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Experimental Infection of Calves with Argentinean Strains of Bovine Herpesvirus Type 4 Belonging to Different Genotypes

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Abstract

Bovine herpesvirus 4 (BoHV-4) is a gamma-herpesvirus which has been isolated from cattle with diverse clinical signs and from apparently healthy animals. Although its pathogenic role is still unknown, the virus has been consistently isolated from vaginal secretions from aborted cows. Phylogenetic analysis of Argentinean BoHV-4 field isolates revealed the existence of three distinct groups. In this study the *in vivo* behaviour of Argentinean BoHV-4 isolates belonging to two different genotypes was analysed (10/154: genotype 1; 07/435: genotype 3) by experimental challenge of calves. Although both BoHV-4 isolates were able to replicate in their host after nasal infection, severe clinical signs were not observed. It was demonstrated that isolate 10/154 replicates better in nasal epithelium whereas isolate 07/435 was consistently detected in ocular secretions. Low antibody titers were detected by virus neutralization and ELISA assays. Neutralizing antibody titers to isolate 07/435 were detected. However, no serological response was detected to the isolate 10/154 belonging to the American genotype. Overall, this information suggests that there are differences in the pattern of *in vivo* behavior of these strains. Further studies are required to understand the immune response to the infections caused by this virus.

Keywords: Bovine Herpesvirus 4; Argentinean Isolates, Immune Response, *In vivo* Behavior

Introduction

Bovine herpesvirus 4 (BoHV-4) is a member of the *Herpesviridae* family, *Gammaherpesvirinae* subfamily, *Rhadinovirus* genus. The members of this genus differ from other herpesviruses isolated from ruminants because they have a reduced set of open reading frames (ORFs) homologous to cellular genes [1]. BoHV-4 was isolated for the first time in 1963 from cattle with respiratory and ocular signs in Europe [2]. Afterwards, virus isolates were classified into two groups by restriction endonuclease analysis [3,4], Movar 33/63-like strains, isolated from Europe, and DN 599-like strains, isolated from North America [5]. In 2007, BoHV-4 was isolated in Argentina from vaginal secretions from cows which had aborted [6] and later from buffy coat fractions in association with bovine viral diarrhea virus (BVDV) [7]. More recently, the virus was also isolated from bovine semen from an artificial insemination center [8].

The pathogenesis of BoHV-4 infection has not been completely elucidated. Susceptible animals are infected by direct contact (aerosols) and active virus replication occurs in the upper respiratory tract, lymphoid organs and the urogenital and alimentary tracts. After initial infection, latency is established in lymphoid organs and mononuclear blood cells [9,10,2]. Isolation of different BoHV-4 strains has been reported from animals showing a variety of clinical signs, such as conjunctivitis, pneumonia, inflammation of the upper respiratory tract [11], metritis [12], skin lesions [13] and tumors of the urinary bladder and rumen [14]. BoHV-4 was also isolated from apparently healthy animals [15]. It is speculated that BoHV-4 is not the causative agent of all these clinical syndromes, but it seems to present itself as an activated latent virus in any place where local immunity is inhibited [16].

Natural infections probably occur through the respiratory and alimentary tracts. Experimental inoculation of BoHV-4 failed to cause any clinical disease in cattle and sheep [17], although it has been reported that BoHV-4 is pathogenic in rabbits [9,18,19] and cats [20]. Previous studies by Verna, *et al.* [7] demonstrated that Argentinean BoHV-4 isolates cluster into three distinct genotypes. Group 1 and 2 strains belong to DN 599- and Movar 33/63-like genotypes, respectively, whereas Group 3 is a novel Argentinean genotype. Most of the Argentinean field isolates correspond to the North American DN 599-like genotypes.

In Argentina there aren't epidemiological studies because there are no effective diagnostic techniques. [21] Quantitatively evaluated the replication properties of different Argentinean BoHV-4 strains and to characterize their phylogenetic relationships. Significant differences were evident among the virus titers of the different BoHV-4 isolates *in vitro*. The results from this study demonstrate that the biological behavior on MDBK cells of BoHV-4 field isolates obtained from aborted cows is independent of their genetic classification. This study might contribute to understand the genetic diversity of BoHV-4 isolates, a virus property which has only been reported in Argentina [7]. This issue is particularly relevant for research purposes, as well as for industry, since obtaining the maximum potential of virus replication is critical for several procedures, for example the optimization of vaccine production.

The *in vitro* replication features of a virus may also have *in vivo* relevance. As a consequence, it is evident that the *in vitro* characterization (biological and molecular) of a viral strain is essential when aspects of the pathogenesis are being evaluated. This observation might be related to the different potential for the *in viv*o dissemination and circulation of the viral strains. The question arises whether this could be a difference in virulence between strains, rather than a difference in their *in vitro* replication on MDBK cells. Considering that BoHV4 has been detected, as an emerging and concomitant agent in reproductive infections of cattle in our country, characterizing the *in vitro* replication kinetics and determining the time point after infection at which a viral strain has its maximal potential of replication is a relevant fact to establish differences among the behaviour of BoHV4 isolates in cell cultures, since it might contribute to the understanding of the pathogenic role of each strain in their natural host.

Moran, et al. [22] prove that, there are differences in the behavioral patterns of the BoHV4 strains used in this *in vitro* study and this variability in the dynamics of replication and biological behaviour is independent of their genetic classification but would be conditioned mainly by the nature of the infected cells. These findings provide an initial step to understand the biology of the Argentinean isolates of BoHV4. The aim of this study was to obtain information on the *in vivo* behaviour of Argentinean BoHV-4 isolates belonging to the Groups 1 and 3. This information will contribute to elucidate whether specific variants of BoHV-4 have different degrees of pathogenicity in cattle.

Materials and Methods

Viruses

BoHV-4 isolates 07/435 and 10/154 were used for animal inoculations. Both strains were isolated in Argentina from samples of vaginal secretions from cows which had aborted. Field isolate 10/154 corresponds to DN 599-like group (Genotype 1) and 07/435 is a Genotype 3 Argentinean isolates [7]. Titres of virus stocks were determined by the end-point titration method and expressed as $TCID_{50}$/ml [23].

Cell Cultures

For viral stocks and virus isolation, Madin–Darby bovine kidney (MDBK) cells grown in minimum essential medium (MEM, Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (Internegocios), L-glutamine (10 µl/ml) (L-glutamine Sigma-Aldrich), penicillin (100 µg/l) (penicillin G sodium salt, Sigma-Aldrich) and streptomycin (200 µg/l) (streptomycin sulfate, Sigma-Aldrich) were used. Cells were incubated at 37 °C in a 5% CO_2 atmosphere. Cells were provided by ABAC (Argentinean Cell Bank) and they are certified as free of contaminating bacteria, mycoplasma and adventitious viruses.

Experimental Design

All procedures for animal handling and experimentation were performed according to the Animal Welfare Committee (CICUAE) of the National Institute of Agrarian Technology (INTA) EEA Balcarce, Argentina. Fifteen calves aged 10 months were divided into three groups of five animals each and were housed in the experimental animal area of EEA Balcarce. All animals tested seronegative for bovine herpesvirus (BoHV)-1 and BoHV-5 in a virus neutralization test (VNT) at the beginning of the experiment and they were evaluated by ELISA (Bio-X Diagnostics, Belgium) for anti-BoHV-4 antibodies. Calves were randomly assigned to one of three groups and intranasally inoculated as follows: In group 1 (n=5) all calves were challenged with 10 ml of isolate 07/435 ($10^{6.87}$ $TCID_{50}$). In group 2 (n=5) all calves were challenged with 10 ml of isolate 10/154 ($10^{4.87}$ $TCID_{50}$) and, in group 3 (mock-infected; n=5) calves were inoculated with 10 ml MEM as placebo.

Differences in the growth kinetics of these field isolates has been previously reported [21]. Thus, calves were inoculated with the highest virus titers reached by each isolate on MDBK cells. Clinical examination of all animals was performed daily (temperature, appetite, respiratory signs and diarrhea). Blood samples with EDTA and without anticoagulant and nasal and ocular swabs which were immediately immersed in 2 ml MEM with antibiotics, were obtained daily from 0 to 9 days post-inoculation (dpi) and then at 11, 14 and 25 dpi. Buffy coats were prepared from anti-coagulated blood and were stored at −80 °C.

Virus Isolation from Buffy Coat Fractions, Nasal and Ocular Secretions

Samples were inoculated in triplicate into MDBK cells in 96-well plates and observed daily for the presence of cytopathic effect (CPE). Blind passages were performed every 48h, and at the third blind passage, samples were routinely tested for the presence BoHV-1 and BVDV by direct immunofluorescence. Because BoHV-4 does not replicate easily in cell cultures, samples were maintained in MDBK cells for up to ten blind passages. When CPE was observed and the presence of BoHV-4 was confirmed by nested PCR, the viral stock was amplified and stored at −80 °C.

Extraction of Viral DNA and PCR

DNA was extracted from infected cells using a commercially available kit (DNeasy Blood & Tissue Kit, Qiagen), according to the manufacturer's instructions. DNA concentration was determined by spectrophotometry at an absorbance of 260 nm. The presence of BoHV-4 DNA was evaluated by PCR assays targeting ORF8 (encoding glycoprotein B) [24] and ORF 25 [25].

Serology

The presence of anti-BoHV-4 antibodies was assessed by VNT on bovine serum samples collected at 0, 7, 11, 14 and 25 dpi, following the protocol previously described by *Frazier, et al.* [26]. Serum samples were also tested by ELISA (Bio-X Diagnostics, Belgium), using an anti-mammalian IgG-horseradish peroxidase conjugate, following manufacturer's instructions. For each sample, the serum coefficient was calculated (OD sample – OD negative serum / OD positive serum – OD negative serum) x 100. A sample was considered negative if its coefficient was less than 30%, and positive if the coefficient was greater or equal to 30%.

Results

Clinical Observation of BoHV-4- Inoculated Calves

Calves challenged with BoHV-4 isolates 07/435 or 10/154 showed moderate muco-purulent nasal discharge which was evidenced in both inoculated groups between 5 to 11 dpi and that persisted for 2-5 days. In the group inoculated with isolate 07/435, two calves showed coughing at 6 and 8 dpi and it persisted for 3 days. As expected, clinical signs were not observed in mock-infected calves. For calves inoculated with isolate 10/154, rectal temperature remained normal (temperature range, 38.0 to 39.5 °C). In the group of calves inoculated with isolate 07/435, temperature ranged from 40.0 to 41.5 °C at 6-8 dpi. Body temperature in mock-infected cattle remained within normal ranges.

Virus Isolation

Virus was consistently isolated from nasal and ocular swabs of all calves, irrespective of the virus strain inoculated. Isolate 07/435 was isolated from nasal swabs at 1, 2 and 9 dpi and from ocular swabs the virus was recovered at 4, 6, 7 and 14 dpi. Isolate 10/154 was recovered from nasal secretions at 2, 4, 5, 7, 11 and 14 dpi. However, isolation from ocular swabs was only achieved at 4 dpi. None of the isolates was recovered from the buffy coats at any time of the experimental period. As expected, the virus was not isolated from mock-infected calves (Table 1).

Time post-infection (dpi)	Isolate 07/435						Isolate 10/154					
	Virus isolation			PCR			Virus isolation			PCR		
	OS	NS	BC	OS	NS	BC	OS	NS	BC	OS	NS	BC
0	-	-	-	-	-	-	-	-	-	-	-	-
1	-	5-Jan	-	-	5-Jan	-	-	-	-	-	-	
2	-	5-Jan	-	-	5-Jan	-	-	5-Mar	-	-	5-Mar	-
4	5-Jan	-	-	5-Jan	-	-	5-Jan	5-Jan	-	5-Jan	5-Jan	-
5	-	-	-	-	-	-	-	5-Feb	-	-	5-Feb	-
6	5-Feb	-	-	5-Feb	-	-	-	-	-	-	-	-
7	5-Feb	-	-	5-Feb	-	5-Jan	-	5-Mar	-	-	-	5-Mar
8	-	-	-	-	-	-	-		-	-	-	-
9	-	5-Jan	-	-	5-Jan	-	-		-	-	-	-
11	-	-	-	-	-	-	-	5-Jan	-	-	4-Jan	-
14	5-Jan	-	-	5-Jan	-	5-Feb	-	5-Jan	-	-	4-Jan	5-Apr
25	-	-	-	-	-	-	-	-	-	-	-	-

Number of positive calves out of the total in each group is indicated at each time point.

OS: ocular swabs, NS: nasal swabs, BC: buffy coat, -: negative.

Table 1: Virus isolation and PCR results from ocular and nasal secretions and buffy coats in BoHV-4 intranasally-challenged calves

Detection of BoHV-4 DNA in Nasal and Ocular Secretions and Buffy Coat Fractions

All isolates from nasal and ocular swabs were confirmed by PCR for ORF 8 and ORF 25. BoHV-4 DNA was detected in buffy coats of calves from both inoculated groups at 7 and 14 dpi (Table 1).

Serology

Serum Neutralization

Independently of the inoculated BoHV-4 isolate, the serologic response of the experimentally-infected calves was weak. The presence of neutralizing antibody titers (highest titer, log 1.8 at 25 dpi) was detected in tests performed using isolate 07/435 for VNT and serum samples from calves in Group 1 (07/435) and Group 2 (10/154). Seroconversion was not detected in animals from any group. It is important to note that at day 0 and in mock-infected calves neutralizing antibodies were detectable when the test was performed with isolate 07/435. These results have also been observed when other Argentinean BoHV-4 strains were used to perform the VNT (data not shown). When isolate 10/154 was used for VNT, all serum samples (homologous and heterologous) were negative (Table 2). Calves in all group had no neutralizing antibody titers to BoHV-1 at any time-point (data not shown).

		Time post-infection (VNT with isolate 07/435)					Time post-infection (VNT with isolate 10/154)				
Isolate	**Calf**	**0**	**7**	**11**	**14**	**25**	**0**	**7**	**11**	**14**	**25**
Group 1 07/435	3	0.9	1.2	1.2	1.2	1.5	-	-	-	-	-
	7	0.9	1.5	1.5	1.5	1.8	-	-	-	-	-
	8	1.2	1.2	0.9	0.9	1.8	-	-	-	-	-
	11	0.9	1.2	1.2	0.9	1.5	-	-	-	-	-
	12	0.9	0.9	0.9	1.2	1.5	-	-	-	-	-
Group 2 10/154	4	0.6	0.9	0.6	0.9	ND	-	-	-	-	-
	9	0.6	1.2	0.6	1.2	ND	-	-	-	-	-
	10	0.6	0.6	0.9	0.6	ND	-	-	-	-	-
	13	0.9	1.2	0.9	0.9	ND	-	-	-	-	-
	14	0.6	0.9	1.2	1.2	ND	-	-	-	-	-
Group 3 mock-infected	1	0.9	1.2	0.6	0.6	ND	-	-	-	-	-
	2	0.9	0.9	0.6	0.6	ND	-	-	-	-	-
	5	0.6	1.2	0.6	0.6	ND	-	-	-	-	-
	6	0.6	0.9	0.9	0.9	ND	-	-	-	-	-
	15	0.9	0.9	1.2	0.9	ND	-	-	-	-	-

ND: not done. Virus titres expressed as log.

Table 2: Virus neutralization titers using isolates 07/435 and 10/154 with homologous and heterologous serum samples of infected and control calves

The serologic response of the experimentally-infected calves was weak, regardless of the inoculated BoHV-4 isolate. The presence of neutralizing antibody titers (highest titer 1:64 (1.8) at 25 days post-infection, dpi) was detected in VN tests performed using isolate 07/435 and in serum samples from calves in the Group 1 (07/435) and Group 2 (10/154) strains (Table 2). Seroconversion was not detected in calves from any group. It is important to note that at day 0 and in mock-infected calves neutralizing antibodies were detectable when the test was performed with isolate 07/435. These results have also been observed when other Argentine BoHV-4 strains were used to perform VN assays (data not shown). When isolate 10/154 was used for VN assays, all serum samples (homologous and heterologous) were negative. Calves in all groups at all times had no neutralizing antibody titers to BoHV-1 (data not shown). Neutralizing antibodies to the 2 strains (07/435 and 10/154) were not detected when sera from colostrum-deprived calves were evaluated.

Elisa

When serum samples were evaluated by ELISA, antibodies were not detected in infected calves. Only one calf infected with isolate 10/154 had anti-BoHV-4 antibodies at 14 dpi. However, 2 calves infected with isolate 07/435 had detectable antibody levels from 4 dpi (Table 3).

		Time post-infection (days)					
	Calf	**0**	**4**	**7**	**11**	**14**	**25**
Group 1 Isolate 07/435	3	23.4	0.1	0.1	0.2	ND	ND
	7	0.1	0.1	0.1	0.1	0.1	0.2
	8	0.1	0.1	0.1	0.1	0.3	0.1
	11	13.2	32.9	35.7	51.5	21.1	23.7
	12	25.3	60.2	50.3	64.9	42.2	30.3

		Time post-infection (days)					
	Calf	**0**	**4**	**7**	**11**	**14**	**25**
Group 2 Isolate 10/154	4	1.5	1.7	1.2	1.1	1.2	ND
	9	0.1	1	0.1	0.1	0.1	ND
	10	0.1	0.1	0.1	0.2	0.2	ND
	13	0.2	1.5	1.6	2.2	1.9	ND
	14	0.3	13.7	15.7	15.8	35.7	ND
Group 3 mock-infected	1	0.1	ND	ND	ND	ND	ND
	2	0.1	ND	ND	ND	ND	ND
	5	0.1	ND	ND	ND	ND	ND
	6	9.3	ND	ND	ND	ND	ND
	15	0.1	ND	ND	ND	ND	ND

ND: not done. Values are expressed as coefficient according at Kit ELISA (Bio-X Diagnostics, Belgium)

Table 3: Anti-BoHV-4 antibodies detected by ELISA serum samples of experimentally challenged and control calves

Discussion

The results of these experiments constitute an initial step to understand the *in vivo* behaviour of Argentinean BoHV-4 isolates. First, both isolates under study were able to replicate in their host after experimental infection by the nasal route. The absence of severe clinical signs after the experimental inoculation of cattle with different BoHV-4 strains has also been reported by others [27]. BoHV-4 was intermittently isolated from nasal and ocular secretions after inoculation. It is apparent that isolate 10/154 replicates better in nasal epithelium than isolate 07/435 since the virus was isolated from more animals and during more days, even when a lower viral dose was used for the experimental inoculation. On the contrary, isolate 07/435 was consistently detected in ocular secretions. BoHV-4 excretion was detected up to 14 dpi. This finding suggests that by this time-point latency was possibly established, in agreement with the findings by [28]. Leukocytes have been recognized as the main site of latency of BoHV-4 [2,18,19,28]. Viral DNA was detected in leukocytes from calves in both inoculated groups.

Nevertheless, the number of animals harboring DNA from isolate 10/154 in the buffy coat fraction was higher when compared with isolate 07/435. Overall, this information suggests that there are differences in the pattern of *in vivo* behavior of these strains. *In vitro* characterization of these viral isolates demonstrated that isolate 07/435 replicates at higher titers in MDBK cells when compared to BoHV-4 10/154. The peak of titers for 07/435 was recorded at 48 h post-incubation whereas 10/154 reached its peak at 120 h of culture [21]. The BoHV-4 isolates belonging to different genotypes have distinct *in vitro* growth kinetics and this is also reflected on their *in vivo* biological properties. This observation provides additional evidence to the fact that BoHV-4 isolates constitute a group of highly divergent strains with genotypic and phenotypic differences. Presently, studies are being conducted to analyze the distribution of BoHV-4 isolates from different phylogenetic clusters in the tissues of cattle.

Low antibody titers have been detected in this study by virus neutralization and ELISA assay. This has also been reported by other authors [26,29-31]. *Kruger, et al.* [20] assumed that absence of neutralizing antibodies might be due to the intrinsic biology of the virus. It is speculated that natural or experimental infection of cattle with BoHV-4 does not trigger a strong immune response mediated by neutralizing antibodies. Similar results were also by *Mohanty, et al.* [11] after the experimental infection of calves with the American prototype strain DN-599. This fact can be explained since glycoprotein domains involved in BoHV-4 infectivity are poorly exposed to the immune system [32]. *Asano, et al.* [33] suggested that as a result of this inefficient humoral immune response, BoHV-4 is able to establish latency or persist in the host. Although the exact mechanism of antibody evasion is not completely understood, it has been shown that O-glycosylation of the major BoHV-4 glycoprotein gp180 is responsible for hiding BoHV-4 vulnerable epitopes [34].

Frazier, et al. [26] suggests that false negative results are a major problem of serologic assays for BoHV-4. Since neutralizing antibody titers were not detected against the field isolate 10/154 (American genotype 2), a relevant finding from this study was that neutralizing activity was detected in calves inoculated with isolate 07/435 (Argentinean genotype 3). This information raises new questions on the nature of the immunological response to BoHV-4. Further studies are required to understand why neutralization titers are detectable in uninfected animals when assays are performed with particular BoHV-4 strains. It is also likely that these strains have cross-reactive antigens with other gamma-herpesviruses, as it has been previously reported by de *Boer, et al.* [35], who suggested BoHV-4 serologic cross-reactivity with bovine lymphotropic herpesvirus (BLHV). Overall, the analysis of the serological results suggests that none of the available tests evaluating the humoral immune response are adequate for assessing the status of BoHV-4 infection in cattle.

Our results clearly demonstrate the differences between the 2 strains under study. The results obtained in the serological analysis can be attributed to differences in the expression of antigenic proteins or to post-translational modifications that mask neutralizing epitopes. Generally, BoHV-4 is recognized as a virus that does not induce an important humoral immune response, which is characterized by the production of low neutralizing antibody levels [29]. However, strain 07/435 was shown to significantly induce the production of high titers of neutralizing antibodies in bovines.

Conclusions

In this study, it is confirmed that there is biological variability between circulating BoHV-4 strains. Future directions should be aimed to find diagnostic assays suitable for detecting BoHV-4 at the herd level and further research should be conducted to understand the immune response to the infections caused by this virus. We describe here in the results of the first comprehensive analysis of the experimental infection with 2 BoHV-4 Argentine strains that were previously characterized as being of genotypes 1 and 2 [7]. While O-glycans contribute to BoHV-4 evasion of neutralizing antibodies, all together, our results suggest that BoHV-4 strain 07/435 has the ability to neutralize the virus in serum samples. The most relevant serological results were observed with alone a strain. In the future, such findings could enhance our understanding of gammaherpesviruses (e.g., BoHV-4) life cycle, including elucidation of the processes of viral entry, assembly, and egress. This may lead to the development of more effective diagnostic tests for use in the detection of BoHV-4 infections.

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