

# First Serbian isolates of bovine herpesvirus 4 (BoHV-4) from a herd with a history of postpartum metritis

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## SUMMARY

Two farms in the Belgrade area have experienced serious problems with postpartal metritis. Serological examination of BoHV-4 infection was done using ELISA test and vaginal swabs were used for virus isolation. Average seroprevalence of BoHV-4 in these farms was 84.37%. BoHV-4 isolation was successful from three vaginal swabs on the MDBK cell line. Rising values of BoHV-4 antibodies were recorded in nine of ten cows with clinical signs of postpartal metritis. PCR and restriction analysis were used for better characterisation and isolate classification. Two isolates showed similarity with MOVAR 33/63 virus type, but one differed in polyrepetitive and other parts of DNA. This was the first isolation and characterisation of BoHV4 from Serbian herds.

**KEY WORDS:** BoHV-4, Postpartum metritis, Seroprevalence, Virus isolation, PCR, Restriction analysis

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Bovine herpesvirus 4 (BoHV-4), a member of the *Gammaherpesvirinae* subfamily, was isolated for the first time in Europe from cattle with respiratory and ocular diseases (Bartha *et al.*, 1966) and later in the United States (Mohanty *et al.*, 1971). BoHV-4 has been isolated from a variety of samples and cells from healthy cattle and from cattle with abortion, metritis, pneumonia, diarrhoea or respiratory infection (Parks *et al.*, 1973; Osorio *et al.*, 1983; VanOpdenbosch *et al.*, 1984; Castrucci *et al.*, 1987; Wellenberg *et al.*, 2000; Frazier *et al.*, 2001). However, only a few researchers have successfully produced experimental disease, and the pathogenic role of BoHV-4 remains unclear (Thiry *et al.*, 1989).

Notwithstanding the unclear BoHV-4 pathogenicity, several researchers suggested BoHV-4 as a major determinant on bovine postpartum metritis (Frazier *et al.*, 2001, 2002), whilst others consider it has a secondary role (Donofrio *et al.*, 2005).

The immune response to BoHV-4 infection is characterized by low level of neutralising antibodies (Thiry *et al.* 1990), so IFAT and ELISA tests are often used for detection of BoHV-4 infection (Edwards & Newman, 1985).

Considering the lack of data regarding the presence of BoHV-4 in Serbia, a serological examination, virus isolation and genotyping of isolates were done.

In a two dairy farms (I-350 and II-2000 milking cows) located in the Belgrade area dairy cattle experienced an increased number of cases of severe postpartum metritis. One hundred and sixty samples from two farms were examined by iELISA (Bio-X Diagnostics, Belgium) for BoHV-

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4 antibodies. In order to complete the epizootical view, serological examination of 80 cows was also done in individual sectors (3-5 cows in stall). Blood samples from ten cows with clinical signs of postpartum metritis were double sampled on the 3<sup>rd</sup> and the 25<sup>th</sup> days postpartum for BHV-4 serological examination by ELISA test.

Ten cows in Farm I between the 3<sup>rd</sup> and the 25<sup>th</sup> days postpartum with metritis and also six without clinical signs of postpartum metritis were intravaginally swabbed and BoHV-4 isolation was attempted. Swabs were 0.22 µm filtered, inoculated on MDBK (bovine kidney cells, ATCC, CCL-22) and cells were observed daily for evidence of cytopathic effect (CPE). Viral DNA was prepared using supernatant from the BoHV-4 infected cell line MDBK as previously described (Donofrio *et al.*, 2000) and 3 different parts (Major DNA Binding Protein, MDBP; Immediate Early 2, IE2; Thymidine Kinase locus, TK) of the BoHV-4 genome based on the published sequence (GeneBank accession number, NC002665) (Zimmerman *et al.*, 2001) were amplified with three pairs of primers: MDBP, sense, 5'-gtggcagataactgtatt-3', antisense, 5'-gactgtttcaggtccaggc-3' and leading to an amplicon of 519 bp; IE2, sense, 5'-acaaacacacagaccagtca-3', antisense 5'-gtttcacaacagattgagca-3' and leading to an amplicon of 1400 bp; TK, sense, 5'-cgaattatagtctaaagt-

catcctc-3', antisense, 5'-gtaaggaccttcacactcttaagc-3' and leading to an amplicon of 2538 bp. In the first cycle, the samples were denatured at 94 °C for 5 min. One µl sample of DNA was amplified during 30 cycles, with each cycle consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and chain elongation with 1 U of *Taq* polymerase (Roche) at 72 °C for 2 min. PCR amplification was performed in a final volume of 50 µl of 10 mM Tris-hydrochloride, pH 8.3, containing 0.2 mM deoxynucleoside triphosphate, 3 mM MgCl<sub>2</sub>, 50 mM KCl, and 0.25 µM concentration of each primer.

Next, to partially genotype these isolates, a restriction fragment length polymorphism method (RFLP) was applied starting from purified viral DNA. Viral DNA was digested with two different restriction enzymes (*EcoRI* and *BamHI*) and run overnight through a 1% agarose gel.

Average seroprevalence of BoHV-4 in two examined farms was 84.37%. Seroprevalence in 51 examined stalls in individual sectors was 3.75%. In nine cows, a significant increase in BoHV-4 specific antibodies was detected on the 25<sup>th</sup> day postpartum (Table 1). Samples 6403, 5650 and 5597 belong to cows from which the BoHV-4 was isolated from vaginal swabs (Table 1).

Four vaginal swab inoculates developed CPE (3-

TABLE 1 - Results of ELISA test for serological examination of paired sera taken 3 weeks apart from cows with clinical signs of postpartum metritis. Sera's degree of positivity are shown in percentages.

Sample ID	Sampling 3 <sup>rd</sup> day postpartum	Sampling 25 <sup>th</sup> day postpartum
6403	0%	76%
5650	0%	102%
6659	37%	125%
6985	44%	138%
5601	34%	26%
5567	56%	89%
6426	96%	169%
5550	98%	161%
5597	21%	135%
5555	4%	97%

4 days p.i.), therefore, they were further analysed for BoHV-4 presence by direct immunofluorescence (Bio-X Diagnostics, Belgium) and three of them were BoHV-4 from the antigenic point of view.

Because cross reactions between BoHV-4 and other bovine herpesviruses were observed (Ludwig *et al.*, 1983; Osorio *et al.*, 1985; Guo *et al.*, 1988), a PCR and restriction analysis were applied for a better characterization.

All three isolates were successfully amplified and compared with the control DNA obtained from two reference BoHV-4 strains (American strain, DN599 and European strain, Movar), confirming the isolation of BoHV-4 (Figure 1). Restriction analysis of whole genome of BoHV-4 showed different fragments scattered through the gel, where the new isolates showed a similarity to each other and to the European reference strain - MOVAR (Figure 2).

Although these Serbian strains did not completely overlap the European or American reference strains, based on the number of fragments with similar size, the Serbian strains seems to be more similar to the European one. However, isolate 5597 differed on the polyreplicative DNA (hyper-molar bands) and also in other parts of DNA compared to isolates 6403 and 5650 and reference strains (Figure 2, marks 1, 2, 3). As previously described (Dubuisson *et al.*, 1987; Frazier *et al.*, 2002; Donofrio *et al.*, 2005), BoHV-4 appears to be capable of significant genomic drift, as demonstrated by variability between isolates from different geographic regions.

Isolates 6403 and 5650 were isolated from seronegative young cows (with clinical signs of metritis) after first calving suggesting primary infection (seroconversion confirmed 22 days later - Table 1, samples 6403 and 5650). Isolate 5597 came from a seropositive 6-year-old cow (Table 1, sample 5597), which indicates reactivation from latent infection.

A significant number of BoHV-4 seropositive animals in the herds (84.37%), seroconversion and increase in specific antibody values in nine out of ten cows (Table 1) and BoHV-4 isolation from vaginal contents, indicate replication of BoHV-4 in the uterus of examined cows in the postpartum period and suggests that BoHV-4 might play a role in the pathogenesis of postpartum metritis in the examined herd, despite the fact that

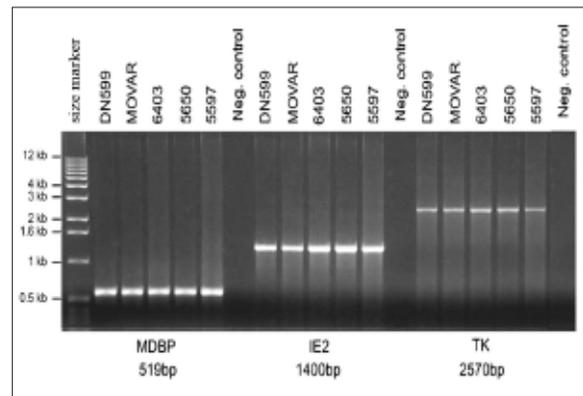


FIGURE 1 - Specific amplification of MDBP, IE2 and TK sequences of BoHV-4 genome. The amplifications were performed using DN 599 and MOVAR 33/63 as BoHV-4 reference strains positive control. Negative control was performed with uninfected cell DNA.

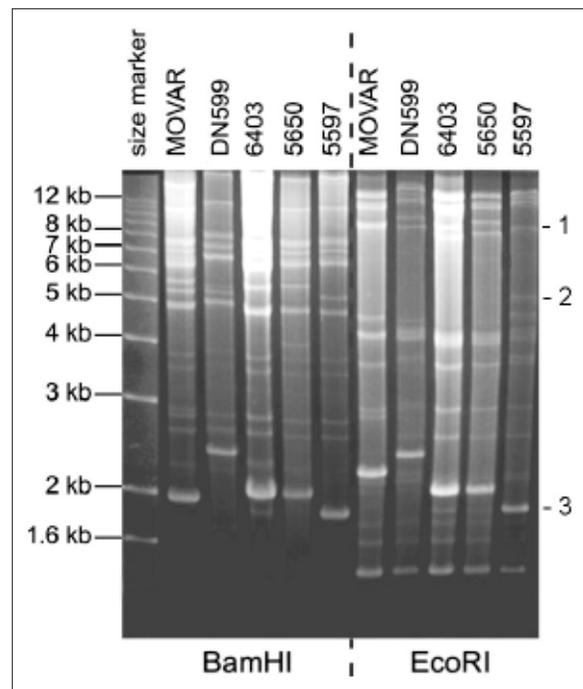


FIGURE 2 - BamHI and EcoRI restriction fragments of MOVAR 33/63 and DN 599 as reference strain and 6403, 5650, 5597 (three field isolates). Molecular size marker determine fragments from 1.6 to 12 kb.

other factors, such as mineral and vitamin deficiencies of cows, hypocalcaemia, uterine contamination with *A. pyogenes* and/or with *Streptococcus sp.* or *E. coli*, etc. might also influence the high rate of postpartum metritis in the examined herd.

On the basis on clinical and laboratory results, it can be concluded that BoHV-4 seronegative young cows were infected before or shortly after delivery, in agreement with other authors (Monge *et al.*, 2005). This period corresponds to the stay of cows in the maternity barn, which can be a critical period for virus transmission. Appropriate conditions for BoHV-4 spreading in the maternity barn can include postpartal reactivation of latent infection and virus dissemination after parturition (Wellemans *et al.*, 1986), increased susceptibility of the uterus for infection in uninfected cows after calving (Wellemans *et al.*, 1986) and close contact among infected and seronegative cows.

Despite the fact that many authors indicate a role of BoHV-4 in the etiology of postpartal metritis (Frazier *et al.*, 2002, Monge *et al.*, 2005), there is a need for further investigation of the pathogenesis of BoHV-4 induced postpartal metritis and also the need for characterisation of the isolates in terms of endometriotropism.

This is the first reported isolation of BoHV-4 in Serbia and also the first seroepidemiological data of BoHV-4 infection in Serbian herds, which adds more on the epidemiology of the virus.

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