

Occurrence of bovine herpesvirus type 4 DNA in Argentinean Holstein cattle from Santiago del Estero, Argentina

Ocorrência de DNA de herpesvírus bovino tipo 4 em gado Holstein argentino de Santiago del Estero, Argentina

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Abstract

Bovine herpesvirus type 4 (BoHV-4) is a gamma-herpesvirus that has been isolated from apparently asymptomatic animals and from cattle with a variety of clinical signs. The pathogenic role of BoHV-4 remains unclear and it is unknown whether the virus acts as a primary pathogen or whether it facilitates secondary infections. After natural or experimental infections, BoHV-4 can establish latency, mainly in cells of the monocyte/macrophage lineage. Latent virus can be reactivated after glucocorticoid treatment or by stress factors. In 2007, BoHV-4 was isolated for the first time in Argentina, from samples of bovine abortions. In the present study, we used viral isolation, nested PCR and restriction endonuclease analysis (REA) to investigate the presence of BoHV-4 in bovine leukocytes from a single herd of dairy cattle with reproductive problems. In this work, we demonstrated that BoHV-4 genome is present in the leukocytes of a high proportion (63.4%) of animals, probably in a latent or persistent state. BoHV-4 was isolated from one out of eleven peripheral blood leukocyte (PBL) samples. By REA we demonstrated the existence of genomic variation among the strains circulating in this particular herd. Furthermore, all PBL samples evaluated in this study differed from the American prototype strain, DN 599. Overall, this work demonstrated that BoHV-4 is present in the leukocyte fraction of dairy cattle and that viral strains present in this herd are genetically divergent. Although BoHV-4 was detected in a herd with a background of reproductive disorders, it is not possible to conclude that the virus is the primary responsible for these conditions.

Keywords: BoHV-4. Leukocytes. nested PCR. REA. Reproductive disorders.

Resumo

O herpesvírus bovino tipo 4 (BoHV-4) é um gama-herpesvírus que foi isolado de animais aparentemente saudáveis e de gado com uma variedade de sinais clínicos. O papel patogênico do BoHV-4 ainda não está claro e não se sabe se o vírus age como um patógeno primário ou se facilita infecções secundárias. Depois de infecções naturais ou experimentais, BoHV-4 pode estabelecer latência, principalmente nas células dos linhagens de monócitos/macrófagos. O vírus latente pode ser reativado após o uso de glicocorticóides ou por fatores de estresse. Em 2007, o BoHV-4 foi isolado pela primeira vez na Argentina, a partir de amostras de abortos bovinos. No presente estudo, utilizou-se o isolamento viral, nested PCR e análise com endonucleases de restrição (REA) para investigar a presença de BoHV-4 em leucócitos de bovinos provenientes de um único rebanho de gado leiteiro com problemas reprodutivos. Neste trabalho, demonstramos que o genoma do BoHV-4 está presente nos leucócitos em uma elevada proporção (63,4%) dos animais, provavelmente em um estado latente ou persistente. BoHV-4 foi isolado de uma de cada onze amostras de leucócitos no sangue periférico (PBL). Por REA nós demonstramos a existência de variações genômicas entre as estirpes circulantes deste rebanho particular. Além disso, todas as amostras de PBL avaliadas neste estudo diferiram da estirpe protótipo Americano, DN 599. Em geral, este estudo demonstrou que o BoHV-4 está presente na fração leucocitária do gado leiteiro e que as estirpes virais presentes neste rebanho são geneticamente divergentes. Embora que BoHV-4 foi detectado em um rebanho com história de distúrbios reprodutivos, não é possível concluir que o vírus é o principal responsável por estas condições.

Palavras-chave: BoHV-4. Leucócitos. Nested PCR. REA. Problemas reprodutivos.

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Introduction

Bovine herpesvirus type 4 (BoHV-4) is a gamma-herpesvirus, which was first isolated in Hungary in 1963 from animals with respiratory and ocular disease¹. Although cattle are the natural host of the virus, several ruminant² and non-ruminant species³ are susceptible to BoHV-4. Sporadic isolates of BoHV-4 from species as diverse as lions, cats and owl monkeys^{4,5} have been reported. The virus has also been isolated from apparently asymptomatic animals⁶ and from cattle with a variety of clinical signs, including diarrhea, metritis^{7,8,9} abortion, vaginitis, mastitis¹⁰, ulcerative mammillitis and skin lesions¹¹. Like other herpesviruses, after natural or experimental infections, BoHV-4 can establish latency^{12,13,14,15,16}. Latent virus can be reactivated after glucocorticoid treatment^{17,18} or by stress factors.

Immune cells of the monocyte/macrophage lineage are the main site of replication and latency of BoHV-4^{19,20}. By nested PCR, BoHV-4 DNA was detected in lymphocytes, lymph nodes and nervous tissue of cattle in absence of clinical signs⁸. In experimentally infected cattle, using the same methodology, Egyed et al.²¹ identified BoHV-4 DNA in bovine peripheral blood leukocytes (PBLs) at 48 days post-infection.

Although BoHV-4 replicates in several cell lines and produces cytopathic effect (CPE), isolation of the virus may be difficult in certain lymphoid and myeloid cells which are resistant to virus infection or in a number of epithelial cells which are poorly permissive to BoHV-4 infection²². In 2007, BoHV-4 was isolated in Argentina from samples of bovine abortions²³. Later, the virus was isolated from nasal swabs, brain, ovocyte granulose cells and from semen from an artificial insemination center. Furthermore, BoHV-4 was isolated from buffy coat fractions in association with bovine viral diarrhea virus (BVDV)²⁴. Restriction endonuclease analysis (REA) of BoHV-4 isolates allowed the classification of the virus into two main groups, Movar-like (European prototype) or DN 599-

like (North American prototype) viruses^{2,6,19,25}. Until now, genetic variations of BoHV-4 field isolates have not been documented. Cross-hybridization with the reference strain DN 599 of 8 American BoHV-4 isolates from cattle with a variety of clinical conditions revealed that only one isolate had a restriction pattern clearly different from the reference strain and no correlation could be established between the origin (disease, sample and herd) and the restriction pattern of the isolates²⁶. By REA, Verna et al.²⁷ have shown that there are remarkable differences among BoHV-4 strains circulating in Argentina. The same analysis revealed that most of these isolates are different from the American prototype, DN 599.

D'Onofrio et al.²⁰ demonstrated that BoHV-4 has tropism for stromal and epithelial cells of the endometrium where it produces rapid CPE. It has been suggested that BoHV-4 recrudescence from latency at the time of parturition is responsible for the development of postpartum endometritis^{7,28}, one of the conditions that has been associated with the virus. In addition, Dubuisson et al.²⁹ showed that BoHV-4 can establish a latent infection in bovine testicles and that the virus can be reactivated by glucocorticoid administration. Therefore, it is apparent that BoHV-4 is a pathogen associated with reproductive problems in cattle.

Nevertheless, the pathogenic role of BoHV-4 remains unclear and it is unknown whether the virus acts as a primary pathogen or whether it facilitates secondary infections. In the present study, we used viral isolation, nested PCR and REA to investigate the presence of BoHV4 in bovine leukocytes from a single herd of dairy cattle with a history of reproductive problems.

Material and Method

Fifty-two Argentinean Holstein cattle from Santiago del Estero Province (Argentina) were used for this study, including 22 cows (2 to 6 year-old) and 30 yearling bulls. This dairy herd has a history of reproductive problems, mainly abortions and repeat-breeding.

Santiago del Estero Province is located in the central-north region of Argentina. It is a semi-arid area with some steppes. The climate is sub-tropical, with high temperatures the entire year and with a dry season.

PBLs were obtained from heparinized blood after centrifugation and lysis of erythrocytes. Briefly, blood was centrifuged at $2,000 \times g$ for 15 min. and the buffy coat was transferred to a 15 ml tube containing 10 ml of cold 1X ammonium chloride (0.15M NH_4Cl , 0.1M Na_2CO_3 , 0.002M EDTA). The cell pellet obtained after centrifugation at $1,000 \times g$ for 7 min. at 4°C was re-suspended in 1 ml of phosphate-buffered saline solution (PBS) [137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4 ; 1.47 mM KH_2PO_4], transferred to a 1.5 ml tubes and centrifuged at $10,000 \times g$ for 2 min. and the supernatant was discarded.

DNA from PBLs was extracted with a commercial kit (ILLUSTRATE tissue 2 cells genomic preparation, Cat. 28-9042-75, Amersham Pharmacia), according to the manufacturer's instructions. DNA concentration was determined by spectrophotometry (LKB Biochrom Ultrospec II, United Kingdom) at an absorbance of 260 nm. Nested PCR was performed as described by Verna et al.^{23,24} using primers that amplify the thymidine kinase (TK) gene of BoHV-4. This nested PCR is highly sensitive, allowing the detection of approximately one viral DNA copy.²¹ For the first amplification round, 3 μl of PBL DNA were added to the PCR reaction mix (25 μl final volume), containing 0.2 μM each primer, 200 μM dNTPs, 25 mM MgCl_2 and 1 U DNA polymerase (T-plus DNA polymerase, Cat. E021, INBIO Highway, Argentina). Primer sequences for the first round PCR are: 5'-GTTGGGCGTCCTGTATGGTAGC-3'; 5'-ATGTATGCCCAAACCTTATAATATGACCAG-3, and the amplification product is 567 bp. Primer sequences for the second PCR round are: 5'-TTGATAGTGC GTTGTGGGATGTGGT-3' and 5'-CACTGCCCGGTGGGAAATAGCA-3' and the amplification product is 260 bp. Amplification was

carried out in a PTC-100 MJ Research, Inc (GMI, USA). thermal cycler, as follows: 95°C 9 min.; [94°C 45 s; 58°C 60 s; 72°C 90s] for 20 cycles and one extension cycle at 72°C for 7 min. For the second amplification round, 2.5 μl of the first round PCR product were used and the annealing temperature was decreased to 55°C for 60 sec. DNA from mock-infected MDBK cells and DNA from cervico-vaginal mucus from an infected cow were used as negative and positive controls, respectively. The identity of the positive control was confirmed by nucleotide sequencing. Amplification products were not obtained when DNA from BoHV-1 (Los Angeles 38) and BoHV-5 (NS569) reference strains were included in the PCR reactions. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$).

Fifty microliters of PBL were re-suspended in 500 μl of Eagle Minimal Essential Medium (E-MEM, Cat. M0643, Sigma-Aldrich, Argentina) supplemented with 10% de bovine fetal serum. Two hundred μl of each PBL suspension were seeded on Madin-Darby bovine kidney (MDBK) cell monolayers, in 24 well-plates. Uninfected MDBK cells were used as controls. Five blind passages were performed every five days and the monolayers were observed daily for CPE. Samples were tested by direct immunofluorescence using an anti-BoHV-4 monoclonal antibody (Animal and Plant Health Inspection Service National Veterinary Services Laboratories, USA).

DNA was extracted by the use of a commercial kit (DNeasy blood & tissue Kit, Cat. 69504, Qiagen). DNA obtained from 13 PBL samples was digested with the restriction enzyme EcoRI (Promega, Cat. R6011), according to the manufacturer's instructions. Digestion products were separated by electrophoresis on 0.8% agarose gels at 50 V using 1X TAE (40 mM Tris, 20 mM $\text{C}_2\text{H}_4\text{O}_2$, 1 mM EDTA) electrophoresis buffer and stained with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$).

Results

Prevalence of BoHV-4 DNA in bovine PBLs

Nested PCR was used to determine the presence of BoHV-4 DNA in PBLs (Figure 1). By this technique, it was possible to demonstrate that 63.4% (33/52) of the animals harbored BoHV-4 DNA in their blood leukocyte fractions. The analysis by animal category revealed that 60% (18/30) of the bulls and 68% (15/22) of the cows were positive for the presence of viral DNA in their PBLs.

Isolation of BoHV-4 from bovine PBLs

Unlike other gamma-herpesviruses, BoHV-4 is able to replicate in a variety of cell lines²², including

MDBK cells.³⁰ BoHV-4-positive and -negative PBLs, as determined by nested PCR, were selected for co-culture with MDBK cells. Only eleven PBLs samples were adequate to attempt viral isolation. Viral CPE was evident in one PBL sample belonging to a yearling bull after five days of co-culture. CPE is characterized by the presence of areas of cell rounding and lysis, which progressively extends to the complete monolayer (Figure 2). No CPE was observed in the remaining samples analyzed (Table 1). The isolation of BoHV-4 in the co-culture was confirmed by direct immunofluorescence using an anti-BoHV-4 monoclonal antibody (Figure 3).

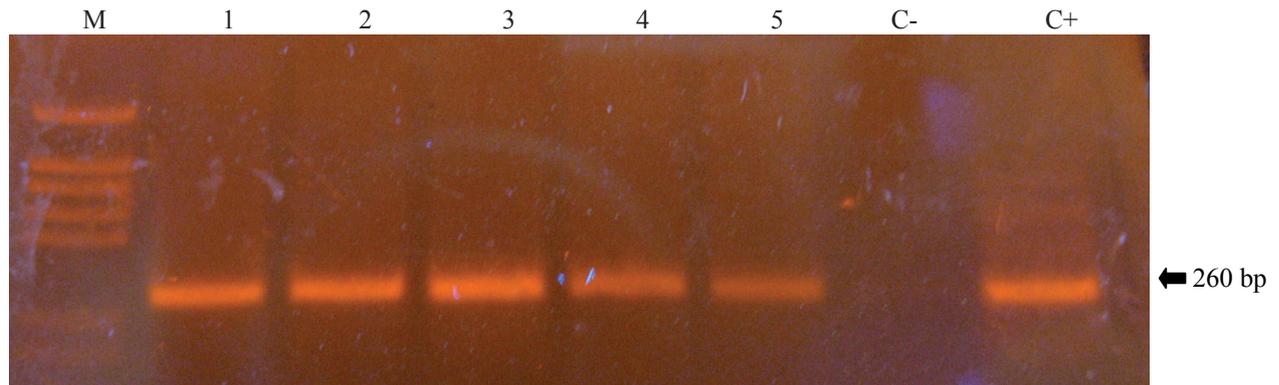


Figure 1 - **Presence of BoHV-4 DNA in PBLs from Argentinean Holstein cattle.** Nested PCR amplifying BoHV-4 TK fragment was performed as described in Material and Methods. The nested PCR amplification product is 260 bp. M: DNA marker (BenchTop pGEM, Promega), Lanes 1-5: BoHV-4 DNA from PBLs. C-, DNA extracted from mock-infected MDBK cells. C+: Positive control, DNA from a cervico-vaginal mucus sample positive to BoHV-4. Place: Tandil (Buenos Aires, Argentina) – 2010

Table 1 - Co-culture of PBLs from Argentinean Holstein cattle with MDBK cells and detection of BoHV-4 genome by nested PCR. Place: Tandil (Buenos Aires, Argentina) – 2010

Sample	Animal Category	Viral isolation	Nested PCR
1	Yearling bull	-	+
2	Yearling bull	-	-
3	Yearling bull	-	+
4	Yearling bull	+	+
5	Yearling bull	-	-
6	Yearling bull	-	+
7	Yearling bull	-	+
8	Yearling bull	-	+
9	Cow	-	+
10	Cow	-	+
11	Cow	-	-

BoHV-4 DNA restriction pattern

Comparison of EcoRI restriction profiles (Figure 4) of BoHV-4 DNA obtained from PBLs revealed the exis-

tence of genomic variation among the strains circulating in this particular herd. All the PBL samples evaluated in this study differed from the American prototype strain,

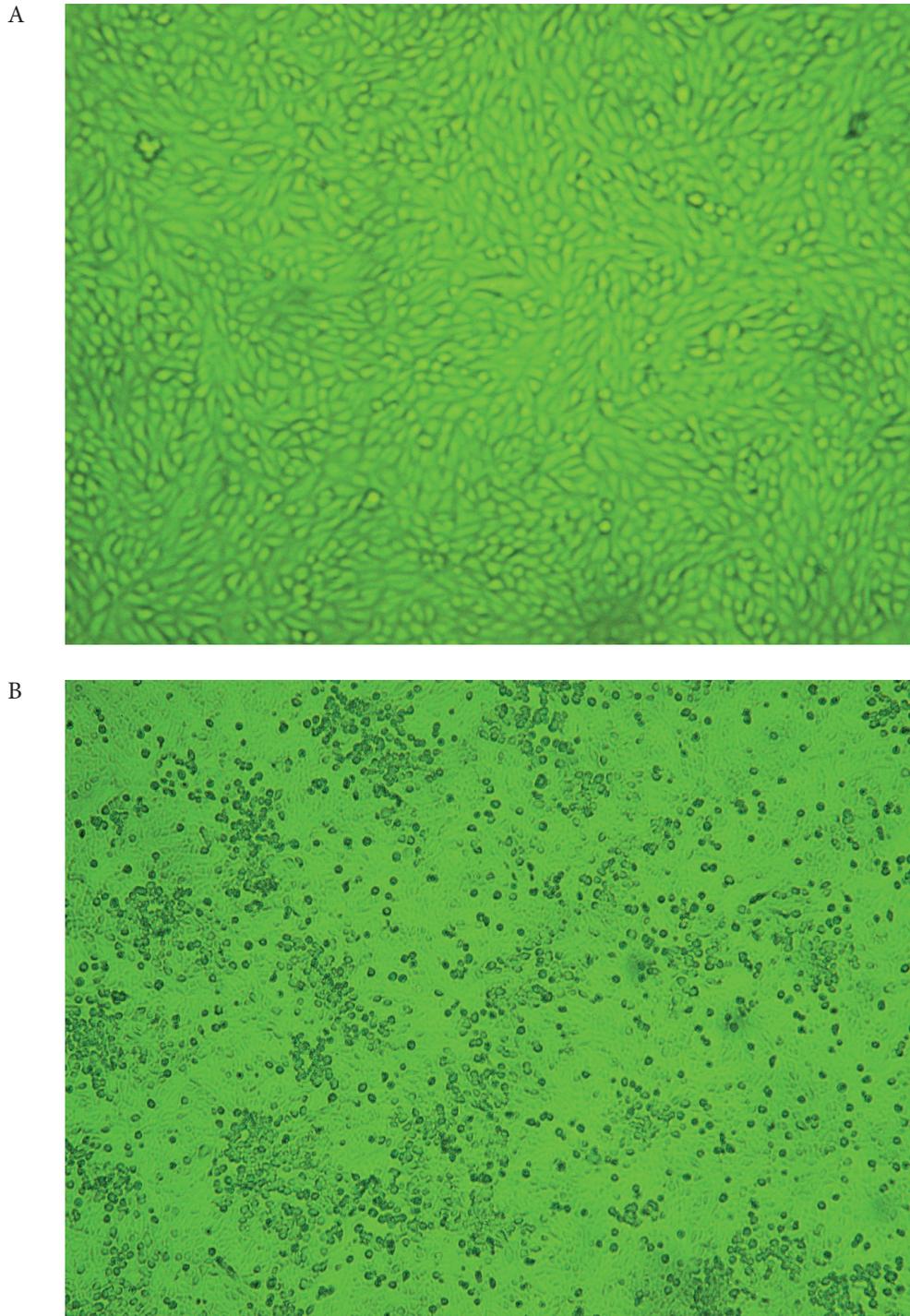


Figure 2 - **Cytopathic effect of BoHV-4 on MDBK cells.** **A.** Uninfected MDBK cell monolayer. Magnification: 20 X. **B.** Cytopathic effect of BoHV-4 on a co-culture of PBLs from a yearling bull with MDBK cells. Magnification: 20X. Place: Tandil (Buenos Aires, Argentina) – 2010

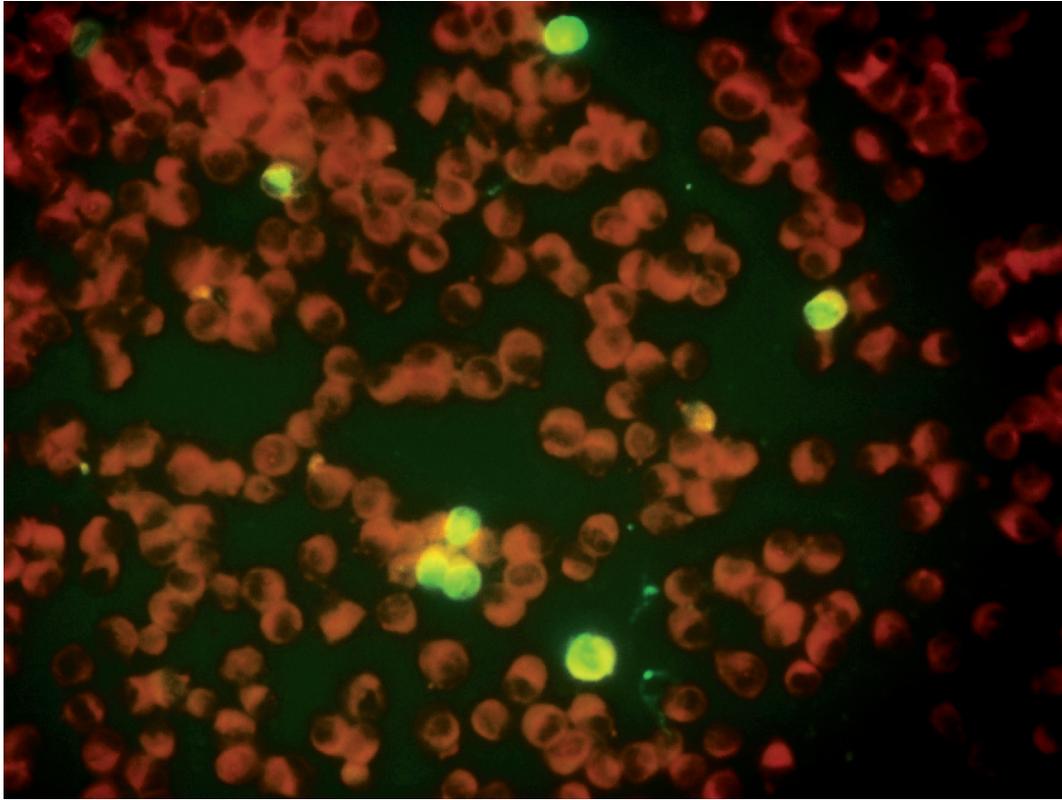


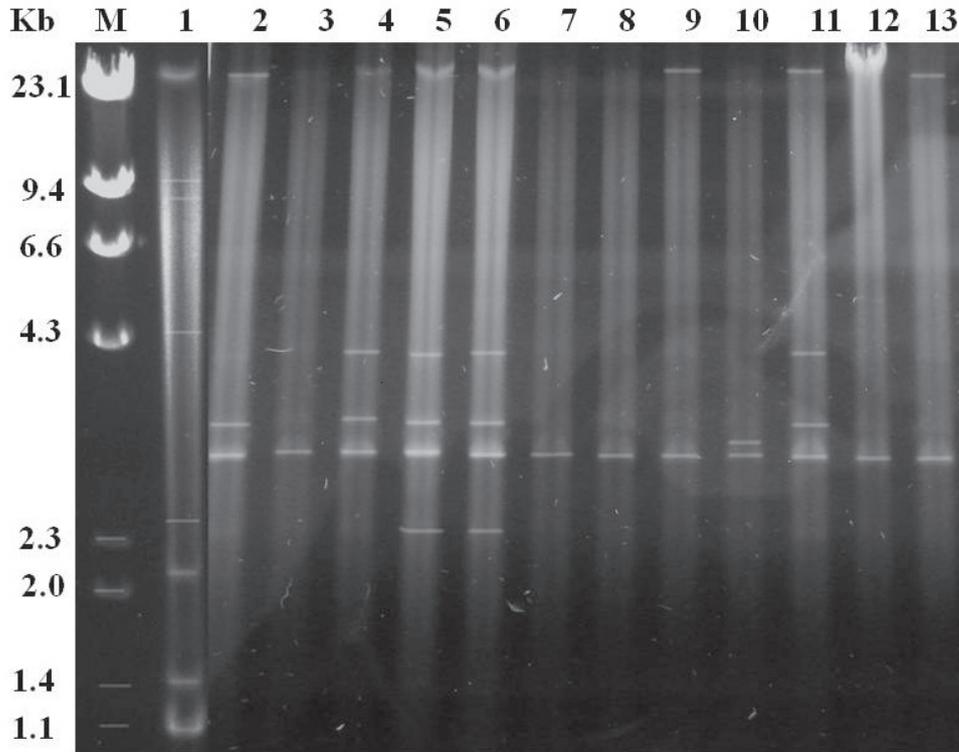
Figure 3 - **Direct immunofluorescence for BoHV-4.** Identification of BoHV-4 in a co-culture of PBLs from a yearling bull with MDBK cells. Magnification: 20X. Place: Tandil (Buenos Aires, Argentina) – 2010

DN 599. According to the REA analysis it is possible to establish the existence of at least five different BoHV-4 restriction profiles in this single dairy herd. Verna, A. (personal communication, 2010) showed that Argentinean BoHV-4 isolates are also different from the European prototype strain. However, the European Movar strain was not included in this study. Therefore, from our results it is not possible to be conclusive in this respect.

Discussion and conclusions

BoHV-4 has been isolated from asymptomatic animals and from cattle with a wide range of clinical manifestations. In the last years, BoHV-4 has been isolated and/or its genome has been identified in several bovine tissues, especially in cases associated with reproductive disorders such as, endometritis^{7,8,9}, abortion^{10,23} and vaginitis¹⁰. It is unknown whether BoHV-

4 has a primary role in the development of post-partum endometritis. However, it has been postulated that the virus might exacerbate the condition when persistently infected macrophages are recruited to the site of inflammation. In the absence of inflammatory processes, it is likely that the pathogenic potential of BoHV-4 is not fully expressed³¹. In addition, Frazier et al.⁷ suggested that dairies where BoHV-4 is endemic and have high incidence of nutritional or metabolic diseases, for example lipidoses, may be more susceptible to clinical cases of metritis associated to BoHV-4. Cattle in Santiago del Estero Province are constantly subject to nutritional and heat stress, which might be an additional factor contributing to the pathogenicity of different agents and facilitating or exacerbating the development of diseases associated with opportunistic or latent pathogens, such as BoHV-4.



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Figure 4 - **EcoRI restriction fragments of BoHV-4 DNA from PBLs of Argentinean Holstein cattle.** M: molecular marker (λ phage DNA digested with HindIII), Lane 1: DN 599 reference strain, Lanes 2-13: PBLs from Argentinean Holstein cattle. Place: Tandil (Buenos Aires, Argentina) – 2010

In this work, we demonstrated that BoHV-4 genome is present in the leukocyte fraction of a high proportion of dairy animals from a single herd. This high percentage of animals from a single herd harboring BoHV-4 DNA in their leukocytes has not been reported before. It is likely that in these animals the virus is in a latent or persistent state. Further studies using quantitative PCR would be conducted to prove this hypothesis.

Unlike other gamma-herpesvirus, BoHV-4 can grow in different cell lines²². Nevertheless, its isolation in cell culture may be difficult. In this work, we attempted to isolate the virus from a reduced number of leukocyte samples. Thus, to determine the frequency of isolation of BoHV-4 from PBLs, it will be necessary to evaluate a larger number of samples. Furthermore, it will be interesting to test whether the stimulation of

leukocytes with mitogens, such as phytohemagglutinin, can be useful to induce or increase the replication of viruses like BoHV-4, which infect and/or establish latency in this cell type. An increase in viral replication by the use of mitogens has been shown, for example, for equine herpesvirus type 1 (EHV-1)³².

Evolutionary studies on BoHV-4 have shown that the African buffalo (*Syncerus caffer*) was the original natural reservoir of the virus and that at least three independent transmission events from buffalo to cattle have occurred, probably via intermediate host species². There is a large divergence between European and North American BoHV-4 strains and American-like isolates have never been described in Europe. Thus, it is speculated a different natural ancestor for the North American BoHV-4 strains; likely, the American bison (*Bison bison*), from which BoHV-4 strains have been isolated³³. The central

part of BoHV-4 genome is well-conserved and the major differences detected by REA patterns between Movar-like and DN 599-like strains are located within the poly-repetitive DNA sequences (prDNA), which flank the unique central part of the genome. This region is highly polymorphic among most of BoHV-4 reference strains. Variations in the REA pattern independent of this region are also apparent³⁴. The analysis of a variety of Argentinean field isolates of BoHV-4 could not detect any clear relationship between the endonuclease restriction pattern and the origin of the isolate. Restriction pattern length polymorphism (RFLP) analysis from 17 Argentinean BoHV-4 field isolates from breeding herds with reproductive problems demonstrated that the strains differed from the American prototype DN 599. Sequencing analysis of these samples revealed that 12 strains could be grouped into the DN 599-like group, 1 isolate belonged to the Movar-like group and the remaining isolates were classified into a yet undefined group Verna, A. (personal communication, 2010). Therefore, the results presented in this study provide additional evidence on the genetic variations of Argentinean BoHV-4 isolates in a specific population of cattle. Differences in the restriction enzyme patterns are probably due to specific point mutations resulting in the loss or gain of certain restriction endonuclease sites. In addition, fragment length polymorphisms were detected in some regions of the genomes Verna, A. (personal communication, 2010). Therefore, sequencing of these regions from several field isolates will be important to explain the heterogeneity observed. It is likely that these genomic differences are the result of variations in the number of repeated sequences, as it has been described for many herpesviruses, such as, Epstein-Barr³⁵, varicella-zoster virus³⁶ and bovine herpesvirus³⁷.

Previous works^{7,9} have shown that BoHV-4 can undergo genomic drift and significant geographic variation has also been observed among strains. From our study, it is also possible to infer that there is considerable variation among strains circulating in animals from a single herd. The low degree of homology between the restriction patterns and BoHV-4 isolates from the same type of sample in a given herd supports the hypothesis that virus pathogenicity depends partly on the strain. Correlation of the viral strain with the clinical manifestations would be less important.

Although the high prevalence of BoHV-4 in the leukocytes of cattle in this particular herd can suggest an involvement of the virus in the reproductive disorders observed, the detection of the viral genome in this cell fraction is not an evidence of the pathogenic potential of BoHV-4. Thus, it would be necessary to isolate or detect the presence of the virus in tissues from aborted fetuses, cervico-vaginal mucus or semen to be conclusive on the pathogenic role of BoHV-4. The main mode of virus transmission is unknown. However, there is evidence of intermittent shedding in milk, saliva and semen^{28,37}. Because BoHV-4 genome has been consistently detected both in cows and bulls, it cannot be ruled out the likelihood of shedding and transmission of the virus during acute infections or reactivation episodes to the offspring and/or by natural service or artificial insemination. Further studies on BoHV-4 pathogenesis are required. Nevertheless, in association with the recent findings by Verna et al.^{23,24,27} and in agreement with the literature, it is possible to infer that BoHV-4 is involved, directly or indirectly, with abortion, endometritis and other reproductive disorders in cattle.

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