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Bovine alphaherpesvirus 1 and 5 in semen from bulls presenting genital lesions under field conditions in Brazil

[*Alfaherpesvírus bovino 1 e 5 em sêmen de touros com lesões genitais, sob condições a campo no Brasil*]

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ABSTRACT

Bovine alphaherpesviruses 1 and 5 (BoHV-1/5) are main pathogens of respiratory, reproductive and neurological diseases in cattle. The aim of this study was to investigate the frequency of neutralizing antibodies against BoHV-1/5 in serum samples and to detect viral DNA in semen of bulls from beef cattle farms located in RS. A total of 372 serum and semen sample from bulls were collected in eighteen farms. Serum samples were submitted to virus neutralization (VN) assay, while semen samples were used to detect BoHV-1 and BoHV-5 DNA by PCR. VN results showed that BoHV-1/5 antibodies were detected in bulls of 66.7% (12/18) of the farms, 295 (79.5%) BoHV positive bulls, 287 for BoHV-1 and 234 for BoHV-; at 43 vaccinated bulls 72.1% (31/43) showing serology negative. BoHV-1/5 DNA was detected in the semen of three bulls; one of the them presenting BoHV-1, one out three presenting BoHV-5 and one BoHV-1/5.co-infection All BoHV DNA positive samples came from animals presenting posthitis and other genital lesions at sampling. Results showed a high seroprevalence of BoHV-1/5 antibodies in bulls as well as strong evidence that these viruses are actively circulating in the cattle farms. A remarkable finding is that in the presence of clinically evident lesions in the genital tract, both BoHV-1 and 5 may found in semen.

Keywords: BoHV-1 and 5, co-infection, DNA, semen

RESUMO

Os alfa-herpesvírus bovinos 1 e 5 (BoHV-1/5) são importantes patógenos de doença respiratória, reprodutiva e neurológica em bovinos. O objetivo deste estudo foi investigar a frequência de detecção de anticorpos neutralizantes contra BoHV-1/5 em amostra de soro e detectar DNA viral em sêmen de touros do rebanho bovino localizado nas fazendas de gado de corte do RS. Um total de 371 amostras de soro e sêmen foi coletado de touros em 18 fazendas, 325 das quais são provenientes de touros não vacinados e 43 de vacinados. Amostras de soro foram submetidas à técnica de vírus-neutralização (VN), enquanto as amostras de sêmen foram submetidas à extração de DNA e posterior PCR (polymerase chain reaction) *para detecção de BoHV-1 e 5. Os resultados da VN demostraram que anticorpos contra BoHV-1/5 foram detectados nos touros não vacinados em 66,7% (12/18) das fazendas, 295 (79,5%) touros mostraram-se positivos para BoHV, 287 para BoHV-1 e 234 para BoHV-5; e para 43 touros vacinados, observou-se que 72,1% (31/43) foram negativos na sorologia DNA de BoHV-1/5, detectado no sêmen de três touros: um deles apresentava BoHV-1, outro BoHV-5 e em um foi detectada coinfecção por BoHV-1/5. Todas as amostras positivas para o DNA viral eram provenientes de animais que apresentavam lesões de postite e outras lesões genitais. Esses resultados demonstram que há uma alta soroprevalência de BoHV-1/5 em touros, bem como uma forte evidência de que esses vírus estão circulando ativamente no rebanho bovino dessas fazendas. Um achado interessante foi a detecção de BoHV-1 e 5 em touros com lesões na região do trato genital.*

Palavras-chave: BoHV-1 e 5, coinfecção, DNA, sêmen

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INTRODUCTION

Herpesviruses are widely disseminated in the host populations and produce unapparent or mild infections, but bovine alphaherpesviruses (BoHVs) are important cattle pathogens. BoHVs are double-stranded DNA enveloped viruses, members of the *Herpesviridae* family, *Alphaherpesvirinae* subfamily, genus *Varicellovirus* (ICTV, 2017). Among the six members of this genus that infect cattle, BoHV-1 and BoHV-5 are by far the most related to economic losses in the world (Thiry *et al.*, 2006).

BoHV-1 is mainly associated to respiratory and reproductive diseases worldwide, with some differences in regional incidence and prevalence (Ackermann and Engels, 2006). Occasionally some neurological disorders have also been reported due to BoHV-1 (Roels *et al.*, 2000). Conversely, BoHV-5 was related to encephalitis since the first isolation in a neurological disease outbreak in cattle herds from Australia (Bagust & Clarck, 1972). Currently, it is considered the main causative agent of non-suppurative meningoencephalitis in young cattle in South America, Europe and Australia (Thiry *et al.*, 2006). Although these viruses have been frequently detected in clinical cases, the effective occurrence under extensive cattle farming is still unknown in several regions (Vonk Noordegraaf *et al.*, 1998).

BoHV-1 and BoHV-5 can be transmitted by direct and indirect contact through nasal, ocular and genital secretions. Respiratory and ocular secretions are considered the main transmission ways in the herds. However, sexual transmission is mainly important because these viruses also replicate in the reproductive tract of bulls and cows (Esteves *et al.*, 2003; Silva-Frade *et al.*, 2014). BoHV in bulls' semen results in extensive viral dissemination by natural mating or artificial insemination (generally occurring when one infected animal is in the acute viral replication phase or after viral recrudescence). Stress due to weaning, transport, and dietary changes are factors usually associated with the occurrence of natural outbreaks (Raaperi *et al.*, 2014).

BoHV-1 and BoHV-5 are highly similar (approximately 85% of identity in the genomes) and share some molecular and antigenic proprieties (Delhon *et al.*, 2003). This similarity represented some difficulties in the taxonomic classification, diagnostic and epidemiology from these agents undifferentiated by virological and serological tests for many years (Vogel *et al.*, 2002; Kunrath *et al.*, 2004). More recently studies demonstrated slight differences in the serological tests and these viruses could be differentiated by glycoprotein B blocking Elisa (Wellenberg *et al.*, 2001) and virus neutralization (VN) assay (Varela *et al.*, 2010).

BoHV-1 and 5 have been detected in bulls from artificial insemination (AI) centers and farms presenting reproductive failures in Brazil. A previous report demonstrated an extremely high occurrence of BoHV-1 and BoHV-5 DNA in bulls' semen from Brazil (Oliveira *et al.*, 2011). Other studies have also reported high frequencies (approximately 30%) of these viruses in semen collected from AI centers in the Minas Gerais State (Rocha *et al.*, 1998; Gomes *et al.*, 2002). However, this is not the real situation in the whole country, since both studies were performed in a limited number of farms and AI centers. The occurrence of BoHVs is still largely unknown in bulls raised in Brazilian commercial herds. The aim of this study was to investigate the occurrence of BoHV-1 and BoHV-5 antibodies and DNA in the serum and semen of bulls in southern Brazil.

MATERIAL AND METHODS

Eighteen farms from the Midwest, Southeast and Southwest regions of Rio Grande do Sul (RS), the southernmost State in Brazil, were included in the present study (Figure 1). The general data related to the sanitary, nutritional and reproductive management of each farm were recovered before performing the clinical evaluation and sampling of each animal. All animals were in the pre-coverage period and did not undergo stress management during the fifteen days prior to the exams. In most establishments, the animals were under field conditions, raised extensively, after the post-breeding period, and they were placed in ryegrass pasture three months before the reproductive period. All properties administered mineral salt and some of them provided balanced foods as a supplement. The study was performed between April and November of 2011.

A total of 325 non vaccinated bulls and 43 vaccinated bulls were selected in the eighteen farms. The following parameters were evaluated in each animal: breed, age, vaccination status against BoHV and bovine viral diarrhea virus (BVDV), period of coverage, body condition and clinical signs of cardiorespiratory alterations. An individual clinical evaluation was also performed, with emphasis on the reproductive characteristics. The clinical exam included prostate palpation and analysis of the seminal vesicles, testicles and penis. Clinical inspection for herpesvirus compatible lesions in bull's genital was performed by direct visual and tactile examination in all reproductive organs. Serum and semen samples were also collected of each animal and stored at -20C until laboratorial analysis

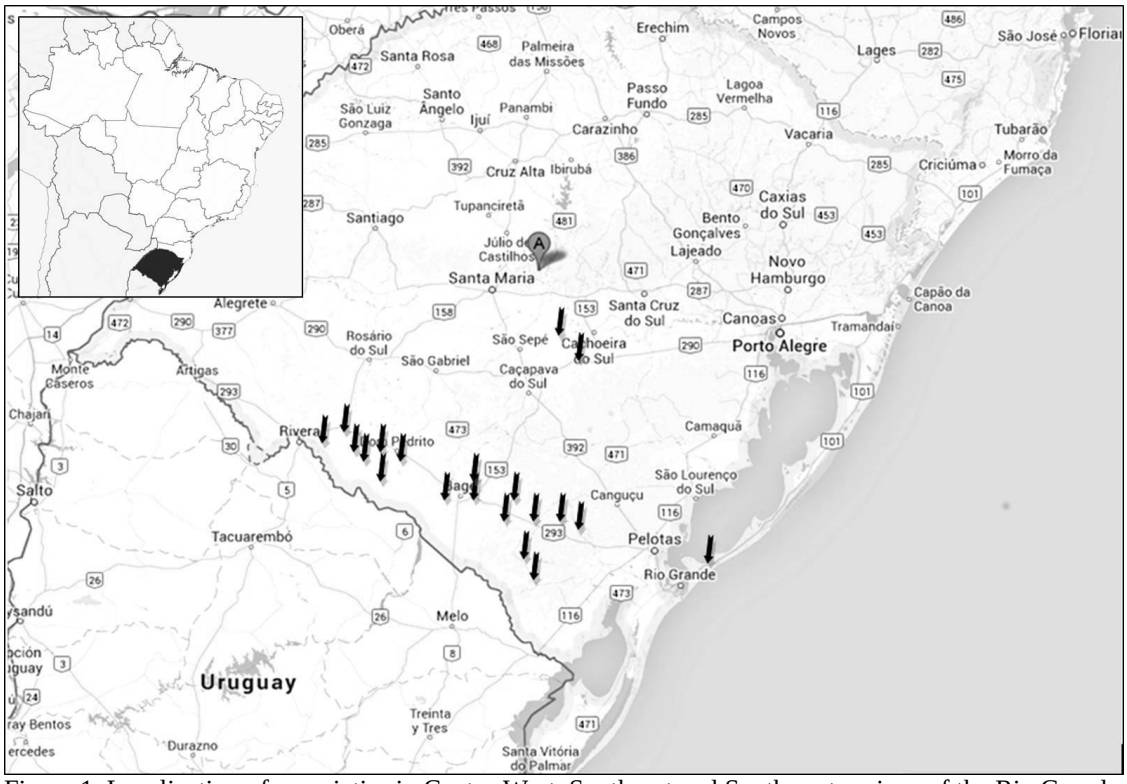

Figure 1. Localization of proprieties in Center-West, Southeast and Southwest regions of the Rio Grande do Sul State.

CRIB cells were used throughout for virus multiplication and quantitation (Flores & Donis, 1995). Cells were routinely maintained in Eagle's minimal essential medium (MEM) containing penicillin (1.6mg/kg), streptomycin (0.4mg/kg) and 10% fetal calf serum (Gibco, BRL). The BoHV-1 EVI 123/98 strain was isolated from a case of rhinotracheitis and the BoHV-5 (EVI 88/95) strain was isolated from a case of encephalitis. Both viruses were Brazilian isolates were previously submitted to molecular and antigenic characterization (D'Arce *et al.*, 2002; Traesel *et al.*, 2014).

The serum samples were tested against $100TCID_{50}$/mL separately to both viruses. The sera were initially inactivated at 56°C for 30min before use. A serial dilution was performed, starting at 1:2 to 1:256 in 96 well microplates, all samples analyzed in duplicates. Serum and virus mixture were incubated at 37ºC for 8h (Guy and Potgieter, 1985) and then a suspension of CRIB cells was added to each well. The plates were incubated at 37ºC and 5% CO_2 for five days. Neutralization titers were calculated as the reciprocal of the highest serum dilution able to avoid cytopathic effect.

DNA was extracted by a silica-based technique (Boom *et al.*, 1990) using Newgene reagents Prep and PreAmp (Simbios Biotecnologia, Cachoeirinha, RS, Brazil). After DNA samples

were submitted to polymerase chain reaction (PCR) to detect genes encoding for glycoprotein C [gC] (Campos *et al.,* 2009) and viral DNA polymerase [DNA pol] (Diallo *et al.,* 2011). Sequences of the primers are described in Table 1.

Amplification of gC was performed in a 25µl PCR reaction with 1mM MgCl (Invitrogen) 0.3µM of each primer (IDT), 10% dimethylsulfoxide (DMSO; Acros Organics), 1U Taq DNA polymerase (Invitrogen), 10% of PCR buffer (Invitrogen) and 0.06mM deoxynucleoside triphosphates (ABgene). Reactions were performed in the Veritti thermocycler under the following cycle conditions: 5min at 94°C; followed by 35 cycles of 1min at 94°C, 1min at 62°C, 1min at 72°C; followed by 5min at 72°C.

Amplification of DNA pol was also performed in a 25µl PCR reaction with 1mM MgCl (Invitrogen), 1µM of each primer, 1.5U Taq DNA polymerase (Invitrogen), 10% of PCR buffer (Invitrogen) and 0.06mM deoxynucleotide triphosphates (ABgene) per reaction. Reactions were also performed in the Veritti Thermocycler under the following cycle conditions: 2min at 95°C; followed by 40 cycles of 20 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C.

Table 1. Sequences and reference of primers used at analyzed of bovine herpevirus 1 (BoHV-1) and 5 (BoHV-5)

Primers	Sequence	Gene	Reference
BoHV-1 *forward*	5'-CTAACATGGAGCGCCGCTT-3'	DNA pol*	Diallo *et al.*, 2011
BoHV-1 *reverse*	5'-GGTACAACATCGTCAACTTC-3'	DNA pol	Diallo *et al.*, 2011
BoHV-5 *forward*	5'-GGTACTTCTTCTTGGTGATG-3'	DNA pol	Diallo *et al*., 2011
BoHV-5 *reverse*	5'-TCGGTCTTCGTCAAGTTC-3'	DNA pol	Diallo *et al.*, 2011
BoHV-1/5 *forward*	5´-CGGCCACGACGCTGACGA-3´	gC**	Campos *et al.*, 2009
BoHV-1/5 *reverse*	5´-CGCCGCCGAGTACTACCC-3´	gC	Campos *et al.*, 2009
BoHV-1 *forward*	5'- CTAACATGGAGCGCCGCTT-3'	gC	Campos *et al.*, 2009
BoHV-1 *reverse*	5'- CGGGGCGATGCCGTC-3'	gC	Campos *et al.*, 2009
BoHV-5 *forward*	5'-GTGGAGCGCCGCTTCGC-3'	gC	Campos *et al.*, 2009
BoHV-5 *reverse*	5'- TATCGCGGAGAGCAGGCG-3'	gC	Campos *et al*., 2009

Legend: *DNA polymerase; ** glycoprotein C.

RESULTS

The clinical exam demonstrated the occurrence of local lesions (caused by trauma) and cutaneous/mucosal changes in the organs of the reproductive system. The main clinical alterations were: lesions (n= 56), asymmetries in the size (n= 29), dermatitis (n= 7), scars (n= 3) and eschar (n= 1) in the in scrotum; tail fibrosis (n= 4), head fibrosis (n= 2) and hyperplasia (n= 4) in the epididymides; hyperemia (n= 22), myiasis (n= 3) and other minor alterations (such as ulcerations, stenoses, papilloma, adhesions, hematoma and edema) in the prepuce; petechiae (n= 45), hyperemia (n= 39) and other abnormalities (hematomas, lesions, ulcers, hair ring, persistent frenulum, varicosities, glans changes, hypospadias, balanoposthitis, corkscrew, papillomas and scars) in the penis. These clinical findings were not criteria for the discard of animals, except for congenital pathologies observed in four bulls (persistent frenulum, hypospadia and penis in corkscrews) and dental wear in other four bulls.

Neutralization antibodies against BoHV-1, BoHV-5 or both were detected in 295 out of 325 (90.8%) of the not vaccinated bulls examined in this survey. BoHV-1 alone specific antibodies were detected in 61 out 295 (20.7%) of the bulls that tested positively, whereas BoHV-5 specific antibodies were present in 8 out of 295 (2.7%). Cross-neutralizing antibodies were detected in 226 out of 295 (76.6%) of the serum samples. Antibodies against BoHV-1 were detected in major number of times, 287f of 295 (97.3%) of sample tested however BoHV-5 in 234 out of 295 (79.3%) of serum samples. Considering of the neutralizing antibodies titers, > 1:256 was the more often detected against BoHV-1, totalizing 84 out of 295 (28.5%), and for BoHV-5 lower levels of antibodies were present was 1:32, in 75 of 295 (25.4%). Thirty-one out of forty-three vaccinated bulls (72.1%) showing negative serology at one or both viruses; three out of 43

(7%) bulls were positive at both, and 7 out of 43 (16.3%) were positive at BoHV-1 and 2 out of 43 (4.6%) at BoHV-5 [data not showed].

From 372 semen samples, in one bull BoHV-1 (#363) was detected once, another sample (#323) showed BoHV-5 DNA and a third sample was positive for both BoHV-1 and 5 (#355). Bulls #323 and #355 were from the same propriety, while bull #363 was from another one. These three bulls positive at BoHV-1/5 DNA came from not vaccinated bulls.

Balanoposthitis was observed in bulls from which these semen samples came. Regarding sample #355, the bull showed posthitis, hyperemia and polyps in the penis; #363 was collected from animals showing posthitis and petechias in the penis. Both animals showed one ovoid nodular mass on the penis; posthitis with polyps in the glans and penis were observed in the donor of sample #323.

DISCUSSION

Herpesviruses are important pathogens in cattle herds around the world. In Brazil the prevalence seems to be high according previous studies (Campos *et al.*, 2009; Oliveira *et al.*, 2011). The present study describes the high frequency of antibodies in field herds as well as the presence of BoHV-1 and 5 DNA in semen of bulls in farms with and without clinical or/and historic of reproductive disorders.

BoHV-1 and BoHV-5 are genetically and antigenically closely related and difficult to be distinguished by routine diagnostic tests. However, it was possible to detect different neutralizing titers of anti-BoHV-1 and anti-BoHV-5 as well as the DNA of both viruses in the present study. Further, BoHV-1 PCR detection in semen showed the occurrence of two positive samples, precisely in one of the farms with higher BoHV-1 antibody titers. This farm has a history of nutritional and health management deficiencies, possibly favoring BoHV-1 shedding in semen of the bulls.

In the evaluation of the neutralizing antibodies titers in the DNA BoHV positive animals, bull #363 (positive for BoHV-1 DNA) had neutralization antibodies of 1:32 for both viruses, bull #323 (positive for BoHV-5 DNA) also presented an antibody titer of 1:32 for this specific BoHV and VN negative against BoHV-1 virus, and bull #355 (positive for both BoHV1 and 5 DNAs), had neutralization antibodies 1:64 at BoHV-1 and 1:32 at BoHV-5. These results suggested that bovine could be infected at BoHV-1 even already infected by BoHV-5, and otherwise is true to, once on time that neutralizing antibodies not protected one against the other. An interesting finding was that 31 of the 43 bulls vaccinated did not present neutralizing antibodies in the VN, demonstrating once again that the vaccine failures occur frequently as already described (Anziliero *et al.*, 2015.)

The presence of BoHV-5 DNA in semen has been reported in other studies. In Iran, DNA was extracted from bull semen samples, and BoHV-5 and BoHV-1 were amplified by PCR assay; showing a high prevalence of BoHV-5 (73.2%) and BoHV-1 (25.89%). Only 18.7% were positive for both viruses in these bull semen samples (Sharifzadeh *et al.*, 2015). In a Brazilian study, a semen sample from an apparently healthy bull, which was identified as BoHV-1 positive during virus surveillance at an artificially inseminated (AI) center in 1996, was re-classified as BoHV-5 after new analyses (Esteves *et al.*, 2003). In Australia, the first evidence of natural transmission of BoHV-5 through infected semen was described in 2009 (Kirkland *et al.*, 2009). The virus was further isolated from a cryopreserved semen from a healthy bull (Diallo *et al.*, 2010). In 2012, Rodríguez *et al.* (2012) observed BoHV-5 in one of several extended semen samples from a healthy donor bull by PCR assay. These studies indicate that animals without evidence of clinical disease and appear to be healthy can be a potential source of venereal virus transmission. Oliveira *et al.* (2011) in Brazil found a high prevalence of virus in semen samples, and in that study, BoHV-5 DNA was isolated in 100% semen samples using multiplex PCR. The study by Oliveira *et al.* (2011) and other studies demonstrate the importance of using molecular biology techniques such as PCR methods for the detection of BoHV-5 in semen samples.

The sampling was performed during pre-mating season them not have been submitted the stress included at sexual activity and without have occurred opportunity of major viral spread in

herd. Date from some years ago showed that only 6% of female bovine are AI in Brazil, then approximately 90% of calves are born from natural mating (Barbosa *et al.*, 2005).

Considering these findings an 70 million dams in Brazil are serviced for approximately 2 million each year bulls, it may be advisable that together with serological monitoring of the herds andrological examination should be performed.

CONCLUSION

The present study showed a low frequency of BoHV-1 and BoHV-5 mainly of compared with others reports. These differences may have been from difference of sensibility at PCR and/or due origins of semen samples and/or period of viral reactivation. Beside these, we can assert that the lesions in penis and prepuce may be important indicatives of bulls infected with BoHV-1 and/or BoHV-5 and probability of them spread virus and infect other susceptible bovines.

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