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Original article

# Prevalence of Bovine herpesvirus type 4 in aborting dairy cows

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## Abstract

Bovine herpesvirus type 4 (BHV-4) is related to many different conditions: infertility, postpartal metritis, vulvovaginitis, mastitis, encephalitis, calf pneumonia, keratoconjunctivitis, cutaneous lesions, digital dermatitis and abortion. In this study a retrospective PCR examination of 100 extracted DNA samples from aborting cows was performed in order to determine: prevalence of BHV-4 in abortive cattle, whether coinfections BHV-4 with other abortifacient pathogens are present in the same sample and to determine the month of gestation when BHV-4 associated abortions were detected. Out of 100 examined samples, the BHV-4 genome was detected in 21 samples (21%). In two samples we detected coinfection of BHV-4 with bovine viral diarrhoea virus (BVDV) and in one with *Neospora caninum*. Most of the BHV-4-associated abortions were detected during the seventh month of gestation. It was concluded that an active BHV-4 infection was present among cows that aborted on the farms examined. The high prevalence of the BHV-4 genome in abortion material suggests that this virus may have caused the abortions. Further studies and examinations are needed to establish causative connection between presence of BHV-4 and abortion.

**Key words:** Bovine herpesvirus type 4, cow, abortion, PCR, Serbia

## Introduction

Bovine herpesvirus type 4 (BHV-4) is a member of the *Herpesviridae* family, *Gammaherpesvirinae* subfamily and *Rhadinovirus* genus and it is different from other herpesviruses isolated from ruminants (Chastant-Maillard 2015). The virus was first isolated in Hungary in 1963 from calves with keratoconjunctivitis and a respiratory disease (Bartha et al. 1966). BHV-4 is related to many different conditions: infertility, postpartal metritis, abortion, vulvovaginitis, mastitis, encephalitis, calf pneumonia, keratoconjunctivitis,

cutaneous lesions, digital dermatitis (Castrucci et al. 1986, Wellenberg et al. 2000, Monge et al. 2006, Costa et al. 2011, Chastant-Maillard 2015). Beside that, the virus may also be isolated from clinically healthy animals (Frazier et al. 2001). Like other herpesviruses, BHV-4 also establishes latent persistent infection (Engels and Ackermann 1996). Given the fact that BHV-4 may be isolated from both healthy and diseased animals, it is difficult to determine its role and significance in the development of pathological processes. *In vitro* investigations suggest that BHV-4 contribute to disease development by stimulation of the inflammatory reaction (Donofrio et al. 2005).

Abortions pose a significant problem for modern dairy cattle husbandry causing both direct and indirect losses, which influence the economy of production (Anderson et al. 1990). Therefore, it is imperative to determine an exact etiological diagnosis in case of abortion in order to eradicate abortifacient agent from the farm. The connection between BHV-4 and abortion could be made upon serological investigations (Czaplicki and Thiry 1998), virus detection in samples (aborted fetuses, placentas and vaginal discharge) collected after abortion (Kirkbride 1992, Deim et al. 2006, Deim et al. 2007, Delooz et al. 2012, Leboeuf 2013) and by virus artificial *in utero* inoculation resulting in abortion (Kendrick et al. 1976). There are no published data about the detection of BHV-4 in samples from aborting cows in Serbia. The only available ones are scarce and focused on the detection of BHV-4 in semen from bulls used in artificial insemination centres (Nikolin et al. 2008) and from dairy herds with postpartum metritis (Nikolin et al. 2007).

The aims of this study were to investigate the presence of BHV-4 genome in the archive of samples originating from aborting cows which had been submitted to the Institute of Veterinary Medicine of Serbia for routine diagnosis, to determine whether coinfections between BHV-4 and some other abortifacient pathogen were present in examined samples and to determine the month of gestation when BHV-4-associated abortions were mainly detected.

## Materials and Methods

### Samples

In order to detect the presence of the BHV-4 genome, a retrospective survey was undertaken on 100 samples of extracted DNA from tissues of aborting cow which had been submitted for routine diagnostic investigation to the Institute of Veterinary Medicine of Serbia in the period between 1 January 2013 and 30 July 2015. Samples used in this study were chosen on the basis of one sample out of three submitted. All of them originated from six commercial farms from the Belgrade city area. The farms held between 700 and 2500 black and white Holstein-Frisian dairy cattle. In order to increase possibility of detecting the etiology of abortion, we insisted that cow's blood, placenta, cervical/vaginal swab and aborted fetus from each aborting cow should be collected for a broad diagnostic investigation. Unfortunately, in the majority of cases this was not possible

and samples varied in type and volume. As a minimum sample for accurate diagnosis, we considered cow's blood and at least one more sample (swab or placenta or fetus) suitable for molecular and bacteriological investigations. Samples of placenta, swabs and aborted fetus from the same cow were treated as one sample. All samples after sampling were stored in a refrigerator at 4°C and were submitted to the laboratory during the same day. Each submitted sample was accompanied by supplementary data which included the date of artificial insemination and date when abortion was observed. For all samples which we used later in our study, routine diagnostic investigation has been conducted as follows. Material from aborted cows were examined by serological (microagglutination test for detection antibodies against *Leptospira* spp., Rose-Bengal test for detection antibodies against *Brucella* spp.), molecular PCR for BHV-1 (Bovine herpesvirus type 1) (Deka et al. 2005), *Brucella* spp. (Leal-Klevesaz et al. 1995), *Neospora caninum* (Baszler et al. 1999), and *Coxiella burnetii* (Berri et al. 2000) genome detection, RT-PCR (reverse transcription polymerase chain reaction) for BVDV (Weinstock et al. 2001) and Schmallenberg virus (Bilk et al. 2012) genome detection and standard bacteriological methods (aerobic and microaerophilic cultivation).

### DNA extraction

For DNA isolation from each aborted fetus, a pool (1 gram) of parenchymatous organs (brain, lung, heart, liver, spleen and kidney) and abomasal fluid was used. The tissues were triturated in a sterile mortar and pestle and mixed with PBS (phosphate buffered saline) in order to prepare 10% suspensions which were mixed well on a vortex for 1 minute and centrifuged at 1500 g for 10 minutes. Placental tissues were processed in the same way. Vaginal and cervical swabs were resuspended in 1 ml of PBS and mixed on a vortex. From each prepared sample, 200 µl of supernatant were used for DNA extraction with a commercial kit for nucleic acid extraction following the manufacturer's instructions (Gene JET Viral DNA and RNA purification kit, Thermo scientific, Lithuania). For positive control, we used DNA extracted from MDBK cell line (Madin-Darby bovine kidney, Izsler, Italy) infected with BHV-4 (field strain isolate originated from investigations by Nikolin et al. 2007) whilst for the negative control we used uninfected MDBK cells. The extracted DNA was stored at -20°C until further analysis.

### Nested PCR

For the detection of BHV-4, nested PCR assay was used. The following oligonucleotides were selected as primers (Fabian and Egyed 2004): The outer primers flanked a 737 bp (base pair) fragment (forward 5'-GACTATGAGGAATGGCACAAG-3'; reverse 5'-TACTCGTAGGCTGGGTCTGG-3'). The inner primers amplified a 271 bp fragment (forward 5'-GGTTGGAAGTGAGCGTATGAT-3'; reverse 5'-GTAGGCGGGTCTGGAAT-3'). The PCR amplifications were carried out in 50 µl reaction mixtures containing 25 µl of TopTaq Master mix solution (TopTaq Master Mix Kit, Qiagen, Germany), 15 pmol of each primer, 5 µl of CoralLoad and 5 µl of the prepared sample. The amplification was carried out in Eppendorf Mastercycler. The steps included in the amplification were: initial denaturation at 95°C for 3 minutes, followed by 25 cycles of denaturation at 94°C for 45 seconds, annealing at 63°C for 45 seconds and elongation at 72°C for 1,5 minutes. For the second, nested PCR reaction, 2 µl of PCR product from the first round were used as a template. The same thermal profile was applied with an exception of 30 cycles and elongation time of 1 minute due to the shorter expected product. The last step, following the last cycle, was final extension at 72°C for 10 minutes for the completion of the amplification. Until gel electrophoresis, the PCR products were stored at 4°C. Ten µl of PCR products were analyzed by electrophoresis in 2% agarose gel using 1 X Tris-borate-EDTA as running buffer at 60V for 33 minutes. The ethidium bromide stained bands were visualized with UV transilluminator. The molecular size of fragments was compared with commercial molecular marker (100 bp FastRuler DNA ladder, Thermo scientific, Lithuania).

### Statistical analysis

The mean value and standard deviation were used in order to determine the average age of gestation when abortion was detected. In order to determine if the coinfection with BHV-4 and some other abortifacient pathogen was significant for occurrence of abortion, Fisher's exact test was applied. Chi-square test was used to determine if BHV-4 positive cows had a tendency to abort at a specific month of gestation.

### Results

Out of 100 samples included into this study, 30 were positive for some of the abortifacient pathogen

(BVDV, BHV-1, *Neospora caninum* or *Coxiella burnetii*) while 70 others were negative to all tested abortifacient pathogens. Blood sera from all samples were negative for antibodies against *Leptospira* spp. and *Brucella* spp. From 100 examined samples, the BHV-4 genome was detected in 21 samples (21%) (Table 1). Detailedly, BHV-4 genome was detected in three out of 30 samples positive to at least one abortifacient pathogen (BVDV, BHV-1, *Neospora caninum* or *Coxiella burnetii*) and in 18 out of 70 samples that were negative to all examined abortifacient pathogens. Out of three samples which, besides BHV-4, were positive to one more abortifacient pathogen, in two samples a coinfection with BVDV and in one sample with *Neospora caninum* were detected. There was no statistical significance ( $p>0.05$ ) that BHV-4 coinfection with other abortifacient pathogens contributed to the development of abortion. In 16 out of 21 BHV-4-positive samples, at least one bacterial species was isolated (Table 1). Considering the age of gestation when abortion was detected, all cases of abortions positive for BHV-4 were detected between the third and the eighth month of gestation. The mean age of gestation when abortion was observed was 5.90 months (SD 1.64). Divided by month of gestation in which BHV-4 positive abortions were detected, we found three cows (14.28%) aborted in the third month of gestation, one (4.76%) in the fourth, four (19.05%) in the fifth, three (14.28%) in the sixth, seven (33.33%) in the seventh and three (14.28%) in the eighth month. There was no significant association ( $p>0.05$ ) between the presence of BHV-4 and the age of gestation when abortion was detected.

### Discussion

The prevalence of BHV-4 in our study was higher than those reported by other authors. Out of 8962 samples, Kirkbride (1992) isolated BHV-4 from 47 samples (0.52%). Given that Kirkbride (1992) in his investigation used only virus isolation for BHV-4 detection, we can presume that BHV-4 prevalence would have been higher if more sensitive molecular methods had been used. Deim et al. (2006, 2007) in their two different investigations examined tissue samples from aborted fetuses and placentas with different diagnostic methods in order to detect BHV-4. Having examined 33 placental samples from aborted cows, Deim et al. (2006) detected BHV-4 DNA in six samples (18.18%). From tissue samples of aborted fetuses, Deim et al. (2007) detected the BHV-4 genome in seven (29%) out of 24 fetuses which were examined. Although Deim et al. in their investigations successfully detected BHV-4 DNA in 18.18% and 29%

Table 1. Description of BHV-4 positive samples.

Cow	Month of gestation when abortion was detected	Type of sample	PCR result for other pathogens	Bacterial isolates
1	7th	Vaginal swab	negative	<i>E.coli</i>
2	5th	Vaginal swab	negative	<i>E.coli</i>
3	6th	Placenta, Vaginal swab	negative	<i>E.coli</i>
4	7th	Placenta	<i>Neospora caninum</i> +	negative
5	7th	Fetus	negative	<i>Streptococcus uberis</i> , <i>E.coli</i>
6	7th	Placenta	negative	negative
7	5th	Placenta	negative	negative
8	4th	Cervical swab	negative	<i>Streptococcus bovis</i>
9	3rd	Placenta	negative	<i>E.coli</i>
10	3rd	Placenta, Cervical swab	negative	<i>E.coli</i>
11	6th	Cervical swab	negative	<i>E.coli</i>
12	3rd	Vaginal swab	negative	<i>Streptococcus intermedius</i>
13	5th	Vaginal swab	BVDV+	<i>Staphylococcus aureus</i>
14	8th	Vaginal swab	negative	negative
15	8th	Placenta, Vaginal swab	BVDV+	<i>E.coli</i>
16	6th	Cervical swab	negative	<i>Streptococcus parasanguinis</i> , <i>Bacillus</i> spp.
17	5th	Vaginal swab	negative	negative
18	7th	Vaginal swab	negative	<i>Streptococcus uberis</i>
19	7th	Fetus, Vaginal swab	negative	<i>Streptococcus uberis</i>
20	8th	Vaginal swab	negative	<i>Streptococcus uberis</i>
21	7th	Placenta	negative	<i>Trueperella pyogenes</i>

samples, respectively, the fact that those investigations were performed on relatively small numbers of samples (33 in first and 24 in second investigation) should be considered. Leboeuf (2013) examined extracted DNA from 639 abortion cases and detected BHV-4 in 6% of samples. Using PCR method Delooz et al. (2012) succeeded in detection of BHV-4 DNA in 1.1% spleens of aborted fetuses. Given the results of our and other authors investigations, it may be concluded that the BHV-4 prevalence detected in samples from aborting cows depends on the diagnostic method used.

During diagnostic investigations in cases of abortion in order to reveal causative agent, the basic principle should be to establish the link between the isolated agent and pathomorphological alterations in tissues of aborted fetuses and/or placenta. This means that the sole detection and/or isolation of some agent do not necessarily mean that detected and/or isolated agent caused the abortion. The link between BHV-4 and abortion could be established with serological investigations. Czaplicki and Thiry (1998) in Belgium reported higher BHV-4 seroprevalence in aborting cows than in cows that never aborted (17.2% and 10.0%, respectively). Deim et al. (2006, 2007) in their investigations detected BHV-4 in aborted fetuses and placental tissues by *in situ* hybridization. In aborted fetuses, BHV-4 was detected in lymphocytes and monocytes of spleen, renal tubular epithelial cells and

in Kupffer cells in the liver (Deim et al. 2006). With evidence of an active replication of BHV-4 in placental cells, Deim et al. (2007) concluded that by destruction of cells, stimulation of inflammation or by interfering with the local immune response, BHV-4 can alter the physiological role of placenta and possibly induce abortion. In our investigation, as well as in those performed by Delooz et al. (2012) and Leboeuf (2013) the presence of the BHV-4 genome was detected by PCR in samples from aborted tissues. With this technique, it is possible only to detect BHV-4 DNA in the examined material. This also pose the main limitation of PCR in abortion cases, given the need for detection of the link between the isolated agent and lesions in tissues.

BHV-4 coinfection with BVDV was also reported earlier (Reed et al. 1979, Frazier et al. 2001). In the available literature, coinfection with BHV4 and *Neospora caninum* has not been described so far. Although the abortogenic potential of BVDV and *Neospora caninum* has been proven, the final outcome of infection is not necessary abortion (Weinstock et al. 2001, Dubey and Schares 2006). Although we did not prove statistical significance, we believe that further investigations may discover whether BHV-4 coinfection with BVDV and *Neospora caninum* contributes to the development of abortion. Beside coinfection with specific abortifacient pathogens, BVDV and *Neospora caninum*, several bacterial species from BHV-4 positive



samples were isolated. Isolated bacteria were mainly opportunistic and ubiquitous and could not be considered relevant findings with respect to the samples from which they were isolated. In order to consider the isolation of opportunistic and ubiquitous bacteria as a cause of abortion, it is necessary to isolate them in moderate to heavy and relatively pure growth from the organs of aborted fetuses and/or placenta with corresponding macroscopic and microscopic lesions (Yaeger and Holler 2007).

The age of gestation in which BHV-4 associated abortions were detected in our investigation was different from results published by Czaplicki and Thiry (1998), who detected BHV-4-associated abortions from the fifth to the ninth month of gestation. Nonetheless, our results of the distribution of BHV-4 associated abortions throughout the gestation period were in concordance with Czaplicki and Thiry (1998), who also detected most of them in the seventh month of gestation. Opposite to this results, Kendrick et al. (1976) inoculated isolate from the cow with metritis into fetuses *in utero* in different stadiums of gestation which resulted with three abortions after inoculation in the third and fourth month of gestation.

Having considered the results of the present investigation, it is concluded that an active BHV-4 infection was present among aborting cows on examined farms. A high prevalence of the BHV-4 genome in abortion material suggested that this virus could be a possible causative agent of abortions. However, in order to provide a clear link between BHV-4 infection and abortion, as well as the potential interaction of BHV-4 with other abortifacient pathogens in the same sample, it is necessary to perform further and more detailed investigations. In the present investigation only samples from aborting cows were included. In order to reveal the total impact of BHV-4 on the cow's health status, additional diagnostic investigations including serological tests and examinations of other categories of cows are needed.

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