Bovine Herpes virus 4 (BHV4) infection induced by stress in imported cows

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SUMMARY

Epidemiological, clinical, pathological, microbiological and viral investigations of native Bulgarian and imported Holland animals with different clinical symptoms in a dairy farm were performed. Viral cytopathic agents with cultural characteristics of BHV 4 were isolated from different organs of imported animals and PCR and nested PCR have confirmed the BHV 4 infection. Serological investigations using 24 hours micro virus neutralisation test using "Godech 1" and "Movar" strains and indirect ELISA showed that titers of circulating anti-BHV 4 antibodies were maximal on the visit day and gradually declined in the Holland imported cows whereas Bulgarian native cows exhibited high circulating antibodies only 30 days after the visit day. These results report clinical manifestations of the BHV 4 infection since the introduction of imported cows in a dairy farm probably caused by stress as a consequence of long lasting transportation and exchanging of the rearing conditions while the viral infection lately propagating to the Bulgarian native cows induced no clinical signs.

Keywords: bovine herpes virus 4, PCR, Micro virus neutralisation test, ELISA, imported cows, stress.

Introduction

Group of bovine herpes virus 4 (BHV 4) is represented from antigenically connected viruses [25]. Morphogenesis and slow replication cycle of the BHV 4 in cell cultures are different from the other bovine herpes viruses. BHV 4 was isolated by BARTHA et al. [2] in Europe and MOHANTY et al. [19] in USA. After infection of cell culture with cytomegalovirus isolated from bison it formed large giant cells, cytoplasmic inclusions and the morphogenesis and ultra structure are all consistent with classification of the virus as a cytomegalovirus [26]. Based on biological properties and restriction enzyme fragment pattern of the slowly replicating herpes viruses the BHV 4 tentatively is classified as a member of Beta - herpesvirinae subfamily [24]. After the investigation of genome structure and gene sequencing is found great similarity with other members from subfamily Gamma - herpesvirinae. The virus has a B type genome according to the classification proposed from the herpes virus study group and now the BHV 4 viruses are classified as a genus Rhadinovirus, in the subfamily Gamma - herpesvirinae in the Herpesviridae family [23].

Infections caused by BHV 4 are the reason for respiratory or ocular clinical symptoms in ruminants and are connected to rhinitis, conjunctivitis, tracheitis, pneumonias, skin damages, gastrointestinal and genital disorders [25]. In female animals the virus causes vulvovaginitis, abortion and endometritis or among male animals, epididymitis or orchitis [12]. BHV 4 is isolated also from animals without clinical symptoms [3]. With exception of some isolates the virus is slightly or non pathogenic for the cattle. In farms with poor condition and hygiene, non competent rearing and feeding, the BHV 4 can be exalted from the latent state and can cause seriously financial losses [13]. Because of the concurrent or subsequent bacterial metritis associated with this viral infection, large losses of productions as a death, delayed breeding and infertility, discarded milk from antibiotic therapy, and decreased milk production are possible [15].
The BHV 4 is isolated from aborted foetuses [18, 21] and cows with reproductive disorders or high incidence of postpartum metritis, pustular vulvovaginitis, abortion, stillbirth, and infertility [7, 17]. Seroepidemiological study in Belgium, in cows with a history of abortion as a result of BHV 4 infection determined that the seroprevalence against BHV 4 infection (17.2%) is significantly higher in comparison with a randomly selected control group (10.0%) [9]. These findings are the premise for an epidemiological research of BHV 4 spreading in Bulgarian farms. The purpose of the study is the isolation, identification and molecular biological characterisation of the viral agents obtained from animals with BHV 4 clinical entity.

Material and Methods

EPIDEMIOLOGICAL AND CLINICAL INVESTIGATION

Epidemiological and clinical researches were performed in a dairy farm. The number and animal’s distribution in the different technological groups, the onset of morbidity and mortality rising, spreading of infection in the herd and clinical symptoms were studied. A total of 84 local cross bred “Holstein” cattle, 35 new imported “Holstein Frisian” pregnant heifers from Netherlands and 14 newborn and growing calves has inhabited in dairy farm. After the import 5 cattle were placed on a disposal in the other dairy farm.

When present, microbiological, paraclinical and mycological investigations of skin lesions were performed.

VIROLOGICAL ANALYSES

Samples from lungs, liver, spleen, kidneys, mediastinal and mesenteric lymph nodes, uterus, rumen mucosa, intestine and draining lymph nodes, tongue epithelium, brain, hoof lesions, skin crusts anduffy coats were collected. Probes from one euthanized and four dead heifers with clinical and pathological symptoms were obtained for viral investigations. Tissue samples prepared as a 10% suspension in phosphate buffered saline (PBS) pH 7.2 with antibiotics were used for the virus isolation.

For the virus isolation and propagation permanent cell cultures of bovine kidney (Madin Darby bovine kidney - MDBK) and embryonic bovine trachea (EBTR) cells were used. As growing Eagle minimal essential medium (EMEM) and antibiotic mix (penicillin 100 UI/mL and streptomycin 100 µg/mL) containing 10% foetal calf serum (FCS) were used. As maintenance the same medium and additives but with 2% FCS was used. The cells were infected at multiplicity of infection 0.2 with a clear supernatant obtained from 10% tissue suspension, treated with antibiotics. Adsorption of the inoculums was carried out at 37°C for 2 hours. After washing of the cell monolayer with PBS pH 7.2 the maintenance medium was added. Cultures without a cytopathic effect for 2 and more passages were accepted as a negative for the viral agents. The infectious titre of viruses was calculated by the method of REED and MUECH [22].

For identification of the viral agents, the neutralization tests with positive hyper immune serum (HIS) against Mucosal disease viral diarrhoea (MD-VD), Bovine herpes virus 1 (BHV 1) [11], commercial ELISA kits for MD-VD, Paramyxovirus parainfluenzae 3 (Pi-3), Adenovirus (Av), Respiratory syncitial virus (RSV) and BHV 1, and polymerase chain reaction (PCR) for demonstration of the gB and TK gens of BHV 4 were performed [14, 28].

For PCR, DNA was extracted by a Roboscreen DNA isolation kit (Germany) according to the firm recommendation from the organ suspension and cell culture isolates. Thermocycler QB - 96 (LKB) and different programs for each pair of primers generating fragments of 615 bp for gB gene (at position 38 – 17 or 555 – 576) and 567 bp for TK gene (at position 132 – 153 or 669 – 698) were used (LKB Vertriebs GmbH, Wien) according to WELLEMBERG et al. [28] and EGYED et al. [14] respectively. Following parameters were used for determination of the BHV 4 gB gene: pre-denaturation at 95°C for 10 minutes followed by 45 cycles composed from denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute and elongation at 72°C for 1.5 minute. The amplification was accomplished by a final elongation for 7 minutes at 72°C. The PCR verification was performed using the respective internal positive and negative controls. DNA samples of MDBK cells were used as negative controls. As a positive control reference BHV 4 “Movar 33/63” strain was applied. Specificity of PCR was determined using the BHV 1 and Swine herpes virus 1 (SHV 1) DNA and the same parameters and primers for BHV 4 amplification. The nested PCR with some modification was performed for the BHV 4 TK gene confirmation [14]. Following parameters of the reaction were applied: pre-denaturation at 95°C for 10 minutes, followed from 30 cycles each composed from a denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and elongation at 72°C for 1.5 minute. The amplification was finished by an elongation step at 72°C for 7 minutes. The PCR product from the first PCR round was amplified in the second one using the same PCR mix, but with the nested oligonucleotides primers (at position 339 - 363 and 577 – 598) (LKB Vertriebs GmbH, Wien), generating fragments of 260 bp [14]. Verification of the nested PCR was performed by using the reference BHV 4 “Movar” strain as a positive control. The PCR products were mixed with gel loading buffer and submitted to electrophoresis on 1.5% agarose gel containing ethidium bromide (1 µg/mL) together with DNA molecular weight marker. The amplified products were visualised as a single compact band of expected size 260 bp under an UV light and documented by gel documentation system (Vilbur Lourmat, DNA photo documentation system, France).

For identification of viral agents, commercial ELISA antigen detection kits (Idexx Laboratories, Switzerland AG...
for the MD-VD, Pulmotest Euroclone, Italy for BHV 1 and Pulmotest Bio X, Belgium kits for the above described other viruses) were performed according to the manufacture description.

**SEROLOGICAL ANALYSES**

Totally of 46 serum samples from imported (30) and Bulgarian (16) animals were obtained in the beginning (visiting of the farms), at 30 and 180 days after the onset of infection. The sera were treated at 56°C for 30 minutes and antibiotics (penicillin 20 UI/mL and streptomycin 20 μg/mL) were added.

Micro virus neutralisation test (MVNT) β – variant with modification [11] was used. Briefly serial two fold dilutions of sera were performed with maintenance medium. Hundred tissue culture infectious dose 50 (TCID₅₀) of reference “Movar 33/63” strain with a titer (10⁶.₆₆ TKID₅₀) and lung isolate "Godech" 1 with a titer (10⁷.₆₆ TKID₅₀) were added. Both serum-virus mixtures were incubated for 2 and 24 hours at 37°C and the indicator system MDBK cells at a quantity 4 x 10⁵ cells/mL were added. The cell culture and used viruses were included as negative and positive controls for the reactions. Evaluation of the results was performed at 72 - 120 hours. The highest dilution of sera giving a complete suppression of the viral growth was accepted as the serum titer. The mean geometrical titres (MGTs) for imported and native animals were determined.

Sera were investigated by a commercial ELISA kit - Bio X Brussels, Belgium according to the firm recommendation. For this purpose from each value recorded for odd columns was subtracted the signal of the corresponding negative control wells. The test was validated if the positive serum was subtracted the signal of the corresponding negative control serum signal and the results was multiplied for each tested samples was divided by the corresponding positive control serum signal and the results was multiplied by 100. For determination the degree of positivity table for QC procedure provided with the kit was used. The signal increasing by two orders in the magnitude (two pulses 2>4, 1>3) was accepted as a clear seroconversion. The samples was adopted as positive if the OD values were greater than or equal to one plus sign (+).

**STATISTICAL ANALYSIS**

Processing of data was performed using descriptive statistics, Kolmogorov Smirnov test for distribution checking of obtained variables and non parametric Mann-Whitney U test for overall testing the difference between antibody titers for both strains. Each hypothesis was tested at a level of significance of 0.05.

**Results**

Epidemiological investigation established that imported 35 Holstein Frisian pregnant heifers from Nederland have been treated with BVDV inactivated vaccine 5 days before travelling, and that they were revaccinated 4 weeks later with the live attenuated Bovilis IBR Marker and Bovilis BVD inactivated vaccines. One month later the heifers were unstable with difficulties in upright stand fall down, became recumbent and later died. Four animals were dead till the visiting in the farm.

After parturition the calves stayed with their mother for 3 - 4 days and later they were moved to the other part of premises in individual boxes. The conditions during pregnancy of imported animals were satisfied, but after the parturition strong loose of body weight and dramatically reduction in milk production were observed. Three to five days after delivery the heifers were with hoof problems lameness, symptoms of pododermatitis with necrosis of the hoof capsule and unpleasant odour. The skin interdigital space was with red colour. The farm specific vaccination against the hoof isolated agents was performed without effect. Almost all imported animals showed the same clinical symptoms. After the inspection 40 - 50% of animals exhibited vaginitis and suppurative discharges. In 7 animals, skin changes, similar to the cattle trichophytia, were observed. The treatment with LT 130 vaccine (produced in Russian Federation) used for prophylaxis and control against bovine trichophyisis (ringworm) was unsuccessful. The native Bulgarian animals were provisionally separated from the imported heifers. The native and imported animals were supplied with the same food. There was high acidity of silage and brewery mash. The clinical status of native breed was satisfied. In native ruminants clinical changes were not recorded.

Strong weakness and body dehydration without any subcutaneous haemorrhages were found after the autopsy of imported heifers. There were not visible changes after opening the abdominal cavity, on the rumen and intestine. The uterus was exaggerated with pus. There was dystrophy and degeneration of liver. Its colour was light brown and necrotic areas (diameter 5 cm) in some places were visible. The lungs were with pale pink colour, dystrophic with reduced size without symptoms of pneumonia. The hearth muscle was without haemorrhages but slightly dystrophic. Kidneys, rumen, reticulum and abomasum exhibited no visible changes. The mucus membrane of abomasum was with red colour, without haemorrhages. The hoofs were with dark colour, interdigital space with pus, dropping from hoof capsule after pressure with an unpleasant odour. After microscopic investigation of skin lesion Trichophiton spores were observed.

With microbiological investigations in samples obtained from three dead and one euthanized heifers *Streptococcus* species, *Proteus* and non haemolytic *E. coli* were found.
Bacterial agents in the other investigated probes were not isolated.

Antigens of MD-VD, Pi-3, Adeno, RSV and BHV 1 viruses were not evidenced by the commercial ELISA kits and cell culture isolation.

Infection of MDBK cells with 10% organ suspensions (lungs, liver, brain, uterus and abomasum) from one euthanized heifer and from 4 dead heifers revealed the presence of cytopathic viral agents. Whereas no cytological changes were observed in control not infected cells (figure 1a), rounding and detachment of cell monolayer at 24 hours (figure 1b) and augmentation of these changes to fully detached cell monolayer at 72 hours were observed (figures 1c and 1d). Suppression of the viral isolates was not observed after the neutralization test with the positive MD-VD and BHV 1 hyper immune sera.

Using PCR, an amplified band of 615 bp corresponding to BHV 4 gB gene (figure 2a) and an amplified band of 567 bp corresponding to BHV 4 TK gene (figure 2b) were evidenced.
in all the extracted DNAs from organ suspensions sampled from the euthanized heifer and from the dead heifers. No amplified fragment was obtained from the negative control uninfected MDBK cells. The PCR specificity was confirmed using other herpes viruses, BHV 1 and SHV 1. Specific amplified products were not obtained from BHV 1 and SHV 1 DNAs (data not shown). After applied nested PCR for the BHV 4 TK gene of the same isolates, the expected DNA fragment of 260 bp was effectively evidenced (figure 3).

Serological investigations were performed by 2 and 24 hours MVNT. The serum titers for both strains are 1-3 log₂ higher in 24 hours MVNT compared with those in 2 hours. No difference in the MGT of native Bulgarian animals for all days of serological investigations was noted. With the “Movar” and “Godech 1” strains, the MGTs on day 30 were significantly lower (“Movar”: 3.27 ± 1.62 log₂ and “Godech 1”: 2.53 ± 2.07 log₂) than in the day of the farm visit (day 0) (“Movar”: 5.0 ± 1.83 log₂ and “Godech 1”: 4.80 ± 2.78 log₂) only for imported animals. The opposite situation was observed for the native Bulgarian animals: the MGT on day 30 was significantly higher than on day 0. Moreover, MGTs on day 0 were significantly higher in imported animals than in the native ones whatever the strains used (“Movar” or “Godech 1”) (figure 4). As shown in figure 5, the mean OD values of ELISA tested sera on days 0 and 30 were significantly higher than on day 80 for Holland animals (p < 0.01). Furthermore, differences between imported and native animals were statistically significant for all days of investigations.
BHV 4 INFECTION IN IMPORTED COWS

Discussion

BHV 4 has not been clearly established as the cause of a specific disease entity. In the present study clear clinical symptoms connected with infection as ocular or respiratory disease such as conjunctivitis, rhinitis, tracheitis pneumonia, gastrointestinal disturbances and similar to malignant catarrhal fever symptoms [25] are not observed with exception of the hyperthermia, weak respiratory symptoms and post natal genital disturbances [8, 19, 27]. The BHV 4 can be reactivated in animals when they are subjects to stress due to long distant transportation, parturition and unexpected changes in atmospheric temperatures [16]. Most probable cause for observed metritis and vaginitis in the investigated animals here is their importation as it is observed by FRAZIER et al. [15].

Observed difficulties in upright stand in imported Holland heifers in the present study most probably are due to the ataxia as a result of elimination or absence of the muscular coordination [16]. The determined lameness and hoofs symptoms of pododermatitis with necrosis are not influenced on the applied specific vaccination against isolated hoof agents as well as skin changes, similar to cattle trichophytha. Both above facts suggest that the BHV 4 is the probable cause for those symptoms. Most probably the weight lose of cattle after the birth is a result of feeding with inadequate ration, no corresponding to physiological needs of the soon delivered animals. Changes of organs established after the autopsy of dead animals as an aggravated uterus with suppurative discharge, dystrophic liver with necrosis, lungs and heart muscle dystrophy, can be connected to pathogen bacterial agents and different viruses, but after microbiological investigations, highly pathogen bacterial agents are not isolated. Virus agents of MD-VD, BHV 1, Ad, Pi-3 and RSV are not proved by the ELISA and neutralization techniques. Although above described organ changes can be connected to inadequate feeding also, the native Bulgarian pregnant animals are feeding with the same ration and their clinical status is satisfied. All those facts suggest that the cause of observed dystrophic changes is the BHV 4.

BHV-4 is isolated from cattle with a variety of clinical symptoms including abortion [10], metritis and vaginitis [4], enteritis, and pneumonia, as well as from the healthy animals [7, 9, 14]. In the present investigation BHV 4 is isolated from heifers with different clinical symptoms only. Nasal and ocular samples, peripheral blood leucocytes and different organs of experimentally inoculated calves were used for isolation of BHV 4 [8, 12, 14]. The present results establish existence of the virus in the lungs, liver, abomasum mucosa membrane and uterus of diseased animals as previously shown by EGYED et al. [14], but the isolation of the viral agents on cell cultures by buffy coats is not successful and not confirmed despite use of highly sensitive PCR for BHV 4 detection. Most probable the reason is the low quantity or the lack of viral DNA in the peripheral blood leucocytes. Independently of the statement of OSORIO et al. [20] that the BHV 4 can not be found in nervous system of experimentally infected rabbits, also confirmed by EGYED et al. [14] for bovine muscle and nervous system, a viral agent with BHV 4 peculiarity was isolated from the brains of two heifers in the present study. Moreover, the presence of viral DNA was confirmed by PCR. Most probably some BHV 4 strains have different capability to impact the nervous system [1]. Typical products for BHV 4 after amplification with the specific primers for gB and TK genes as described by WELENBERG et al. [28] and EGYED et al. [14] were evidenced. The lack of the bands with DNAs obtained from BHV 1 and SHV 1 strains confirmed the specificity of PCR. The use of single and nested PCRs for determination of TK genes, permit to perform more precise studies on in vivo distribution of BHV 4 in organs and peripheral blood leucocytes of animals.

Serological investigations were performed by 2 and 24 hours MVNT. As the serum titers for both strains are 1-3 log, higher in 24 hours MVNT compared to those in 2 hours [6], incubation of 24 hours were used in the following investigations. Described clinical symptoms were observed only in imported animals, suggesting that the BHV 4 infection was circulating among them. Evidence for this statement was the significantly lower MGTs determined on the day of visiting than 30 days later with “Godech 1” and “Movar” strains (p <0.05). On the other hand, there was not observed difference in the MGTs of native Bulgarian animals for all days of serological investigations except for the day 30 with the “Movar” strain. Significantly lower MGTs observed on the day of visiting than on day 30 evidenced that the infection with BHV 4 was appearing later in the native animals than in the imported ones. Significant differences in MGTs on day 0 (visit day) between the 2 groups of animals also corroborated this assertion.

The indirect ELISA is successfully used for the determination of seroprevalence against BHV 4 in dairy cows where they found 54% seroprevalence (range 33.1% - 69.6%) and in the cows with reproductive disorders (56.8%) than in the cows without reproductive disorders (44.9%) [4]. Using commercial ELISA technique, 69.6% of cows with metritis in dairy herds positively reacted [5]. After investigation by the indirect ELISA test, significant decreases in the titers of circulating antibodies on days 30 and 180 were observed in the imported animals whereas circulating anti-BHV 4 antibody titers were maximal on day 30 (compared to the days 0 and 180) in the Bulgarian native animals, confirming in this way that the BHV 4 infection has circulated within different time periods for the both groups of animals.

As a conclusion, the present study evidenced that the imported Holland animals exhibited BHV 4 infection since their introduction in the dairy farm probably because of the weakness of the immune system as a consequence of the stress caused by long lasting transportation and exchanging of the rearing conditions and that lead to later exaltation of BHV 4 infection in the Bulgarian native animals.
REFERENCES


