, lungs, granulosa cells, oocytes (González Altamiranda et al. 2015) and bovine semen (Lomónico et al. 2009, Morán et al. 2013). To date, more than 50 isolates have been obtained in our country, mainly associated with abortions in bovine females. Genomic analyses of these Argentinean strains demonstrated that they are highly divergent and can be classified in three different groups: Genotype 1 comprises Movar-like strains (European group), Genotype 2 includes DN599-like strains (American group) and Genotype 3 corresponds to a novel genotype (Verna et al. 2012).

Considering that there are not previous data about the characteristics of the in vivo infection by Argentinean BoHV-4 strains, the aim of this study was to evaluate the tissue distribution, the presence and type of microscopic lesions of three phylogenetically different Argentinean BoHV-4 strains, in experimentally-infected calves. Information about the biological behaviour of this pathogen might contribute to the understanding of the pathogenic role of each strain or phylogenetic group in cattle.

MATERIALS AND METHODS

Cell Culture

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 (MEM) with Earle salts (E-MEM) (Sigma-Aldrich, Saint Louis, MO, USA), supplemented with 10% fetal bovine serum (Bioser, Buenos Aires, Argentina) and antibiotic-antimycotic solution (Gibco, Langley, OK, USA). Cells were incubated at 37 °C in a 5% CO₂ atmosphere.

Virus Strains and Experimental Inoculation of Calves

BoHV-4 strains belonging to different phylogenetic groups were selected for the experimental assay. Strains 09/508 (genotype 1), 08/330 (genotype 2) and 07/435 (genotype 3) (Verna et al. 2012), displayed distinctive in vitro activity when their biological behavior was analyzed in different cell cultures (Verna et al. 2016, Morán unpublished results). Viral stocks were propagated in MDBK cells, in T-25 flasks (NEST Biotechnology, China) (1 × 10⁶ cells/ml), for 48 h. Supernatants were harvested and frozen at −80°C. Virus titres were determined by the endpoint titration method and expressed as tissue culture infective doses (TCID₅₀), according to Reed & Muench (1938). Four crossbred, male, 6 month-old calves were used in this study. All animals were free of detectable antibodies to BoHV-1. Low neutralizing antibody titres to some Argentinean BoHV-4 strains were detected prior to animal inoculation. This is a frequent finding even when a high number of animals is evaluated (Verna, unpublished results). Therefore, calves which were not harboring BoHV-4 genome in peripheral blood leukocytes, as demonstrated by PCR, were selected for this study. Calves were intranasally inoculated by aerosolization with 20 ml of 10⁶ TCID₅₀ of BoHV-4. Ten ml of the inoculum was distributed in each nostril. The animals were inoculated as follows: Calf 1 (C1) 09/508 strain, Calf 2 (C2) 08/330 strain, Calf 3 (C3) 07/435 strain and Calf 4 (C4) mock-infected, inoculated with MEM as placebo. Calves were maintained in isolated pens and fed on grass hay and commercial concentrate foodstuff and water ad libitum. They were euthanized at 21 dpi. All animals were monitored daily for rectal temperature, presence of nasal and ocular discharges and any other clinical sign. Calves were deeply anesthetized and euthanized according to the regulations of the Institutional Committee for Care and Use of Experimental Animals of INTA and the Animal Welfare Committee of the University of the Center of Buenos Aires Province (Res. 087/02), Argentina, where these experiments were carried out.

Sample Collection

Nasal and ocular secretions, leukocytes from peripheral blood and serum samples were collected on 0, 7, 14 and 21 dpi for viral genome detection, virus isolation and determination of neutralizing antibody titres. At necropsy, on 21 dpi, tissues were aseptically and individually collected for virus isolation, genome detection and histopathology. The following tissues were evaluated: lung, spleen, nasal epithelium, liver, medulla oblongata, pulmonary lymph node, tracheal lymph node, palatine tonsil, frontal and piriform cerebral lobes, trigeminal ganglia, trachea, retropharyngeal lymph node and kidney. Samples were frozen at −80 °C or stored in 10% buffered-formalin for histopathology.

Serology

The presence of neutralizing antibodies was evaluated by microtitration (Van Der Maaten et al. 1985) on MDBK cells, in 96-well plates. The serum samples of the inoculated calves were tested with the homologous BoHV-4 and the BoHV-1 reference strain Los Angeles. Neutralizing antibody titres were determined
as the highest dilution that completely inhibited cytopathic effect (CPE) after 72 h of incubation. A three-fold or a higher increase in the titre of neutralizing antibodies was considered as an indicator of seroconversion due to infection.

**Virus Isolation**

Nasal and ocular secretions were diluted (1:5) in MEM supplemented with penicillin G (200 U/ml) and streptomycin sulfate (200 µg/ml). Tissue samples were homogenized in MEM (10% w/v), and the suspensions were centrifuged at 3,000 ×g for 30 min at 4 °C. Twenty microliters of supernatant were inoculated in quadruplicate into monolayers of MDBK cells in 96-well plates (NEST Biotechnology, China) and incubated at 37 °C. Samples were passaged 4 times, 3 days for each passage, and monitored daily for CPE. Virus titres in the samples were evaluated at the first detection of virus CPE and determined, as mentioned above, by the end-point titration method, in 96 well-plates and expressed as TCID$_{50}$/ml.

**Detection of BoHV-4 DNA by Nested PCR**

DNA from tissue samples and peripheral blood leukocytes and nasal and ocular secretions was extracted by the phenol chloroform method. DNA concentration was determined by spectrophotometry in a Nano Drop 2000 (Thermo Scientific-USA) at an absorbance of 260 nm.

The presence of BoHV-4 DNA was evaluated by nested PCR assay, adapted from Fabian & Egyed (2004), targeting the ORF 25 (major capsid protein). The PCR amplifications were carried out in 25 µl reaction mixture containing 1 µl of 5 mM deoxynucleotides triphosphate (Promega), 1µl of each 15 pmol primer, 1.25 µl of 50mM MgCl$_2$ (PB-L, Argentina); 0.25 µl of 5U/µl Taq DNA polymerase (PB-L, Argentina), 2.5 µl of 10 x buffer (PB-L) and water. For the second amplification round, 2 µl of the first round PCR product were used. The primers used for the amplifications were BoG3 5’GACTATGAGGAATGGCACAAG3’ and BoGe 5’TACTCGTAGGCTGGTGCTG3’ in the first round, and B4up 5’GGTTGGAAGTAGGAGGTATGAT3’ and B4low 5’GTAGGGCGGGGTCTGGAAT3’ in the second (Thermo Fisher Scientific, Argentina). The PCR products were 737 bp and 271 bp for the first and the second round, respectively. The amplifications were carried out in a Veriti Thermal Cycler (Applied Biosystem - USA) as follows: first round consisted of 94 °C 15 min.; (94 °C 45 s; 56 °C 45 s; 72 °C 90 s) for 25 cycles. The second amplification round was (94 °C 45 s; 56 °C 45 s; 72 °C 60 s) for 30 cycles and one extension cycle at 72 °C for 10 min.

DNA from uninfected MDBK cells and PCR mix without DNA sample were used as negative controls. DNA from BoHV-4 12/365-infected MDBK cells was used as positive control. The PCR products were separated by electrophoresis in 1.2% agarose gels and visualized by SYBR® Safe DNA gel stain (Thermo Fisher Scientific, Argentina) under ultraviolet light.

**Histopathology**

Tissue samples were fixed in 10% neutral buffered formalin, paraffin-embedded and stained with hematoxylin and eosin (H&E) using routine methods.

**RESULTS**

**Clinical Findings**

Clinical signs were not observed in any animals, except for mild nasal secretions which were present in C1 at 24 hpi. Individual rectal temperatures were recorded. The highest rectal temperature (41.7 °C) was recorded at 14 dpi in C2, inoculated with strain 08/330.

**Isolation of BoHV-4**

BoHV-4 strains 08/330 and 07/435 were isolated from nasal secretions at 7 and 14 dpi and the strain 09/508 was isolated at 14 dpi. Strain 08/330 was isolated from ocular secretions at 7 dpi. The identity of all isolates was confirmed by nested PCR. The highest virus titres (6.41 and 5.79) were recorded for strain 08/330 in nasal and ocular secretions at 7 dpi (Table 1). Nevertheless, in comparison with the other strains, isolate 09/508 had a higher virus titre in nasal secretion at 14 dpi, the only time point at which the virus was isolated. None of the strains were isolated from tissues or leukocytes from inoculated or mock-infected calves.

**Genome Detection**

Viral DNA was detected in leukocytes from C1 at 21 dpi and C2 at 14 and 21 dpi. As shown in Table 2, viral DNA was also detected in spleen, medulla, trigeminal ganglia, trachea and piriform cerebral lobe of C1; spleen, trachea, kidney, retropharyngeal lymph node, frontal cerebral lobe of C2 and from pulmonary lymph node, frontal and piriform cerebral lobes of C3. Viral genome was not detected in samples from ocular and nasal secretions. As expected, viral DNA was not detected in tissues from mock-infected calf.

**Microscopic Findings**

At the time of euthanasia, macroscopic lesions were not observed in any calf. Microscopic observation of tissues revealed mild mononuclear inflammatory response in tracheal sub-mucosa and in the capsule of retropharyngeal lymph node of obtain basic information on the tissue distribution and the
### Table 1. Isolation and virus titres (expressed as log_{10} TCID_{50}/ml) of BoHV-4 from ocular and nasal secretions

<table>
<thead>
<tr>
<th>Date dpi</th>
<th>C1 (09/508) OS</th>
<th>NS</th>
<th>C2 (08/330) OS</th>
<th>NS</th>
<th>C3 (07/435) OS</th>
<th>NS</th>
<th>C4 OS</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
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<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>+ 5.79</td>
<td>+ 6.41</td>
<td>-</td>
<td>+ 2.36</td>
<td>-</td>
<td>-</td>
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<tr>
<td>14</td>
<td>-</td>
<td>+ 4.05</td>
<td>-</td>
<td>+ 1.97</td>
<td>-</td>
<td>+ 2.36</td>
<td>-</td>
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<td>21</td>
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</tr>
</tbody>
</table>

OS: ocular secretions, NS: nasal secretions

### Table 2. Detection of BoHV-4 DNA by nested-PCR and isolation from tissue samples

<table>
<thead>
<tr>
<th>Tissue Sample</th>
<th>C1 (09/508)</th>
<th>C2 (08/330)</th>
<th>C3 (07/435)</th>
<th>C4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCR</td>
<td>Isol</td>
<td>PCR</td>
<td>Isol</td>
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<tr>
<td>Lung</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spleen</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Nasal epithelium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Medulla</td>
<td>+</td>
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<tr>
<td>Pulmonary lymph node</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
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<tr>
<td>Tracheal lymph node</td>
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<tr>
<td>Tonsil</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>CNS- frontal lobe</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Trigeminal ganglia</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Trachea</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Retropharyngeal lymph node</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CNS - piriform lobe</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ND: Not done

### Table 3. Antibodies titres to BoHV-4 strains and BoHV-1 determined by VNT

<table>
<thead>
<tr>
<th>Calf</th>
<th>C1 (09/508)</th>
<th>C2 (08/330)</th>
<th>C3 (07/435)</th>
<th>C4</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpi</td>
<td>0 7 14 21</td>
<td>0 7 14 21</td>
<td>0 7 14 21</td>
<td>0 7 14 21</td>
</tr>
</tbody>
</table>

### Strain

- **08/330**: 32 32 32 32 64 64 128 128 128 128 64 128 128 128 64
- **07/435**: 16 16 8 8 4 8 8 16 16 16 8 32 - 8 - 8
- **09/508**: - - 8 32 - 4 4 4 8 8 4 8 - 4 4 4
- **BoHV1**: - - - - - - - - - - - - - - - -
of C1. Furthermore, mild gliosis, shrunk neurons with pyknotic nuclei, eosinophilic cytoplasm and chromatolysis were observed in trigeminal ganglia from the same calf (Figure 1). In C3, slight mononuclear cell infiltrate was present in the capsular connective tissue of the pulmonary lymph node. Neuronal hyperchromatosis in the frontal cerebral lobe and mild satellitosis in the piriform lobe were also observed (Figure 2). Histological lesions were not present in samples from C2 and C4.

Serological Findings

Significant changes in antibody titres were not observed when the sera from all calves were tested with the homologous strains. As shown in Table 3, seroconversion to the homologous strain was only detected in C1 at 21 dpi. Antibody titres at 0 dpi were detected in calves challenged with strains 08/330 and 07/435. Nonetheless, it is important to highlight the highest neutralizing antibody titres to strain 08/330 detected in all animals. Low antibody titres (4 - 32) to 09/508 and 07/435 strains were detectable in C2, C3 and C4. Neutralizing antibodies to BoHV-1 were not detected.

DISCUSSION AND CONCLUSIONS

Previous studies on BoHV-4 have shown an irregular pattern of in vivo replication; the virus replicates in different tissues of experimentally-infected animals, both in cattle and non-ruminant species (Naeem et al. 1991, Egyed et al. 1996, 1997, Boerner et al. 1999). The objective of this work was to presence of lesions of field Argentinean BoHV-4 strains in their natural host. Even when caution must be taken in reaching general conclusions, due to the low number of animals used, it is important to highlight that specific organs where the virus genome can be detected were identified in this in vivo model and slight differences among isolates were observed.

Viral isolation in cell culture is the gold standard method for diagnosis of a virus infection; however, in some circumstances the variability in the rate of replication and the presentation of CPE of the different viral strains make this methodology laborious and difficult to perform. In contrast to other gammaherpesviruses, BoHV-4 grows efficiently in cell culture. However, a significant variation in the kinetics of replication of different BoHV-4 isolates has been reported by our research group (Verna et al. 2016). Although only the strain 08/330 was isolated from ocular secretions, all BoHV-4 strains were isolated from nasal secretions after inoculation. In agreement with Fábián et al. (2005), isolation of BoHV-4 from leukocytes was not demonstrated in this in vivo study; nevertheless two out of three infected calves harboring viral DNA in leukocytes were detected. None of the strains was isolated from tissue samples. Given the complexity of virus isolation, the use of highly sensitive techniques, such as nested PCR, provides greater efficacy for the detection of the virus.
Unlike previous works involving experimentally-infected calves (Egyed et al. 1996), clinical signs were not observed in the animals inoculated with these Argentinean strains, with the exception of a slight nasal discharge in only one calf. While mild microscopic lesions in tissues from calves inoculated with the strains 09/508 and 07/435 were observed and no microscopic findings were present in the tissues of the calf infected with the strain 08/330, the PCR results clearly demonstrate that these BoHV-4 strains are able to disseminate throughout the body, reaching different organs, mainly the respiratory tract. Furthermore, it is important to underline that viral DNA from the three strains was detected in the central nervous system. While these results differ from other experimental inoculations of cattle (Egyed et al. 1996, Boerner et al. 1999), they are consistent with other previously published reports (Asano et al. 2003, Costa et al. 2011, Campos et al. 2014). Moreover, the detection of the viral genome in spleen agrees with the finding of Lopez et al. (1996), regarding the ability of BoHV-4 to produce a persistent infection in a particular zone of this organ in cattle.

Although none of the strains were isolated from peripheral blood leukocytes, the presence of viral DNA during the second and third weeks post-infection confirms that BoHV-4 infects and persists in lymphoid cells (Egyed & Bartha 1998, Asano et al. 2003, Fábián et al. 2005). It is likely that these cells are in part responsible for the tissue distribution of the virus, as it has been previously suggested (Egyed et al. 1996).

Regarding the techniques used to determine the immune status of BoHV-4-infected animals, it can be considered that VNT results are controversial in the diagnosis of BoHV-4. The humoral immune response is not efficient in infected cattle; it appears to be associated with a deficient exposure of glycoprotein domains involved in the stimulation of viral neutralization antibodies production, due to the presence of a barrier of glycans (Dubuisson et al. 1990). It has been shown that O-glycosylation of the major BoHV-4 glycoprotein gp180 is responsible for hiding BoHV-4 vulnerable epitopes (Machiels et al. 2011). BoHV-4 prevalence is usually low in countries where the virus has been detected; seropositivity rates vary in a range of 16 to 30%, with low neutralizing antibody titres (1: 8 to 1:64) (Wellemans et al. 1991, Wellemberg et al. 1999, Frazier et al. 2002, Elhassan et al. 2011, Motta Giraldo et al. 2013). Coincident with the findings by these authors, low neutralizing antibody titres were also detected by VNT in this experiment.

The detection of variable levels of neutralizing antibodies to any of the BoHV-4 strains in all animals used in this experiment, including the uninfected control calf, makes this finding a point of analysis. On the basis of these results it can be hypothesized that there could be a non-specific neutralizing factor or cross-reaction with other viral agents such as bovine lymphotropic herpesvirus (BLHV), another member of the ruminant rhadinovirus group. BLHV has also been associated with reproductive failure in cattle (Banks et al. 2008, Gagnon et al. 2010) and the genome of this virus has been detected in animals with positive serology for BoHV-4 (de Boer et al. 2014).

It can also be inferred that this non-specific neutralizing activity, present in the serum of animals, could be the reason why the virus is completely or partially inhibited and unable to develop its pathogenic potential. This could explain the presence of mild injuries in infected tissues and the absence of severe clinical manifestations. Since VNT can result in the detection of non-specific neutralizing action for some strains, as mainly observed with strain 08/330, it would be advisable to use alternative serological techniques for laboratory diagnosis as a tool to survey herds. Currently, a commercial indirect ELISA for detection of BoHV-4 antibodies is available. However, the possibility of detecting antibodies stimulated by BLHV has also been reported and further validation of this test is suggested (de Boer et al. 2014). An immunoperoxidase monolayer assay (IPMA) for detection of BoHV-4 antibodies has been developed by Wellemberg et al. (1999). By this technique it is possible to detect antibodies to BoHV-4 earlier than by ELISA. However, compared to the ELISA test, IPMA readings are usually subjective and need to be interpreted by more than one person to reduce subjectivity.

Overall, the findings of this study suggest that, although slight, there are differences in the behavioural patterns of the strains used in this in vivo model using the natural host of BoHV-4. The strain 08/330 was isolated in ocular and nasal secretion, showing the highest viral titres when compared with the other challenge strains. This data agrees with results of previous in vitro experiments, in which it was observed that this strain replicates at high titres in different cell lines (Morán unpublished results).

Furthermore, the genome of this strain was detected in leukocytes more frequently than the other strains. Virus DNA from strains 09/508 and 08/330 was also more frequently detected in tissue samples (5/14) whereas the genome of the strain 07/435 was only detected in 3/14. Nonetheless, despite the differences observed in the in vivo activity of BoHV-4 strains and the results of cross-neutralizing antibodies among homologues strains, it is not possible to establish association between the phylogenetic groups and patterns of biological behavior of each strain. Further evaluation involving a higher number of inoculated animals will be required. Nevertheless, the findings of this study provide an initial step to understand the biology of the Argentinean isolates of BoHV-4. The high variability among strains isolated in Argentina (Verna et al. 2012, 2016) highlights the significance of research on the role of this virus as an infectious agent capable of causing reproductive disease in cattle. This is the first report in Argentina on histopathological findings and tissue distribution of BoHV-4 in its natural host after an experimental infection.
REFERENCES


with local strains. Therefore, this study provides information for a better understanding of the pathogenesis of infection by BoHV-4 and the means by which these strains circulate in the herds. Currently there are not sanitary measures and rapid diagnostic techniques to conduct an epidemiological study to determine the local situation of this emerging virus. Thus, in view of the importance of the presence of BoHV-4 in Argentina and its association with reproductive losses and different clinical manifestations in cattle, further studies of this viral agent, both at the epidemiological and immune-molecular level, are required.


Funding
This study was funded by grants from ANPCyT, PICT 2008-1421 and PICT 2011-0312, Argentina.

Conflict of Interest
Statement the authors declare that they have no conflicts of interest.

Ethical Approval
All applicable national and institutional guidelines for the care and use of animals were followed. The study was approved by the Animal Welfare Committee of the University of the Center of Buenos Aires Province (Res. 087/02) and INTA Balcarce, Argentina.