



INVESTIGATIONS ON BOVINE HERPESVIRUS 4 INFECTIONS IN CATTLE IN BULGARIA

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Summary

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Cattle infected with BHV 4 show respiratory symptoms, gastrointestinal and postnatal genital disorders. The virus is a cofactor of bacterial infections. This paper reflects data on the prevalence of BHV 4 infections in several herds, among breeding animals and bulls from artificial insemination stations (AIS). One hundred and twenty six serum samples from bulls were investigated by MVNT and 45 (35.7%) were positive. Epizootiological and clinical studies on the prevalence of BHV 4 infections were limited to bovine farms with suspected infection with this virus. To establish seroconversion to the virus, double serum samples from 264 cattle – 165 native-born cows and 99 imported pregnant heifers were investigated by the microvirus neutralisation test (MVNT) and commercial ELISA for the detection of antibodies against BHV 4. Ninety-nine samples (37.5%) were positive in the MVNT and 110 samples (41.6%) – in ELISA. One out of the seven milk samples from cows with signs of mammary glands involvement was positive in the polymerase chain reaction (PCR) performed to detect the *gB* and *Tk* virus genes. Four of the ten whole blood samples from acutely ill animals were positive for BHV 4 DNA by PCR. The results of the present study confirmed that the BHV 4 circulated within the Bulgarian cattle population.

Key words: BHV 4, bulls, cattle, ELISA, microvirus neutralisation test, PCR

INTRODUCTION

Based on biological characteristics, a wide range of hosts, genome structure and genetic content, bovine herpesvirus 4 (BHV 4) is classified in the subfamily *Gamma herpesvirinae*. It differs from the members of the subfamily *Beta herpesvirinae* due to its smaller genome and the characteristic type B structure of *Gamma herpesvirinae* (Roizman & Pellet, 2001).

Isolation and characterisation of BHV 4 was performed in Hungary in 1963 from calves with respiratory symptoms and keratoconjunctivitis (Bartha *et al.*, 1966), and in the United States from heifers with respiratory symptoms (Mohanty *et al.*, 1971). The virus has been isolated from cattle exhibiting various clinical signs, such as conjunctivitis, inflammation of the upper respiratory tract, enteritis and

pneumonia (Smith *et al.*, 1972). BHV 4 has also been isolated from the reproductive organs of animals with orchitis, epididymitis, vaginitis, abortion, metritis and mamillitis, (Thiry *et al.*, 1981; Wellemans *et al.*, 1983; Wellemans & Van Opdenbosh, 1987; Wellenberg *et al.*, 2001; Frazier *et al.*, 2002; Peshev & Sirakov, 2020). Cattle infected with BHV 4 showed mild respiratory symptoms and postnatal genital disorders (Wellemans *et al.*, 1983; Castrucci *et al.*, 1987). BHV 4 can be reactivated after stress due to prolonged transport, calving and changes in ambient temperature (Izumi *et al.*, 2006; Peshev & Christova, 2013; Chastant-Mailard *et al.*, 2015). Dubuisson *et al.* (1987) detected BHV 4 in bulls with orchitis and demonstrated the possibility of virus spreading through the semen.

BHV 4 is a cofactor of bacterial infections; urinary and mammary tract symptoms are more severe in mixed viral-bacterial infection.

This paper reflects data on the prevalence of BHV 4 infections in several farms, among breeding animals and bulls from artificial insemination stations (AIS) in Bulgaria.

MATERIALS AND METHODS

Studies on the prevalence of BHV 4 infections were limited to cattle farms with suspected infection with this virus. Various clinical symptoms leading to infection with BHV 4 were observed. The animals in some of the farms were immunised against mucosal disease-bovine viral diarrhoea viruses (BVDV), infectious bovine rhinotracheitis (IBR) and ringworm.

Serum samples

Serum samples from diseased animals (264 cattle including 165 native cows and

99 imported pregnant heifers) with clinical signs of BHV 4 were investigated. Two consecutive serum samples (during the acute phase of the infection, and after a 3–4 week interval) were examined to establish seroconversion to the virus.

Two cow farms with indigenous and imported animals, with increased mortality among the imported cattle were visited and 64 serum samples were collected from clinically ill animals on the day of the visit (0) day; 21 and 90 days after that.

Investigations on the prevalence of BHV 4 infections were carried out in breeding bull herds and AIS in Sofia, Sliven and Smolyan. Totally, 126 serum samples from males from AIS was collected.

Microvirus neutralisation test (MVNT) for the detection of antibodies against BHV 4

The study was conducted with MVNT β -variant (Dilovski *et al.*, 1982) with modification (extended time of contact of the virus with serum antibodies – 24 h incubation). The reference BHV 4 strain "Movar 33/63" was used in the reactions.

Sera were treated at 56° C for 30 min and antibiotics (penicillin 20 IU/mL and streptomycin 200 μ g/mL) were added. The sera were diluted twice with MEM Hanks, and 100 tissue culture infectious dose 50 (TCID₅₀) from the reference strain "Movar 33/63", with titre of 10^{6.66} TKID₅₀ were added. The serum-virus mixture was incubated for 24 h at 37° C. As indicator system, a Madin Darby bovine kidney (MDBK) or embryonic bovine trachea (EBTR) cells at amounts of 4×10⁵ cells/mL were added. Two controls from uninfected cell culture (CC) and cell culture infected with the virus containing 100 TCID₅₀/mL were included. The results were read at 72–120 h. The highest dilution of serum, giving complete inhibition

of the development of the indicator virus, was taken as the serum titre.

Enzyme-linked immunosorbent assay (ELISA)

The sera were tested using a commercial ELISA kit (Bio X Brussels, Belgium) according to the manufacturer's instructions. Serum samples and positive control serum (100 μ L, diluted 1:100) were added to the antigen-coated wells and to the control antigen-free wells, and incubated for 1 h at 18 °C to 25 °C or 37 °C. The plates were washed 6 times and 100 μ L of conjugate (anti-bovine IgG1 monoclonal antibodies conjugated to horseradish peroxidase) was added to each well. The plates were incubated again for 1 h at 18 to 25 °C or 37 °C and washed 6 times. Then a substrate-chromogen (H_2O_2 -tetramethylbenzidine) was added (100 μ L) to each well. The colour reaction was stopped after 10 min by adding 50 μ L of 1M phosphoric acid. Optical density (OD) was determined on ELISA plate reader at a wavelength of 450 nm.

From the OD₄₅₀ values recorded for odd columns, were subtracted the corresponding OD₄₅₀ values of the even negative control wells. The test was considered valid if the positive serum gave a difference in optical density (OD₄₅₀) after 10 minutes higher than the OD₄₅₀ given in the table accompanying the kit.

The OD₄₅₀ value of each sample was divided by the OD₄₅₀ value of the corresponding control, and multiplied by 100 to obtain it as a percentage: $PP\% = (OD_{450} \text{ sample} / OD_{450} \text{ control}) \times 100$. The table accompanying the kit was used to determine the positivity of each serum as followed: (+) from 0 to 11.33%; (++) from 11.33% to 35.27%; (+++) from 35.27% to 59.21%; (++++) from 59.21% to 83.14% and (+++++) from 83.14% to 107.08%.

The seroconversion was considered to be clear if the signal increased by two orders of magnitude, for example from (++) to (+++++) or from (+) to (+++).

Molecular biology tests

Seven milk samples from cows with signs of mammary gland involvement were examined by the polymerase chain reaction (PCR) to detect the *gB* and *Tk* virus genes. Ten samples of whole blood from acutely ill animals from both farms were investigated by PCR. The preparation of BHV 4 DNA from milk and whole blood was performed by a Gia amp DNA cadorn mini kit. The PCR was performed by the methods described by Wellenberg *et al.* (2001).

Statistical analysis

Differences between groups were evaluated by means of χ^2 test, Fisher exact probability test and non-parametric test for independent samples (Mann-Whitney U test).

RESULTS

Epizootiological and clinical studies on the prevalence of BHV 4 infections

Studies on the spread of BHV 4 infections were conducted in several cow farms with suspected disease and in breeding bulls and bulls from AIS.

In the comparative serological tests performed on 264 serum samples, 99 samples or 37.5% were positive in the MVNT, and 110 samples or 41.6% in ELISA. Animals with different clinical symptoms pointing to BHV 4 infection were found in two visited cow farms with native and imported pregnant cows. In imported animals mortality was increased, and owners observed clinical signs associ-

ated with movement disorders, collection of pus with an unpleasant odour in the hooves and drooping of hooves in some animals 3–4 days after parturition. During our visits, calved heifers had difficulty getting up, moving, some of them did not eat and their milk secretion was significantly reduced.

In imported cows redness of the skin over the hooves, hair loss, lameness and pododermatitis were found (Fig. 1A). The body skin demonstrated varying degrees of damage, similar to ringworm, affecting not only the head and neck, but also much larger body areas (Fig. 1B). The animals were very weak. In some of them the hair coat in the area of the tail and especially the tip of the tail had shed (Fig. 1C) and they had dermatitis of different severity and area size.

Between the 3rd and 7th day post partum, some of the recently calved imported

heifers had endometritis, initially with haemorrhagic and later purulent discharge from the vulva. In some of the animals there was an increase in temperature, affecting one or more udder quarters and a change in the consistency of the milk secretion, which was mixed with white flocculi when infected with secondary microflora. The mammary gland was enlarged, warm and painful to the touch. Due to the mammary gland involvement, there were also difficulties in the movement of the hind limbs.

Serological studies on the prevalence of BHV 4 infections

In the MVNT serological study of 64 serum samples obtained from cattle from both farms for antibodies against BHV 4 strain “Movar 33/63”, the mean geometric titre (MGT) was significantly lower ($3.27 \pm 0.42 \log_2$) ($P=0.0264$, Mann-Whit-



Fig. 1. Macroscopic changes in a cow with BHV 4 infection. **A.** redness of the skin over the hooves; **B.** varying degrees of ringworm-like skin damage in larger areas of the body; **C.** fallen tip of the tail.

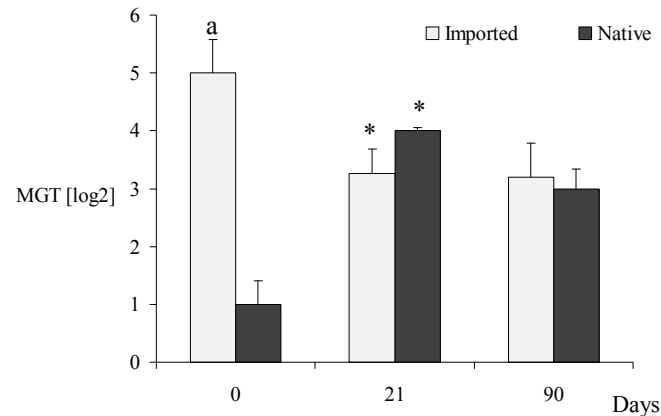


Fig. 2. Mean geometric titres (mean \pm standard errors) of sera tested by using MVNT for antibodies against BHV 4 strain “Movar 33/63”. Significant differences between MGT values on day 0 and 21: * $P=0.0264$, $P=0.0284$ for imported and native groups respectively; significant differences between both groups: ^a $P=0.0303$.

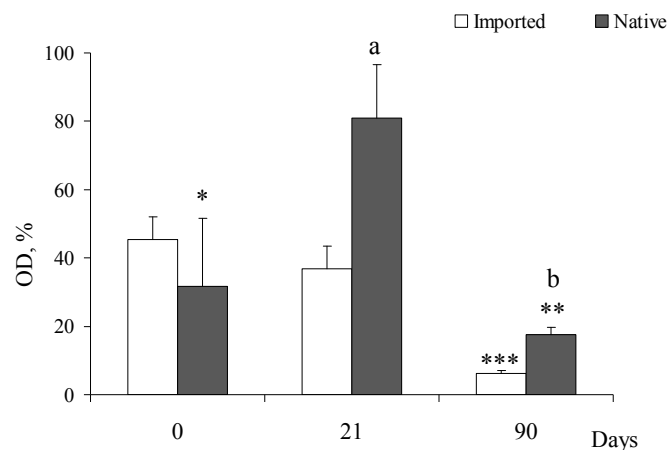


Fig. 3. Mean optical density of sera tested by ELISA (Bio X Brussels, Belgium) for BHV 4 antibodies. Significant differences: between day 0 and day 21: * $P=0.0342$; between day 0 and 90: *** $P=0.0001$; ** $P=0.0019$; significant differences between both groups: ^a $P=0.0273$; ^b $P=0.0001$.

ney U test) on the 21st day after the visit, than on the day of the visit (0 day; $5.0 \pm 0.58 \log_2$) only for imported heifers. In contrast, MGTs were significantly higher ($4.0 \pm 0.05 \log_2$) ($P=0.0284$, Mann-Whitney U test) on day 21 after the visit, than on day of visit (0 day) ($1.0 \pm 0.41 \log_2$) for native animals (Fig. 2). Signifi-

cant difference in MGTs on the day of the visit to the farms was found between the two groups of animals. On the 90th day of the study, no significant difference was found in the neutralising titres for native and imported cattle (Fig. 2).

The mean optical density (OD), obtained by ELISA on day 90 after farm

visit was significantly lower (6.17 ± 0.88) than on day 0 (45.4 ± 6.55 ; $P=0.00099$) and day 21 (36.86 ± 6.59 ; $P=0.000129$ (Mann-Whitney U test) for imported animals. For native animals the average OD on the 21st day was significantly higher (80.95 ± 15.68) than on the day 0 (31.72 ± 19.96 ; $P=0.0342$) and day 90 (17.66 ± 2.05 ; $P=0.0019$) (Fig. 3). The difference in OD for the respective days of the study between imported and native animals was statistically significant for day 21 ($P=0.0273$) and day 90 ($P=0.00066$) of the study (Fig. 3).

In the study of 126 samples of bulls by MVNT for BHV 4 antibodies against "Movar 33/63" strain for a period of four years 45 samples (35.7%) were positive for antibodies against BHV 4. The percentage of positive serum samples in 2012 was significantly lower (5.9%) compared to the previous three years ($P=0.001$, $P=0.02$ and $P=0.0000$, respectively; χ^2 test) while for 2011, the percentage of po-

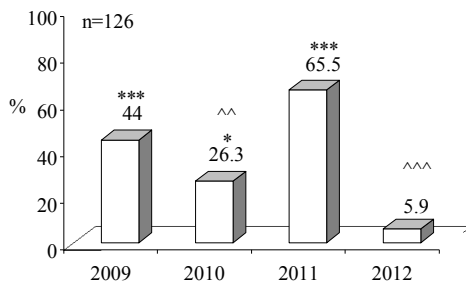


Fig. 4. Percentages of positive serum samples from bulls tested by using MVNT for BHV 4 antibodies against "Movar 33/63" strain over the four-year period. * $P=0.02$; *** $P=0.001$, $P=0.0000$): significant difference between three previous years vs the last year; ^^ $P=0.002$; ^^^ $P=0.0000$ between the third year (2011) and the other years.

sitive animals was significantly higher (65.5%) than both 2010 (26.3%; $P=0.002$; χ^2 test) and 2012 (5.9%; $P=0.0000$; χ^2 test) (Fig. 4).

No changes of the reproductive system were clinically observed in breeding bulls and bulls from AIS.

Molecular biological investigation

BHV 4 DNA was detected in one of the 7 milk samples from cows with involved mammary glands using PCR for the *gB* and *Tk* virus genes. Examination of 10 whole blood samples by PCR revealed BHV 4 DNA in 4 of them.

DISCUSSION

It is known that BHV 4 infections cannot be associated with characteristic clinical signs such as those found in other bovine viral diseases. However, various clinical signs have been described, such as abortion (Deim *et al.*, 2007), metritis, vaginitis, enteritis and pneumonia (Thiry *et al.*, 1986; Castrucci *et al.*, 1987; Egyed *et al.*, 1996; Czaplicki & Thiry, 1998). In our epizootiological studies in cow farms with suspected BHV 4, some of the animals had high fever, mild respiratory disorders, musculoskeletal disorders and severe postpartum genital disorders. In newly introduced animals, the development of metritis and mastitis was found, similarly to a study performed in Spain, where postpartum metritis was observed in 83% of cows infected with BHV 4 (Monge *et al.*, 2006). The hoof involvement, leading to lameness and more severe limb injuries found in this study, resulted in difficulty in the standing up and moving, most probably is due to ataxia as a result of elimination or lack of muscle coordination in imported animals (Izumi *et al.*, 2006).

Pododermatitis and hoof necrosis that did not respond to treatment and specific vaccination against isolated hoof pathogens were observed. The specific skin infection similar to ringworm in the animals was not affected by the applied ringworm vaccination. These two facts suggest that the most likely cause of these symptoms was an infection with BHV 4. The virus circulation was confirmed after performing the PCR, which revealed the presence of BHV 4 DNA in one milk and in four whole blood samples, obtained from the diseased animals.

Dermatitis, pyrexia and haemorrhagic syndrome (DPHS) is a rare bovine syndrome with unclear etiology. Bellino *et al.* (2015) described two animals with DPHS in Italy, with clinical pathological findings suggesting a potential pathogenic role of BHV 4. Similar clinical pathological syndromes were observed by us at one of the farms which confirmed our statement that these symptoms resulted from the circulation of BHV 4.

It is known that BHV 4 can exist in a latent state in the infected animals (Thiry *et al.*, 1986) and can be reactivated under the influence of external factors, such as stress in the animals caused by prolonged transport, calving (Wellemans *et al.*, 1986; Frazier *et al.*, 2001; Peshev & Christova, 2013; Chastant-Mailard *et al.*, 2015), as well as from unexpected changes in ambient temperature (Izumi *et al.*, 2006). The most probable reason for the observed postpartum metritis and vaginitis in the studied imported animals is the stress due to the prolonged transport during their import, as well as calving.

The observed weight loss in imported animals post partum may result from inadequate diet that did not meet their physiological needs, but the local animals were fed the same ration and their clinical

status was satisfactory, which gives us reason to assume that the probable cause of this weight loss in animals was BHV 4 infection.

To make a correct diagnosis of the investigated animals it was necessary to establish a clear seroconversion against BHV 4. This was achieved by examining consecutive serum samples, the first obtained during the acute phase of infection and the second: after 3–4 weeks. In farms with imported pregnant and local cows, samples were collected during the first visit, and samples from the same animals were tested after about three weeks and after three months. The serological tests were performed through 24 h MVNT. Serum titres using a 24 h incubation of the serum-virus mixture were by 1–3 log₂ higher than serum titres in 2 h incubation assay similar to that established by Bitsch (1978) for bovine herpesvirus 1 (BHV 1). This result gave us the reason to use 24-hour incubation in subsequent studies to have greater accuracy and higher sensitivity in reading the reaction.

Obtained significantly lower ($P < 0.05$) MGT with strain "Movar 33/63" ($1.0 \pm 0.41 \log_2$) on the day of farm visit compared to the 21st day of the study ($4.00 \pm 0.05 \log_2$) proved that the infection with BHV 4 appeared later in native than in newly introduced animals. This was confirmed by the significant difference of MGT on day 0 between the two groups of animals – $5.0 \pm 0.58 \log_2$ for the newly introduced and $1.00 \pm 0.41 \log_2$ for the native.

Frazier *et al.* (2002) found 36% positive blood samples (with antibody titres of 3–4 log₂) in the second week after parturition, while such titres were not detected prepartum: an evidence that BHV 4 can be reactivated under stress condition, such as the parturition. The same authors de-

scribed negative serological status in all cows in the tenth week post partum. In our studies, a negative serological status was not found, but only a decrease in antibody titres. Higher antibody titres for newly imported cows on the day of the visit to the farm compared to the other days of the study are evidence of circulation of BHV 4 infection, confirmed by the observed clinical signs only in recently imported animals.

The 41.6% positive sera by ELISA among the 264 serum samples was rate similar to that obtained by the commercial ELISA to detect antibodies against BHV 4 from cows with metritis (40% – Fridgut & Stram, 2006 and 69.6% – Bilge-Dagalp *et al.*, 2010) as well as from cows with reproductive disorders (54 to 56.8 % – Bilge-Dagalp *et al.*, 2007).

Indirect ELISA used for the detection of antibodies against BHV 4, found out a significant decrease in antibody titres at the 21st (36.86±6.59 %) and 90th day (6.17 ±0.88%) in newly imported animals. In contrast, in native cows the antibody titres at the 21st day were significantly higher (80.95±15.68 %) compared to the serum titres on the day of the visit to the cow farms (31.72±19.96 %) and at the 90th day (17.66±2.05 %). This reaffirms the active circulation of BHV 4 infection at different time periods for both groups of animals. Most probably the reason for the earlier onset of infection in imported animals, in which clinical signs similar to those of BHV 4 were observed, was a consequence of the stress caused by prolonged transport, calving and change of housing conditions and nutrition (Peshev & Christova, 2013). The cause for the later appearance of the infection in the local Bulgarian animals is their contact with the imported animals, which were a source of infection as proved by applied molecular biology

and serological methods.

The study of cattle blood samples by ELISA and MVNT showed a higher percentage of the seroreagents by the ELISA (41.66%) compared to MVNT (37.5%). This is attributed to the higher susceptibility of the ELISA compared to MVNT for this virus (Edwards & Newman, 1985).

The high percentage (35.7%) of positive sera antibodies against BHV 4 from bulls in three AIS by MVNT is evidence for the virus circulation among this population. In a study by Simeonov (1992) in bulls from six AIS for antibodies against BHV 4, 22.8% positivity rate was found out. The extent of infection in different stations varied from 11.76 to 38.46 %. The higher percentage of positive animals found in our research may be attributed to the fact that in recent years a larger number of animals have been concentrated in a smaller number of AIS, the contacts between them – closer and the possibility of virus circulation among breeding animals – higher.

Studies of bulls from AIS by ELISA to detect specific IgG 1 antibodies to BHV 4 were performed in Serbia by Nikolin *et al.* (2008) and 36% positive animals were found. The authors confirmed the circulation of the virus by PCR. In our studies, a lower percentage of positive sera was detected. There may be two probable reasons: first, the MVNT used by us to detect antibodies against BHV 4, which is less sensitive than the ELISA (Edwards & Newman, 1985), and second: the smaller number of animals included in our study.

The fact that antibodies were found in sera from breeding and AIS bulls showed that the virus circulated among the bulls, despite the lack of clinical changes in the reproductive system and semen quality. This is confirmed by the results of serological tests, which proved that the infec-

tion was more active at the beginning of the four-year study period (2009–2011), when the number of positive sera was higher as did the titres compared to later studies.

The results of the present study confirmed that the BHV 4 circulated within the Bulgarian cattle population.

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