Acta Veterinaria (Beograd), Vol. 58, No. 2-3, 267-273, 2008.

DOI: 10.2298/AVB0803267N UDK 619:616.988:636.2

PRESENCE OF BOVINE HERPESVIRUS TYPE 4 (BHV-4) INFECTION IN BULLS FOR ARTIFICIAL INSEMINATION IN SERBIA

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(Received 5. September 2007)

Bovine herpesvirus type 4 (BHV-4) is a worldwide distributed gammaherpesvirus, with different antigenic and biologic properties from all the other bovine herpesviruses. In vivo distribution of BHV-4 was examined by testing various organs of experimentally infected calves. However, limited data regarding excretion and transmission of BHV-4 from naturally infected cattle are available. In this report, we describe the detection of BHV-4 in the semen of bulls used for artificial insemination. Blood and semen of bulls from two major centers for artificial insemination (A.I. center) were examined in this study. In order to detect the presence of BHV-4 infection in the studied herds, 35 serum samples from Center I and 15 from Center II were examined using indirect ELISA for detection of specific anti-BHV4 IgGl immunoglobulins. Serological examination showed that 18 out of 50 serum samples were positive for BHV-4 antibodies. Despite the fact that BHV-4 was not isolated from semen samples the, presence of BHV-4 was detected in one sample using the nested PCR. This fact can be used to presuppose that artificial insemination is a potential route for BHV-4 transmission.

Key words: BHV-4, PCR, semen, virus isolation

INTRODUCTION

Bovine herpesvirus type 4 (BHV-4) is a worldwide distributed gammaherpesvirus, with different antigenic and biologic properties from all the other bovine herpesviruses. Characteristics of the virus, especially the genomic structure, classify this virus in the same group as Epstein-Barr virus and the Herpesvirus Saimiri.

Like with other herpesviruses, animals infected with BHV-4 develop a latent infection, which can be reactivated by dexamethasone treatment, or induced by different stress factors. Target cells for BHV-4 survival in the infected organism are essentially blood mononuclear cells (Lopez *et al., 1996;* Osorio *et al., 1985*), but it seems that other cells could also be included in the mechanism of virus persistence (Asano *et al.,* 2003).

BHV-4 has been isolated from several groups of clinical entities, but experimental infection of susceptible animals with field isolates seldom elicited the typical clinical signs. Nevertheless, BHV4 has also been isolated from apparently healthy cattle (Belak and Palfi, 1974). In Belgian herds, most BHV-4 isolations were from uterine or peritoneal exudates of cows with postpartal clinical problems *(*Wellemans *et al.,* 1984). Two years later, Wellemans *et al. (1986)* produced similar clinical symptoms by experimental intravenous inoculation of field strains in pregnant and non pregnant cows. Still, BHV4 has been isolated from cases of bovine abortions at different stages of pregnancy *(*Crandell *et al.,* 1976; Reed *et al.,* 1979).

The study by Drolet *et al.* (1986) is a good example of the diversity of infection caused by BHV-4. In this 28-month study, 33% of viral isolates were associated with abortion, 25% with pneumonia, 17% with diarrhoea and 25% with other symptoms. Although 3 isolates were from animals yielding no other pathogens, 75% of the isolates were from animals with concurrent bacterial, fungal, or other viral infections. Those authors speculated that BHV-4 might be somewhat similar to that of BHV-1 in cattle. A BHV-4 strain isolated in Belgium from a case of oedematous orchitis and azoospermia *(*Thiry *et al.,* 1981) produced inconsistent lesions following intratesticular inoculation in bulls. Infiltration of interstitial tissue by mononuclear cells was more frequently observed in the epididymis than in the testicle. Strains of "non-syncytia forming" herpesviruses have also been isolated from semen *(*Loretu *et al.,* 1974).

In vivo distribution of BHV-4 was examined by testing nasal and conjunctival exudates, peripheral blood leukocytes, uterine exudates from cows with postpartal metritis, various organs of experimentally infected calves and clinical samples *(*Egyed *et al.,* 1996*;* Osorio *et al.,* 1985). However, little information about excretion and transmission of BHV-4 from naturally infected cattle has been published. In this report, we describe the detection of BHV-4 in the semen of bulls for artificial insemination and hence presume that semen may be one of the potential BHV-4 transmission routes.

MATERIAL AND METHODS

Samples

Blood and semen of bulls from two major centers for artificial insemination (A.I. center) near Belgrade were examined in this study.

Blood samples were taken aseptically from v.jugularis and kept at room temperature for 2 hours. Serums were collected and transferred into 1.5 mL sterile tubes. All the blood samples were negative for Bovine Herpesvirus type 1 (BHV-1) Blue Tongue virus (BTV), Bovine Viral Diarhoea Virus (BVDV), Brucelosis, Leptospirosis and Listeriosis antibodies.

Semen samples from 50 bulls from two centers were collected in March 2007 and kept at -70C until further examination. All the semen samples were taken as raw semen and before inoculation on MDBK cells, semen samples were diluted 1:50 and 1:100.

All the semen samples from these centers were PCR tested for the presence of BHV-1, BVDV and BTV. All samples tested negative.

Subsequent samplings of semen from BHV-4 positive bull were performed in April, May and June of the same year.

Serological test

In order to detect the presence of BHV-4 infection in examined herds, 35 and 15 serum samples from Center I and II (respectively) were examined for the presence of specific anti-BHV4 IgGl immunoglobulins (Bio-X, Belgium). The cut-off value of optical density, was calculated by the difference in optical density between antigen coated and control wells from a positive control supplied by the manufacturer.

Cell culture

Monolayers of Madine Darby Bovine Kidney (MDBK) cells were used for the isolation of the virus. Cell line was grown in Eagle's minimum essential medium (EMEM, Sigma-Aldrich, USA) supplemented with 10% foetal bovine serum (FBS, Gibco, Scotland), 100 I.U/mL penicillin and 100 μg/mL streptomycin. The cells were grown at 37^{o}C (5% CO_2) on cell culture (24-wells) plate, with 1,5 mL of cell culture media in each well.

Virus isolation

Raw semen samples were previously diluted 1:50 and 1:100 in Eagle's minimum essential medium (EMEM, Sigma-Aldrich, USA), in order to avoid negative effects of sperm to cells.

Diluted semen samples were inoculated by 0.3 mL in each well and incubated 1h at 37 ^{o}C. After incubation, samples were discarded and cells were washed once with MEM and incubated further in fresh medium at 37^{o}C (5% CO_2) for 7 days. During this incubation period, cell cultures were examined daily to detect the appearance of cytopathic effects (CPE). In the absence of CPE, the samples were given a further two passages. BHV-4 field isolate strain was used as a positive control, while non-inoculated MDBK cell culture served as a negative control.

Extraction of viral DNA

DNA extractions were performed using a Qiagen DNA mini kit, using *Blood and body fluid spin protocol*. Purified DNA at the end of this process was free of proteins, nucleases and other contaminants or inhibitors. Starting with 200 μL of undiluted semen and adding proteinase K and AL buffer reagents, the extraction spin protocol was performed as suggested by the manufacturer. Finally, we were supplied with 4-15 μg total DNA in 200 μL volume.

BHV-4 nested PCR

For the detection of BHV-4 a nested PCR assay was used. Following oligonucleotides were selected as primers *(Fabian and Egyed,* 2004):

The outer primers flanked a 737-bp fragment (forward 5'-GACTATGAGGAATGGCACAAG -3'; reverse 5'- TACTCGTAGGCTGGGTCTGG -3'). The inner primers amplified a 271-bp long product (forward 5'-GGTTGGAAGTGAGCGTATGAT-3'; reverse 5'-GTAGGCGGGGTCTGGAAT-3'). The PCR amplifications were carried out in 50 μL reaction mixtures containing 25 μL of Qiagen master mix solution (HotStarTaq Master Mix Kit, Qiagen), 15 pmol of each primer, 5μl of Cresol Red and 5 μg of sample. The amplification was carried out in an Eppendorf, Mastercycler. The amplification included denaturation at 94^{o}C for 45 s, annealing at 63^{o}C for 45 s, and synthesis at 72^{o}C for 1.5 min. The first round of PCR consisted of 25 cycles, while to increase yield and sensitivity we applied 30 cycles in the second round. The first step in both rounds was initial denaturation and polymerase activation at 94^{o}C for 15 minutes. From the first PCR, 1 μL of the reaction mixture was transported into the second reaction. Due to the smaller size of the products, the synthesis period was shortened to 1 min in the second assay. The tubes were kept at 72^{o}C for 10 min after the last cycle to complete the extension, than the mixtures were cooled to 4^{o}C. DNA extracted from purified BHV-4 added to negative semen served as positive controls, while distilled water was applied as the negative control in each PCR assay. The PCR products were examined by electrophoresis in 1% agarose gels using 1 × Tris–borate–EDTA as running buffer at 60V for 33 minutes. The ethidium bromide-stained bands were visualized with UV light. The molecular sizes of fragments were compared with those of a 100 bp ladder (FastRuler DNA ladder, Fermentas).

RESULTS

Among 35 serum samples, taken from Center I, in 6 samples were detected BHV-4 antibodies, and 12 out of 15 samples from Center II were positive for BHV-4 antibodies. In total, serological examination revealed 18 BHV-4 antibody positive serum samples.

Results of ELISA testing of blood samples showed 18 of 50 samples to be BHV-4 positive (Table 1).

Table 1. ELISA testing for BHV-4 antibody presence

	Total Number of blood samples	BHV-4 positive blood samples	BHV-4 negative blood samples
A.I. Center I	35	6	29
A.I. Center II	15	12	3

During a twenty-one days incubation period, CPE was not observed on MDBK cells and all samples were declared negative by virus isolation. However, using a nested PCR we detected that one semen sample was positive for BHV-4 (Figure 1). But, results of PCR assay of semen samples taken from the same animal 30, 60 and 90 days after the first (initial) sampling were negative.

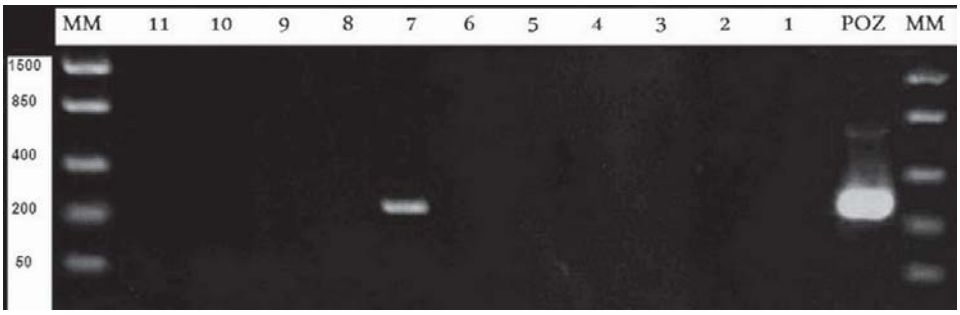

Figure 1. Detection of BHV-4 in semen of bulls by nested PCR. First round of PCR did not show any specific amplification. MM-Molecular size marker, POZ-positive control, 1-11 examined samples. Specific amplification observed on sample No.7 with size of 271bp, the same as the positive control. Numbers on the left side represent positions of fragment sizes of 1500, 850, 400, 200 and 50 base pairs

DISCUSSION

Considering the lack of data showing the presence of BHV-4 in bovine semen and the potential role of BHV-4 spreading by artificial insemination, we examined the seroprevalence and possible presence of BHV-4 in bovine semen. Truman *et al*. (1986) detected the specific BHV-4 antibodies in 38% of bulls for artificial insemination in Germany. Considering the nature of herpesvirus infections and latent infection which BHV-4 establishes in infected organism, reactivation and excretion can occur after immunosupresive treatment or stress factors. These data and lack of literature data regarding the presence of BHV-4 in semen encouraged us to start examination of BHV-4 infection in bulls for artificial insemination in Serbia.

In addition, previous reports showed that the majority of seroconversions happened after first calving (Van Opdenbosch *et al.,* 1988). In that report was shown that 38% of young calves under 3 months of age, reacted positive for BHV-4 antibodies, while calves older than 3 months showed seroprevalence under 3%, which suggested transfer of maternal antibodies against BHV-4. Examination of different age categories, showed rapid increase of number of seropositive animals older than 2 years, which corresponds with time of first pregnancy and calving.

Our results showed that 18 of 50 bulls were infected with BHV-4. That means that these previously infected bulls have the potential to shed the virus, so they are candidates for examination of semen by virus isolation and PCR. Negative results of virus isolation for the semen which was positive by PCR can be explained by reduction of sensitivity of virus isolation caused by usage of diluted semen.

Semen samples taken from bulls whose semen was positive for the presence of BHV-4 in March were taken three more times, in 30 days intervals and

tested by PCR. All the semen samples taken from that bull later were negative for the presence of BHV-4. That means that semen of infected bulls can be aviremic for longer periods of time, but in the reactivation phase BHV-4 could be present in the semen.

Considering the fact that this is a first report of BHV-4 presence in semen, further investigations should be done in terms of experimental reactivation of BHV-4 in latently infected (seropositive) bulls, in order to understand the incidence of BHV-4 excretion via semen during this period.

Thus, it can be supposed that AI can be one of the possible routes for BHV-4 transmission. Despite that it is important to note that other infection routes, such as the postpartal period in the maternity barn (Nikolin *et al*., 2007), or milking (Donofrio *et al*., 2000), are also possible transmission routes of BHV-4. Finally, our results suggest that the presence of BHV-4 in semen should be considered as a possible threat for BHV-4 infection for inseminated cows and heifers.

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REFERENCES

1. *Asano A, Inoshima Y, Murakami K, Iketani Y, Yamamoto Y, Sentsui H*, 2003, Latency and Persistence of Bovine Herpesvirus Type 4, Strain B11-41, in Bovine Nervous Tissues, *J Vet Med Sci*, 65, 1, 87-93.
2. *Belak S, Palfi V*, 1974, Characterization of a herpesvirus isolated from spontaneously degenerated bovine kidney cell culture, *Acta Vet Acad Sci Hung,* 24, 249-53.
3. *Crandell RA, Sells A DM, Gallina AM*, 1976, The isolation and characterization of a new bovine herpesvirus associated with abortion, *Theriogenology*, 6, 1-19.
4. *Donofrio G, Flammini CF, Scatozza F, Cavirani S*, 2000, Detection of bovine herpesvirus 4 (BoHV-4) DNA in the cell fraction of milk of dairy cattle with history of BoHV-4 infection, *J Clin Microbiol,* 38, 4668-71.
5. *Drolet R, Werdin R, Goyal S*, 1986, The role of bovine herpesvirus type-4(DN 599) infection in Minnesota cattle, Proceeding of the annual meeting of American Association of veterinary laboratory diagnosticians, 29, 335-46.
6. *Egyed, J, Ballagi-Pordany A, Bartha A, Belak S*, 1996, Studies of *in vivo* distribution of bovine herpesvirus type 4 in the natural host, *J Clin Microbiol*, 34, 1091-5.
7. *Fábián K, Egyed L,* 2004, Detection of bovine gammaherpesviruses by a nested duplex PCR, *J Virol Meth*, 115, 93-8.
8. *Lopez OJ, Galeota JA, Osorio FA*, 1996, Bovine herpesvirus 4 (BHV4) persistently infects cells on the marginal zone of spleen in cattle, *Microb Pathogen*, 21, 47-58.
9. *Loretu K, Marinov P, Genov I, Bohnel H*, 1974, Virus isolations from cases of infectious bovine pustule-vulvoginitis and posthitis (IBR-IPV) in cattle in Tanzania, *Bull Epizoot Dis Afr*, Dec, 22, 4, 303-10.
10. *Nikolin V, Donofrio G, Milošević B, Taddei S, Radosavljević V, Milićević V*, 2007, First Serbian Isolates of Bovine Herpesvirus 4 (BoHV-4) from a Herd with a History of Postpartum Metritis, *New Microbiologica,* Jan, 30, 1, 53-7.

11. *Osorio, FA, Rock DL, Reed DE*, 1985, Studies on the pathogenesis of a bovine cytomegalo-like virus in an experimental host, *J Gen Virol*, 66, 1941-51.
12. *Reed DE, Langpap TJ, Bergeland ME*, 1979, Bovine abortion associated with mixed Movar 33/63 type herpesvirus and bovine viral diarrhea virus infection, *Cornell Vet*, 69, 54-66.
13. *Thiry, E, Pastoret PP, Dessy-Doise C, Hanzen C, Calberg-Bacq CM*, 1981, Isolement d'un herpesvirus chez un taureau infertile, *Ann Med Vet*, 125, 143.
14. *Truman E, Ludwig H, Storz J*, 1986, Bovine herpesvirus type 4: studies on the biology and spread in cattle herds and in insemination bulls, *J Vet Med,* B 33, 485-501.
15. *Van Opdenbosh E, Wellemans G, Ooms LAA, Degryse ADAY*, 1988, BHV-4 (bovine herpesvirus 4) related disorders in Belgian cattle: a study of two problem herds, *Vet Res Commun,* 12, 347–53.
16. *Wellemans G, Antoine H, Broes A, Chalier G, Vanopdenbosch E*, 1984, Varied symptoms of cases of chronic metritis associated with a herpesvirus in cattle, *Ann Med Vet,* 128, 65-74.
17. *Wellemans G, Vanopdenbosch E, Mammerickx M*, 1986, Inoculation experimentale du virus LVR 140 (herpes bovin IV) a des vaches gestantes et nongestantes, *Ann Rech Vet,* 17, 89–94.

ZASTUPLJENOST INFEKCIJA BOVINIM HERPESVIRUSOM TIPA 4 (BHV-4) KOD BIKOVA U CENTRIMA ZA VEŠTAČKO OSEMENJAVANJE U SRBIJI

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SADRŽAJ

Bovini Herpesvirus tip 4 (BHV-4) je široko rasprostranjen gamaherpesvirus u populaciji goveda, sa potpuno različitim antigenim i biološkim karakteristikama od ostalih goveđih herpesvirusa. *In vivo* distribucija BHV-4 je ispitivana testiranjem različitih organa eksperimentalno inficirane teladi, kao i kliničkih uzoraka. Za sada ima malo informacija o karakteristikama BHV-4 i putevima infekcije. U ovim ispitivanjima su uzorci krvi i semena uzorkovani od bikova iz dva centra za veštačko osemenjavanje. Sa ciljem da se utvrdi prisustvo BHV-4 infekcije, 35 krvnih seruma iz Centra I i 15 iz Centra II ispitivano je na prisustvo specifičnih anti BHV-4 IgG_1 klase, pomoću iELISA testa. Serološkim ispitivanjem je u 18 od ukupno 50 krvnih seruma utvrđeno prisustvo specifičnih antitela na BHV-4. Virusnom izolacijom nije utvrđeno prisustvo BHV-4, ali je pomoću nested PCR tehnike detektovano prisustvo virusa u jednom ispitivanom uzorku. Imajući u vidu ove rezultate, može se pretpostaviti da veštačko osemenjavanje ima ulogu u prenošenju ovog virusa.