Detection of bovine herpesvirus 1 and 5 in semen from Brazilian bulls


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Abstract

Bovine herpesvirus 1 (BoHV-1) and 5 (BoHV-5) are important pathogens of the respiratory and genital tract of cattle and may also affect the central nervous system and cause meningoencephalitis. Both virus types are estimated to be widely distributed in Southern Brazil. In the present study, BoHV-1 and/or BoHV-5 DNA were detected in bovine semen samples from two states of Brazil by two species-specific nested polymerase chain reactions (nPCRs). These nPCRs were used to assay 53 samples of fresh semen and 23 samples of frozen semen from breeding bulls. Viral DNA was detected in all 76 semen samples: all were positive for BoHV-5, whereas 34 of these were positive for BoHV-1 as well. Moreover, in five fresh and in 13 frozen semen samples—of a total number of 40 samples suitable for virus isolation—infectious BoHV-1 and/or BoHV-5 virus were detected. In conclusion, both BoHV-1 and BoHV-5 were detected in bovine semen in Brazil highlighted the importance of examining bull semen in search for both agents to reduce the risk of transmitting these viruses.

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1. Introduction

Bovine herpesvirus 1 (BoHV-1) and 5 (BoHV-5) are pathogens of major veterinary importance, responsible for substantial economical losses worldwide [1–3]. Bovine herpesvirus 1 causes predominantly respiratory and genital disease in cattle [4], although it can also be associated with meningoencephalitis [5]. The closely related BoHV-5 is a major cause of viral meningoencephalitis in cattle, but can also infect the genital tract [6]. Both viruses have been associated with reproductive failure, e.g., early embryonic death and abortions, which probably account for the most significant losses linked to BoHV-1 and BoHV-5 [7–9]. Due to their high degree of antigenic similarity, BoHV-1 and BoHV-5 are highly cross-reactive in standard serological assays. Therefore, type-specific prevalences have not, to date, been determined [10,11].

It has been estimated that BoHV-1 infects 50–90% of the Brazilian cattle population [12,13]. However, the prevalence of BoHV-5 infections, as well as how much

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of the estimated BoHV-1 prevalence is actually due to BoHV-5 is unknown. Like other alphaherpesviruses, BoHV-1 and BoHV-5 can cause latent infections in trigeminal and paravertebral ganglia, from where they may eventually be reactivated [14,15]. During episodes of reactivation, viral shedding may take place and lead to contamination of semen [7,16].

We recently conducted a study to detect latent BoHV-1 and or BoHV-5 DNA in trigeminal ganglia of slaughtered cattle [17]. The objective of the present study was to identify BoHV-1 and BoHV-5 DNA in samples of semen from breeding bulls employing two species-specific nPCRs.

2. Materials and methods

2.1. Semen samples

Fifty-three samples of fresh semen were obtained from bulls from a farm in the Brazilian state Rio Grande do Sul (34) and a farm near the city of Goianópolis in the state Góias (19). In addition, 23 frozen semen samples were obtained from an artificial insemination center in Rio Grande do Sul. The bulls from the two farms were Nelore (Zebu) and ranged from 2–3 y old, whereas the AI centre bulls were Bos taurus (e.g., Angus, Hereford and Holstein) and ranged from 3–7 y. Although no serological data regarding BoHV-1 or BoHV-5 infections were available for any of these bulls, none showed obvious signs of a BoHV infection.

2.2. DNA extraction from fresh and frozen semen

Extraction of DNA from fresh and frozen semen was performed as described by van Engelenburg and others [16] and Smits and others [18], with small modifications. In brief, 50 μL of each semen sample were centrifuged (Eppendorf centrifuge 5415C) at 8500 g for 10 min. To each supernatant, 150 μL of TE buffer [10 mM Tris (Invitrogen, Carlsbad, CA, USA), pH 7.5; 1 mM EDTA (Invitrogen)] were added. Samples were then mixed with 22 μL of 10% sodium dodecyl sulfate (SDS, Promega, Madison, WI, USA), 0.5 μL transfer RNA (10.3 mg/mL, Sigma Chemical, St. Louis, MO, USA ), 1 μL proteinase K (20 mg/mL, USB Corporation, Cleveland, OH, USA), 200 molecules of an internal control (IC) plasmid [17] and incubated at 37 °C for 1 h. Extraction of DNA was performed by adding one volume of buffer-saturated phenol (pH 8.0, Invitrogen) and 13 μL of 5 M NaCl (Nuclear, Diadema, SP, Brazil), followed by incubation at room temperature for 30 min. After centrifugation at 8500 g for 12 min, 0.6 volumes of isopropanol (Pro Analysis, Indústria Quimica Ltda, Rio de Janeiro, RJ, Brazil) were added to the supernatant. Subsequently the mixture was incubated at -20 °C for 30 min and centrifuged for 20 min at 8500 g. Pellets were resuspended in 100 μL of TE buffer and 1 mL of n-butanol (Vetc, Rio de Janeiro, RJ, Brazil) was added. After vigorous mixing, the samples were centrifuged at 8500 g for 10 min. The DNA containing pellets were air dried and resuspended in 50 μL TE.

2.3. Nested polymerase chain reactions (nPCRs)

The nPCRs were performed as described by Campos and others [17]. In brief, for the first PCR, primers were used that recognize both BoHV-1 and BoHV-5 and were expected to give rise to amplicons of 575 bp (BoHV-1) and 572 bp (BoHV-5). To each reaction, 200 molecules of an internal control (IC) template were added to detect false negative reactions. The IC template is a plasmid containing the amplified region of BoHV-1, with a 135 bp internal deletion leaving the primer binding sites intact. The IC template gives an amplification product of 440 bp. To identify false positive reactions, three negative control samples, i.e., samples with 50 ng of BoHV negative bovine genomic DNA, were added to each set of 12 PCRs. Reactions were performed in a Mastercycler Eppendorf thermocycler and the products were analyzed by electrophoresis on a 1.5% agarose (Invitrogen) gel stained with ethidium bromide (0.5 μg/mL; Promega) and compared with pUC19 x HinfI (New England Biolabs, Ipswich, MA, USA) as a molecular weight marker.

To differentiate between BoHV-1 and BoHV-5, the products of the first PCR were used in two species-specific nPCRs, as described by Campos and others [17]. These nPCRs give rise to a BoHV-1 specific product of 161 bp and a BoHV-5 specific product of 236 bp.

2.4. Virus isolation from semen

Virus isolation was carried out following an OIE (World Organization for Animal Health) standard protocol [19]. In short, the semen samples were diluted in 90% fetal bovine serum (FBS, Gibco, Grand-Island, NY, USA) supplemented with amphotericin B (2.0 μg/mL, Cristália, Itapira, SP, Brazil), enrofloxacin (10.0 μg/mL, Baytril®, Bayer, São Paulo, SP, Brazil) and gentamicin (50.0 μg/mL, Gentamax®, Marcolab, Duque de Caxias, RJ, Brazil). Samples were added in quadruplicate into 96-well cell culture plates seeded
24 h prior with CRIB cells (Madin Darby bovine kidney cells resistant to BVDV) [20]. For each sample, 50 μL were added per well. Samples were incubated on cells for 1 h at 37 °C for adsorption. Subsequently, monolayers were gently washed twice with 100 μL of Eagle’s minimal essential medium (EMEM, Gibco) supplemented with 5% FBS (Gibco) plus antibiotics as above, and replenished with a fresh volume (100 μL/ well) of medium. Plates were then incubated for 72 h at 37 °C in a 5% CO₂ atmosphere (Forma Scientific). Negative control wells in each plate were mock infected with medium only, following the same protocol. Monolayers of CRIB cells were examined and compared with negative control cells for cytopathic effect (CPE). When no CPE was observed, plates were frozen and thawed, the cell medium was harvested and inoculated into new culture plates. If no CPE was observed up to the third blind passage, a sample was considered negative for virus isolation.

2.5. Isolation of viral DNA and restriction enzyme analysis

Seven of the 18 CPE positive semen samples were submitted for DNA isolation to confirm the presence of BoHV virus and to determine the species (BoHV-1 or BoHV-5). Recovery of viral DNA from BoHV-1 or BoHV-5 infected cells was performed as described by Green and others [21] and Pignatti and others [22], with some modifications as follows. Each of the seven samples was used to inoculate a confluent monolayer of CRIB cells in a 75 cm² tissue culture flask. When CPE was evident in approximately 90% of the cell monolayer, the supernatant was removed and the remaining infected monolayer was treated with 5 mL of 2.5% trypsin (Gibco) and incubated at 37 °C for 5 min and harvested carefully so as not to disrupt the cells. Next, 5 mL of cold PBS [8.5 g of NaCl, 1.84 g of Na₂HPO₄·2H₂O, 0.26 g of NaH₂PO₄·H₂O, H₂O q.s.p.1000 mL, pH 7.2] were added to the tissue culture flask to remove the residual cells and the 10 mL of cells were centrifuged for 10 min at 1000 g (Cientec CT 5000). The pellets were treated with 2 mL of lysis buffer [100 mM Tris-HCl (Invitrogen) pH 7.9, 100 mM EDTA (Invitrogen), 0.25% Triton X-100 (Roche Diagnostics Brasil Ltda., São Paulo, SP, Brazil)]. This solution was mixed gently, incubated for 10 min at room temperature and NaCl was added to a final concentration of 0.2 M. Next, this solution was centrifuged for 10 min at 1000 g to pellet the cell nuclei. To the supernatant 5 μL proteinase K (20 mg /mL, USB Corporation) and 40 μL 10% SDS were added to final concentrations of 50 μg/mL and 0.2%, respectively. This mixture was incubated 1 h at 56 °C followed by a phenol extraction and an isopropanol precipitation. The obtained pellet was resuspended in 10 μL of TE (10 mM Tris-HCl pH 7.9 and 1 mM EDTA) per tube supplemented with 3 μL of RNase (20 mg / mL, Qiagen Inc., Valencia, CA, USA). After incubation for 15 min at 37 °C, 3 to 7 μL (depending on the concentration of viral DNA) were used for restriction enzyme analysis (REA) with HindIII enzyme (New England Biolabs, Ipswich, MA, USA), which allows differentiation between BoHV-1 and 5. The reactions were analyzed by overnight electrophoresis on 0.7% agarose (Invitrogen) gel stained with ethidium bromide (0.5 μg/mL) and compared with Lambda DNA (Promega) x HindIII molecular weight marker.

3. Results

3.1. Detection of BoHV-1 and BoHV-5 DNA in semen samples

The 57 semen samples from Rio Grande do Sul (34 fresh semen samples from a breeding farm and 23 frozen semen samples from an artificial insemination (AI) centre) and 19 fresh semen samples from a breeding farm in the state Goiás, were all subjected to a first PCR, based on primers that recognized both BoHV-1 and BoHV-5. The products of this first PCR, however, were not always visible on the agarose gel, perhaps due to the low amount of viral DNA in the sample (Fig. 1A). The products of the first PCR were subjected to two species-specific nPCRs that were able to discriminate between DNA of BoHV-1 and DNA of BoHV-5. The BoHV-1 specific nPCR gave rise to a product of 161 bp, whereas the BoHV-5 specific nPCR gave rise to a product of 236 bp (Fig. 1B).

All 76 semen samples were positive for BoHV-5 DNA, whereas 34 (44.7%) of these were also positive for BoHV-1 (Table 1). Of the 57 semen samples collected from bulls of European breeds in the state of Rio Grande do Sul, 25 were positive for both BoHV-1 and BoHV-5. Among the semen samples of the AI center, fewer samples were positive for both virus species. Of the 23 frozen semen samples from the AI center, five were positive for both virus species. Conversely, of the 34 fresh semen samples of a farm in this state 20 samples were positive for both BoHV-1 and BoHV-5. Of the 19 fresh semen samples collected from bulls of the Zebu breed in the state of Goiás, nine samples were positive for both BoHV-1 and BoHV-5.
3.2. Virus isolation and REA

To determine whether the BoHV-1 and/or BoHV-5 DNA-positive semen samples contained infectious virus, virus isolation was performed. However, 36 samples could not be assayed due to lack of sufficient semen (16) or due to cytotoxicity (20). Of the tested remaining 40 samples, 18 produced a typical herpesvirus like cytopathic effect (CPE) and were considered positive (45%) (Table 1). Thirteen of these 18 semen samples with infectious BoHV were from bulls of the AI centre in Rio Grande do Sul (European breeds) and five were from bulls from the farm in Goiás (Zebu breed).

To confirm that the CPE was really due to a bovine herpesvirus infection and to determine which of the two viruses (BoHV-1 or BoHV-5) was isolated, restriction enzyme analysis (REA) of the first seven of the 18 virus isolation positive samples was performed. The REA of the isolated viral DNA with the HindIII enzyme[23,24] showed the presence of BoHV-1.2 DNA in all seven samples. A BoHV-5 HindIII restriction enzyme fragment pattern was difficult to observe, however typical BoHV-5 bands (with approximate sizes of 50 and 57 kb) were present in all samples (Fig. 2). The pattern of the BoHV-1 fragments is typical for the BoHV-1.2 genotype [23,24]. The upper band (arrow) represents the typical two largest HindIII fragments (~57 and 50 kb) of BoHV-5.

4. Discussion

In this study, 100% of 76 semen samples of Brazilian cattle were positive for BoHV-5 DNA and 44.7% of these samples were positive for BoHV-1 DNA as well.
The percentages of BoHV-1 and BoHV-5 infections of the bulls of European breeds and those of Zebu breed did not differ significantly. From 18 of the 40 samples that could be tested (45%), infectious BoHV-1 and/or BoHV-5 could be isolated. Seven of these 18 viral isolates were analyzed by restriction enzyme analysis. All seven contained both BoHV-1 and BoHV-5 genomic DNA and all BoHV-1 genomes were of the BoHV-1.2 genotype.

The presence of BoHV-1 and BoHV-5 in semen samples of Brazilian cattle was demonstrated before. In that regard, BoHV-1 DNA was found in 31.7% of semen samples (n = 101) from an artificial insemination centre [25]. Bovine herpesvirus type 1 DNA (n = 5) and BoHV-5 (n = 2) were detected in semen samples of healthy bulls [26]. Bovine herpesvirus type 1.2 was also found in semen of a bull without clinical signs [27]. Likewise, BoHV-5 DNA was found in 30% (n = 20) of semen samples and in semen of a bull without clinical signs [27–29]. In addition, in Australia, BoHV-5 was detected in semen of a healthy donor bull [30].

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The percentage of 31.7% BoHV-1 positive samples found by Rocha and others [25] seemed comparable to the 44.7% found in this study. The highest percentage of BoHV-5 positive semen samples reported before was 30% [by Gomes and others, 28]. The 20 semen samples used by these authors all contained infectious BoHV-1 [25]. The observed 30% BoHV-5 DNA positive samples were thus positive for both BoHV-1 and BoHV-5. None of the earlier studies showed such a high percentage of BoHV-5 positive semen samples as found in the present study. The differences between our and earlier results may have been due to differences in sensitivity of the PCR assays used and/or due to the origins of the semen samples. It is noteworthy that DNA of both BoHV-1 and BoHV-5 were recently found by our group in ganglia of cattle from South Brazil [17]. In that study, the percentages of cattle carrying BoHV-1 DNA or BoHV-5 DNA or both, were 82.8, 93.1, and 75.9%, respectively. These numbers differed from those obtained here, but the high numbers of BoHV-5 infected animals found in both studies corroborate each other.

This study also showed that fresh as well as frozen semen samples contained infectious virus. The low percentage (45%) of semen samples from which BoHV-1 or BoHV-5 virus could be isolated, despite the fact that they were all positive by PCR, reflected the fact that PCR of BoHV-1 or BoHV-5 was generally more sensitive than virus isolation [16,18]. This is because viral DNA from non-infectious particles can also be detected by PCR [31]. Furthermore, anti-BoHV antibodies in semen may hinder the infectivity of the virus [31]. Starting ~3 wk after an artificial infection with BoHV-1, a PCR assay scored four to nine times more BoHV-1 DNA positives than BoHV-1 virus isolation [32]. In this study, however, the BoHV-1 specific nPCR failed to detect viral DNA in semen samples that were BoHV-1 virus isolation positive. Our BoHV-1 specific nPCR is thus less sensitive than virus isolation. This was probably due to the annealing temperature that was optimized to discriminate between BoHV-1 and BoHV-5 at the expense of its sensitivity. We inferred that the number of samples positive for BoHV-1 was actually higher than detected by our nPCR.

The seven virus samples that were used to perform a restriction enzyme analysis (REA) were the first 7 of the 18 virus batches obtained by virus isolation. As it happened, these seven samples were obtained from only four bulls from the same artificial insemination centre. The REA showed the presence of genomic DNA of both BoHV-1 and BoHV-5, although the amount of BoHV-5 DNA was ~20 times lower than the amount of BoHV-1 DNA. Presumably BoHV-1 grows better than BoHV-5 in the CRIB cells that were used for virus isolation. This may reflect biological differences between these viruses. The observed pattern of the BoHV-1 DNA fragments showed that all BoHV-1 genomes had the BoHV-1.2 genotype, which is normally found in South America [27].

The presence of infectious BoHV-1 and BoHV-5 virus in semen is a matter of concern. Primary BoHV-1 and BoHV-5 infections of cattle are an animal health concern, with expected economic losses. Consequently, it may be advantageous to control BoHV-1 and BoHV-5 infections and eventually eliminate these viruses from herds. A growing number of countries already started to control BoHV-1 and BoHV-5 infections [33]. In addition, more and more countries created national and international trade barriers for BoHV-1 positive products [33]. However, management of cattle in Brazil is quite different from the countries that already started BoHV-1 control programs. Most cattle in Brazil are managed extensively, roaming free over immense areas and the herds are very large, making it hard to implement a control program. The use of serologically differentiating or diva vaccines [34] would be the method of choice, but is not yet put in practice in Brazil [35]. There is a diva vaccine available against BoHV-1, but not against BoHV-5. Although one might expect that a
BoHV-1 vaccine also protects against BoHV-5, this is still a controversial issue [36–38].

However, even without a specific control program, the spread of BoHV-1 and 5 by contaminated semen can be reduced. Bovine herpesviruses latently infect their host, occasionally reactivate and intermittently emerge in the semen, often without reappearance of clinical signs [39]. To avoid spreading of BoHV-1 and BoHV-5 by artificial insemination, all semen samples, including those from apparently healthy bulls, should be tested for their presence. Due to the intermittent secretion of these viruses not all batches of semen are contaminated with infectious virus. If two straws of each batch for artificial insemination are tested negative, the risk of transmitting these viruses to inseminated cows is substantially reduced [39,40].

In conclusion, we investigated the presence of BoHV-1 and BoHV-5 in fresh and frozen semen samples from healthy Brazilian bulls using two species-specific nPCR assays and virus isolation. We found BoHV-5 DNA in all samples (N = 76) and in 44.7% of these samples BoHV-1 DNA was detected as well. Of 40 tested samples, 45% were also positive by virus isolation. These results highlighted the importance of testing semen samples, even from healthy bulls, for the presence of BoHV-1 and BoHV-5, especially in regions where these viruses are endemic.

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