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Clinical and reproductive consequences of using BVDV-contaminated semen in artificial insemination in a beef herd in Argentina

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ABSTRACT

The current report was prompted by an atypical outbreak of mucosal disease that occurred in a beef herd in the southwestern part of Buenos Aires Province, Argentina, where a total of 9/41 (21.9%) yearling bulls died. Blood samples from 73 bulls and 189 heifers were tested for evidence of persistent BVDV infection with Bovine Viral Diarrhea Virus (BVDV). Non-cytopathic BVDV was isolated from 7 (9.6%) 24- to 36-month-old bulls, and 3 (1.6%) 36-month-old heifers. Non-cytopathic BVDV was also detected in the seminal plasma of three of six persistently infected (PI) bulls. Furthermore, a 171 bp genomic fragment of BVDV was consistently detected by nested RT-PCR in one of the two samples of the commercial semen used for artificial insemination, indicating that this semen could be a possible source of infection for the whole herd. To evaluate the possible reproductive consequences of PI heifers and bulls, ovaries and semen were obtained from PI cattle for *in vitro* assays. The *in vitro* fertilization of oocytes with semen from PI bulls was associated with decreased cleavage and embryo development rates. Additionally, non-cytopathic BVDV was isolated from the follicular fluid of PI heifers. Genetic typing revealed that all isolates BVDV from the present study had a high percentage of homology and that all of the fragments from the RT-PCR clearly fit with the BVDV 1b cluster. These findings confirm the negative impact that BVDV can have on the reproductive performance of cattle and the importance of applying the proper sanitary controls to minimize the risk of BVDV infection.

1. Introduction

Bovine viral diarrhea (BVD) is an economically important disease in cattle with a worldwide distribution. This disease is caused by the Bovine Viral Diarrhea Virus (BVDV), a *Pestivirus* of the *Flaviviridae* family (Bachofen et al., 2010). *Pestiviruses* are considered to be some of the most successful viral agents in nature as a result of their capability to cause disseminated disease and to persist undetected within a herd. Acute infections (horizontal transmission) with prevailing non-cytopathogenic (ncp) BVDV strains are characterized by transient viremia with variable shedding of viruses (Houe, 1995). Trans-placental (vertical) transmission of the virus can lead to the birth of a persistently infected (PI) animal (Viet et al., 2007). Persistently infected cattle are immune-tolerant to BVDV and contribute significantly to the continuous spread of the virus through a

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herd. Therefore, the identification and removal of PI cattle is essential to control BVDV infections. Persistently infected cattle are susceptible to mucosal disease (Sandvik, 2005).

Mucosal disease (MD) occurs when PI cattle become super-infected with cytopathogenic (cp) biotypes of BVDV (Bolin et al., 1985). Because PI cattle comprise approximately 1% of the cattle population in an infected herd, mucosal disease is characterized by a low case attack rate coupled with a high fatality rate (Brownlie, 1990). Cohorts of PI animals infected with the same strain of BVDV often succumb to mucosal disease in a narrow span of time, which occurs when the ncp-BVDV infecting one PI animal develops a mutation that yields a cp-BVDV, which is then subsequently spread to the rest of the PI cohort (Bolin et al., 1985). In Argentina, the strategy for the prevention of BVDV is based on the immunization of cattle using inactivated virus vaccines. The current case report was prompted by an atypical outbreak of mucosal disease, which occurred in a ranch in the southwestern region of Buenos Aires Province, Argentina, where a high number of PI animals were subsequently detected.

2. Materials and methods

2.1. Animals and history

An Angus beef herd suffering reproductive losses located in Buenos Aires Province, Argentina, comprising 560 cows, 201 heifers, 81 adult bulls, and a group of 41 yearling (virgin) bulls, was used in this study. A routine vaccination program, consisting of two doses of an inactivated BVDV vaccine in heifers and cows prior to the mating season, and artificial insemination were performed yearly. Every two years, females were artificially inseminated (AI) with commercially available frozen semen.

By the beginning of 2009, an outbreak of abortions and calves with congenital defects was detected by the practitioner. In May 2009, two yearling bulls were found dead without previous clinical signs of disease. One month later, another two bulls exhibited signs of disease including ptyalism and diarrhea. After that, the Specialised Veterinary Diagnostic Service (SDVE) of INTA Balcarce visited the herd to assist with the diagnosis. Clinical specimens were obtained from three affected yearling bulls, and full *post-mortem* examinations of two of the three affected bulls were performed. One week after the SDVE visits, two new cases were observed. A total of nine (22%) yearling bulls died. All affected animals belonged to the herd of bulls born by means of AI using commercially available semen from two bulls, purchased from the same AI center. Because a presumptive outbreak of mucosal disease was diagnosed by the SDVE, screening of the entire herd was recommended to detect other PI animals on the premises.

2.2. Pathology

Tissues obtained at necropsy were fixed by immersion in 10% neutral buffered formalin for 48 h, followed by standard methods for the preparation of histological sections stained with hematoxylin and eosin (H + E) (Campero et al., 2003). To investigate the presence of concurrent bacterial infections, tissue and fluid samples collected aseptically during postmortem examinations were submitted for aerobic and microaerophilic bacterial isolation.

2.3. Laboratory assays: samples, culture and technical procedures

Blood and tissue samples obtained from 3 bulls at necropsy (samples 09/342 and 09/366) were processed for virus isolation, virus neutralization assays, and microscopic examination using previously described routine methods (Odeón et al., 2003).

The detection of persistent BVDV infections was performed using heparinized blood samples from 73 bulls (09/464) and 189 heifers (09/731V). This technique involved virus isolation in microplates and detection by a direct fluorescent antibody test (DFAT) using conjugated porcine polyclonal antiserum (American Bioresearch, Sevierville, TN).

To evaluate the possible reproductive consequences of having PI heifers and bulls in the herd, ovaries and semen were obtained from PI cattle. The surgical castration of three PI heifers and the collection of semen by electroejaculation from six PI bulls were performed by licensed veterinarians using a Hauptner castration device (Ecraseur, Hauptner, Germany) to remove both ovaries from each animal and an Electrojac V (Ideal Instruments, Chicago, IL, USA) to obtain semen. After routine semen evaluation, the ejaculates from five bulls were frozen in liquid nitrogen *via* standard procedures.

BVDV titration was performed using the follicular fluid of the ovaries from three PI heifers and the seminal plasma of six PI bulls. The virus titration procedures involved multiple ten-fold dilutions using the endpoint method (Reed and Muench, 1938). BVDV viral neutralization was evaluated in seminal plasma from PI yearling bulls.

2.4. Processing and analysis of semen

Routine semen analysis, including the measurement of the semen volume and of the concentration and percentage of motile spermatozoa, was performed on the ejaculates of the six PI bulls according to the recommendations of the Society for Theriogenology (Kenney et al., 1983). Briefly, sperm concentration was performed by cell counting with a Neubauer chamber after dilutions of semen samples. Motility after thawing was determined by direct observation with a white light microscope of semen samples.

Semen straws from two bulls used for AI were also obtained to check for the presence of BVDV.

2.5. Nested PCR (nPCR) (RT-PCR universal Pan Pesti)

A nested PCR assay was performed on all semen samples from PI bulls and on the commercially available frozen semen from two bulls as previously described Hyndman et al. (1998). Two minor modifications were made: the denaturation conditions were 94 °C for 1 min, and 30 cycles were used in the first round. The expected amplified product was 171 bp long. The amplified products were

Table 1
Details of the field strains. WNC: without accession number.

Strain	Date of isolation	Biotype	Accession number GenBank
09/342	18/06/2009	Cp	JQ646075
09/366	25/06/2009	Cp	JQ646074
09/464	31/07/2009	Ncp	JQ646073
09/731V	10/12/2009	Ncp	WNC
09/731S	10/12/2009	Ncp	WNC

separated by electrophoresis in a 2% agarose gel, and the DNA bands were visualized by ethidium bromide staining.

Universal Pan *Pestivirus* primers (324/326) were used in the RT-PCR, with an annealing temperature of 51 °C in the case of virus isolation, to obtain 288 bp sequences (Vilcek et al., 1994).

2.6. Phylogenic analysis

The amplified fragments from the RT-PCR reactions belonging to samples 09/342, 09/366, 09/464, 09/731S, and 09/731V were sequenced by the Biotechnology Department of INTA Castelar and analyzed with BioEdit software (version 7.0.9.0) to obtain 150 bp sequences (Table 1). The Clustal X program (version 1.8.3) was used to align the sequences. Distance matrices and dendrograms were calculated using the Kimura 2-parameter method (Kimura, 1980) and Neighbor Joining (Saitou and Nei, 1987) using the Mega program (version 4) (Tamura et al., 2007) with a bootstrap test with 1000 resamples. For visualization and modeling, Dendroscope software (version 2.7.4) (Huson et al., 2007) was used.

2.7. In vitro fertilization and embryo evaluation

Cumulus-oocyte complexes (COCs) were recovered by aspiration of 2–10 mm follicles in ovaries collected at a local slaughterhouse and were cultured *in vitro*. Fertilization was performed with frozen–thawed semen from PI bulls after purification *via* a discontinuous Percoll density gradient (30–60–90%). The final sperm concentrations were 2×10^6 sperm/ml. The gamete co-culture was performed in IVF-SOF supplemented with 50 μg/ml heparin.

After 24 h, presumptive zygotes were stripped and then cultured in SOF-citrate at 38.5 °C and 5% CO_2, 5% O_2, and 90% N_2 with maximum humidity for seven days. Cleavage and embryo production were assessed at days two and seven, respectively.

2.8. Statistical analysis

The data collected from IVF procedures (cleavage and embryo production) were analyzed by the Chi square test ($P < 0.05$). A P value <0.05 was considered indicative of statistical significance.

3. Results

3.1. Diagnosis of mucosal disease and persistent infections in the herd

Cytopathic BVDV was isolated from the intestines, spleen, lymph nodes, skin, and buffy coat of the two cases analyzed (09/342 and 09/366). A total of 9/41 (21.9%) 18-month-old bulls were confirmed to have died from mucosal disease. Non-cytopathic BVDV was isolated from the buffy coat collected from seven (09/464) of 73 (9.6%) 24- to 36-month-old bulls and three (09/731V) out of 189 (1.6%) 36-month-old heifers. All positive animals were confirmed as persistently infected by virus isolation from a second serum and buffy coat sample taken 20 days after the first sampling. Including the nine bulls that died from mucosal disease, the overall prevalence of PI bulls was 14%.

3.2. Histopathological findings

The microscopic changes found in the three analyzed yearling bulls were compatible with mucosal disease. Histological analysis revealed hemorrhages and lymphoid depletion as well as follicular lymphocytolysis of primary lymphoid follicles in the peripheral lymph nodes. Individual cell necrosis and vacuolation were present in the stratum basale and stratum spinosum of the squamous epithelia in the tongue and esophagus. Minimal non-suppurative inflammation was shown to be associated with these epithelial lesions. The lamina propria of the small intestine also had several hemorrhages and exhibits signs of moderate inflammatory caused by macrophages, lymphocytes, and plasma cells. The degeneration and necrosis of the intestinal crypt epithelium were prominent findings in the small intestine. The affected crypts were dilated and contained large amounts of cell debris with mixed neutrophils and macrophages. Lymphoid necrosis in Peyer's patches and primary follicles of the spleen was also a common finding.

3.3. Isolation and titration viral

Non-cytopathic BVDV was detected in follicular fluid from PI heifers by viral isolation. The viral titers in the follicular fluid and serum of the PI heifers were determined and were on the order of $1 \times 10^{8.3} \pm 0.75\ TCID_{50}$/ml and $1 \times 10^{4.1} \pm 1.1\ TCID_{50}$/ml, respectively. Moreover, the presence of ncp-BVDV was detected in seminal plasma from three of the six PI bulls by viral isolation, and the viral titers in the seminal plasma of the PI bulls were on the order of $1 \times 10^4 \pm 0.28\ TCID_{50}$/ml. Virus was isolated from the frozen–thawed semen sample from five of six PI bulls (one of the samples was not used because of poor quality). In the three bulls negative for viral isolation from seminal plasma, we confirmed the presence of neutralizing antibodies against the reference cytopathic strain (Singer).

3.4. Detection of viral RNA by nested RT-PCR in commercial semen

BVDV was not detected in either of the two samples of commercially available frozen semen by viral isolation.

Table 2
Microscopic analysis of PI bulls spermatozoa. ND: not done.

Bull no.	Concentration ($\times 10^6$ spermatozoa/ml)	Sperm motility (%)
PI 1	348	50
PI 2	72	40
PI 3	208	35S
PI 4	80	35
PI 5	280	60
PI 6	ND	–

However, an amplified fragment of approximately 171 bp of BVDV genomic RNA (5′UTR) was consistently detected by nested RT-PCR in one of the two samples of the commercial semen used for AI (09/731S). Moreover, the same genomic fragment was detected in all five semen samples from PI bulls.

3.5. Sequence analysis of severe acute and persistent BVDV strains

Genetic typing revealed that all isolates of BVDV from the present case had a high percentage of homology and that all of the fragments from the RT-PCRs clearly fit into the BVDV 1b cluster (Fig. 1). Four of the sequences examined clustered in a monophyletic group, composed only of Argentinian isolates. The remaining sequence (09/464) grouped with other Argentinian isolates that are closely related to the Osloss reference strain, but both groups showed low bootstrap values. Nucleotide identity among the five sequences studied ranged between 92% and 99%, and the range of the percent identity among all type 1b sequences was 90–100%.

3.6. Analysis semen from PI bulls and fertilization in vitro

The concentration and percentage of motile spermatozoa in each ejaculate were assessed (Table 2). The concentration and percentage of motile spermatozoa were within the normal ranges. These ranges include: concentration from 300 to 2500 million per ml, with an average of 1200 million of sperm. Motility after thawing should be greater than 50%.

A summary of the results of the *in vitro* fertilization with semen from PI bulls is shown in Table 3. The fertilization of oocytes with this semen was associated with significantly lower cleavage and embryo development rates compared with *in vitro* fertilization with semen free of BVDV (Table 3).

Table 3
In vitro **e**mbryo development after fertilization with semen from BVDV PI bulls. Media values with different letters show significant differences $P < 0.05$.

Group	Cleaved oocytes/total oocytes (%)	Day 7 embryos/total oocytes (%)
Control	75/108[a] (69.4)	26/108[a] (24.1)
PI	174/461[b] (37.8)	50/461[b] (10.8)

PI, persistent infected.

4. Discussion

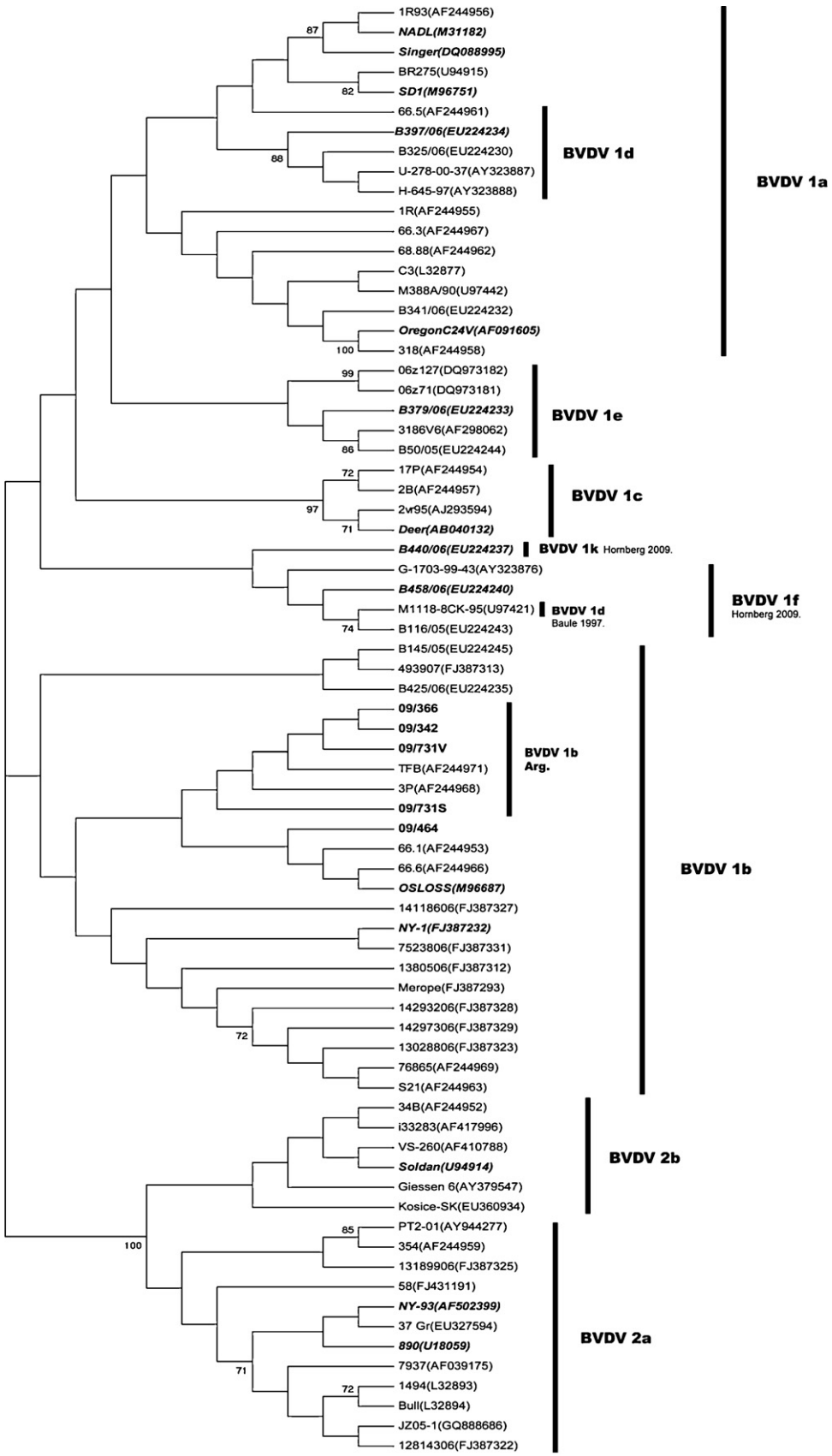
The current study revealed an unusually high outbreak prevalence of MD and persistent infection with ncp-BVDV in the herd for which the most probable source of infection was AI using contaminated semen. The transmission of a BVDV within a herd, or between herds, depends primarily on contact between susceptible individuals and PI animals (Kirkland et al., 1994). Sometimes the introduction of the virus into a herd may remain unrecognized for many months. The infection can be introduced in a covert way, and the appearance of disease can be delayed, making it extremely difficult to subsequently trace the source of the infection. In this report, an exhaustive analysis of the management of the herd was performed. Other sources of BVDV infection were ruled out because the addition of new cattle was not registered during the study and the whole herd was screened for the presence of BVDV infection. Therefore, because the yearling bulls were sired by artificial insemination with commercial semen from the same AI center that was confirmed to be contaminated with ncp-BVDV, is likely that the source of the infection was the semen used in the AI.

Several outbreaks of mucosal disease have been reported previously (Odeon et al., 2003; Lunardi et al., 2008). However, in this case, an association between mucosal disease and an unusually high prevalence of persistently infected animals in a herd was established. The outstanding points in this case report are the high percentages of mortality (22%) due to mucosal disease among yearling bulls, the high prevalence (12%) of PI cattle in the herd and the link between these events and AI using contaminated semen.

Interestingly, a higher prevalence was observed in bulls than in heifers. This fact can be explained by the selection criteria used by the cattlemen; heifers were removed from the herd for sale after weaning, and the offspring of cows were selected for replacement according to three selection criteria: frame, biotype, and ovarian activity. Given that PI cattle often exhibit low rates of development and weight gain, this type of management could explain the differences in prevalence between PI bulls and heifers because PI heifers were culled from the herd early because of their poor development or body condition.

The circulation of BVDV in herds leads to two important problems: the generation of PI animals and economic loss associated with decreases in the fertility and reproductive efficiency. The detection of several isolates of BVDV with a high percent homology within the same herd indicates a common source of the virus, such as contaminated semen, and the widespread circulation of these strains among different categories of cattle. Genotyping revealed that the strains involved in this outbreak were BVDV type 1b. In Argentina, the circulation of BVDV types 1a, 1b, 1c, and 2 has been reported (Jones et al., 2001). The most recent results regarding the circulation of BVDV in Buenos Aires Province revealed that most isolates belong to BVDV type 1b (personal communication, Pecora A and Malacari D).

Although reproductive disorders were observed in the current study, it was not possible to associate the clinical signs with the genotype of the strains. This lack of


1R93(AF244956)
NADL(M31182)
Singer(DQ088995)
BR275(U94915)
SD1(M96751)
66.5(AF244961)
B397/06(EU224234)
B325/06(EU224230)
U-278-00-37(AY323887)
H-645-97(AY323888)
BVDV 1d
BVDV 1a
1R(AF244955)
66.3(AF244967)
68.88(AF244962)
C3(L32877)
M388A/90(U97442)
B341/06(EU224232)
OregonC24V(AF091605)
318(AF244958)
06z127(DQ973182)
06z71(DQ973181)
B379/06(EU224233)
3186V6(AF298062)
B50/05(EU224244)
BVDV 1e
17P(AF244954)
2B(AF244957)
2wr95(AJ293594)
Deer(AB040132)
BVDV 1c
B440/06(EU224237)
BVDV 1k Hornberg 2009.
G-1703-99-43(AY323876)
B458/06(EU224240)
M1118-8CK-95(U97421)
B116/05(EU224243)
BVDV 1d
Baule 1997.
BVDV 1f
Hornberg 2009.
B145/05(EU224245)
493907(FJ387313)
B425/06(EU224235)
09/366
09/342
09/731V
TFB(AF244971)
3P(AF244968)
09/731S
BVDV 1b
Arg.
09/464
66.1(AF244953)
66.6(AF244966)
OSLOSS(M96687)
14118606(FJ387327)
NY-1(FJ387232)
7523806(FJ387331)
1380506(FJ387312)
Merope(FJ387293)
14293206(FJ387328)
14297306(FJ387329)
13028806(FJ387323)
76865(AF244969)
S21(AF244963)
BVDV 1b
34B(AF244952)
i33283(AF417996)
VS-260(AF410788)
Soldan(U94914)
Giessen 6(AY379547)
Kosice-SK(EU360934)
BVDV 2b
PT2-01(AY944277)
354(AF244959)
13189906(FJ387325)
58(FJ431191)
NY-93(AF502399)
37 Gr(EU327594)
890(U18059)
7937(AF039175)
1494(L32893)
Bull(L32894)
JZ05-1(GQ888686)
12814306(FJ387322)
BVDV 2a

association could be explained by the fact that the isolates were obtained from PI animals, and therefore, the clinical manifestations have little to do with the BVDV infection *per se*. The inability of PI animals to handle normal bovine pathogens can result in clinical presentations more closely linked to the secondary pathogen than to the BVDV infection (Tajima and Dubovi, 2005). In these cases, the experimental infection of susceptible animals or other prospective studies should be performed to elucidate the clinical signs associated with this PI strain.

The high titers of ncp-BVDV detected in the follicular fluid of PI heifers are consistent with the results of other studies that showed virus titers of $1 \times 10^{6.97} \pm 0.17\,TCID_{50}$/ml in ovarian tissue collected from PI heifers (Fray et al., 2000). The presence of ncp-BVDV within the ovaries, uterus, and corpus luteum of heifers has been documented in previous studies (Booth et al., 1995; Grooms et al., 1996) and is in agreement with our results, confirming the affinity of BVDVs for reproductive tissues. In addition, the virus concentration in the serum from the three PI heifers was similar ($1 \times 10^{4.0}\,TCID_{50}$/ml) to those reported previously (Brock et al., 1998), being within the range for PI cattle. The differences observed between the viral titers in the follicular fluid and serum could be attributed to fact that the serum concentrations of BVDV decline gradually in all persistently infected animals over a period of at least 2 years, whereas the follicular fluid concentrations remain more stable due to the abovementioned affinity of the virus for reproductive tissues (Brock et al., 1998).

In this report, it was possible to correlate the presence of neutralizing antibodies with the absence of virus in both the serum and the seminal plasma of PI bulls. This observation could have important implications for the identification of PI bulls by viral isolation from serum samples, a method that is used frequently in herd screening programs. Similar cases have been reported previously, in which the level of viremia was undetectable by virus isolation from the serum for one of the seven PI animals that had neutralizing antibodies to the reference strain, BJ (Brock et al., 1998). Another report presented a particular case of an immunocompetent, non-viremic bull from which BVDV was not isolated from a range of tissues and blood samples. This bull had a high neutralizing antibody titer to a specific BVDV isolate, confirming that this bull was not a classical PI animal (Vogues et al., 1998). High titers of virus in semen have been reported, usually at least 100 times higher than the titers in the blood. To date, there have been no reported studies determining the dose required to establish the infection through the reproductive tract. However, the levels in the semen are thought to be well above the threshold for infection, at least by this route. The high virus titers detected in the seminal plasma of PI bulls are comparable with the data presented in other studies (Kirkland et al., 1991) and not only reflect the presence of the virus in the reproductive tract but also indicate the ability of BVDV to replicate in those tissues.

As was clearly demonstrated in this study, the semen of persistently infected bulls is a risk factor and a potential source of BVDV infection. PI bulls usually exhibit normal growth and development, and the results of the macroscopic analysis of semen are normal. However, the bulls included in this study had high titers of BVDV in their semen ($1 \times 10^{7.6}\,TCID_{50}$/ml), and this virus can survive processing and cryopreservation (Kirkland et al., 1994; Givens and Waldrop, 2004). In this study, the use of semen from PI bulls in IVF procedures significantly decreased the cleavage and embryo development rates. These results agree with to those reported by other authors who have found that the use of frozen–thawed semen from PI bulls results in significantly lower fertilization, cleavage, and blastocyst development rates when used for *in vitro* embryo production (Guerin et al., 1992). Other authors have also noted that the effect of the virus on fertilization and early embryonic mortality could be associated with specific viral strains (Wentink et al., 1989). Our results indicate that semen is a direct input *via* the *in vitro* production system and highlight the impact of the virus on the rates of cleavage and embryo development.

The detection of viral RNA in un-preconditioned semen samples using a *nested* RT-PCR protocol in the current work enhanced the diagnostic quality by improving the time required and the sensitivity of the assay. In most diagnostic techniques, the semen sample should be processed before virus isolation or RNA extraction due to the deleterious effects on viral RNA, the cell culture cytotoxicity and the inhibition of reverse transcriptase enzymes caused by seminal plasma (Revell et al., 1988).

The results of this study confirm the severe negative impact that BVDV can have on the reproductive performance of cattle. The importance of applying the proper sanitary controls to minimize the risk of BVDV infection and the deleterious effects on reproduction were also demonstrated. Taking into account the limitations of inactivated vaccines and the genetic and antigenic diversity of BVDV, we should implement biosecurity practices to prevent the introduction and circulation of this virus in herds. Protocols for the detection of BVDV in the biological material used for AI and for elimination of contaminated materials are also needed. To ensure efficient control, it is important that all parties involved in the management and health supervision of livestock are familiarized with basic information on BVDV control, including the biology and epidemiology of the infection. The circumstances of the outbreak of MD described in this study and the association of this outbreak with the high prevalence of PI animals supports the implementation of routine screening when BVDV infection is suspected.

Fig. 1. Phylogenetic tree showing the genetic relationship of the BVDV isolates. The amplified fragments of the RT-PCR products belonging to protocols 09/342, 09/366, 09/464, 09/731S, and 09/731V, were sequenced and analyzed whit Bioedit 7.0.9.0 software to obtain 150 bp sequences. Clustal X program, version 1.8.3., was used to align the sequences. Distance matrix and dendrogram were calculated under Kimura 2 parameters method and Neighbor Joining using Mega program, version 4 with 1000 resample of bootstrap test. The isolates from this study are printed in bold. Reference strains are shown in cursive.

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