DETECTION OF BOVINE HERPESVIRUS 1 FROM AN OUTBREAK OF INFECTIOUS BOVINE RHINOTRACHEITIS

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Abstract

Clinical symptoms from the respiratory tract were observed in cattle after the introduction of pregnant heifers into the dairy herd. Sera and nasal swabs from all animals and tissue samples from two dead animals were tested for BHV1. Specific antibodies against BHV1 were found in serum samples of 24 animals. Only one sample reacted doubtfully in gB ELISA. The virus was isolated only from nasal swabs and lungs collected from 2 weeks old calf. The remaining samples were negative in virus isolation test. PCR with external primers detected the presence of BHV1 in 11 nasal swabs and in lung and liver samples of 2 weeks old calf. In nested PCR almost all tested samples were positive. Restriction enzyme analysis confirmed the specificity of amplification. Results of laboratory diagnosis revealed that introduction of newly purchased animals into the herd initiated the outbreak of disease caused by BHV1.

Key words: cattle, bovine herpesvirus 1, infection, diagnostics.

Infectious bovine rhinotracheitis (IBR) is a highly contagious infectious disease that is caused by bovine herpesvirus 1 (BHV1). Apart from respiratory disease, this virus can cause other clinical syndroms such as infectious pustular vulvovaginitis or balanoposthitis, conjunctivitis, encephalitis and generalized systemic infections (6, 8, 19). In adult cows infection is associated with a severe and prolonged decrease in milk yield, reduced fertility and abortions (12, 13). Many infections run with a subclinical course (7, 21).

Cattle of all ages and breeds are susceptible to infection with BHV1. The virus is usually introduced into a herd with acutely infected animals. Such animals shed large amounts of the virus with nasal and ocular secretions and infect susceptible individuals. The semen of infected bulls may contain BHV1 and the virus can be transmitted to cows by natural mating or artificial insemination (4, 9, 10, 15, 17). BHV1, like other alphaherpesviruses, can set up a latent infection in neurons of sensory ganglia (2, 3). Due to stressful conditions or corticosteroid treatment latent BHV1 can be reactivated and re-excreted at irregular intervals. Therefore, cattle latently infected with BHV1 can also be a source of infection (16).

Laboratory examinations are required to make a definite diagnosis of BHV1 infection. Virus isolation in cell culture is the most frequently used method to detect BHV1 in samples. However, this method is laborious, can take up to 7 days, and samples of good quality are required. Therefore, samples such as nasal swabs should be collected as soon as possible after the outbreak of the disease. Currently, new techniques like PCR to detect the presence of the virus in nasal secretions and tissues are available (5, 11, 14, 20). The major advantages of PCR assay are its sensitivity, specificity, and rapidity.

The purpose of the study was to investigate the cause of respiratory disease outbreak in dairy cattle by means of virus isolation and PCR assays.

Material and Methods

Clinical signs and course of infection. The first symptoms of the disease were observed after the introduction of pregnant heifers into the holding. The heifers kept under compulsory quarantine in Poland, were examined for BHV1 and were found negative in ELISA. Before their introduction, none of the animals present at the farm at that time showed any symptoms of disease although the serological status of the herd was unknown. Shortly after the arrival, one of the introduced heifers developed fever (41.5°C), dyspnoea, hyperaemia of the nasal mucosa, and serous discharge from eyes and nose. Subsequently, similar symptoms occurred in other animals in the herd.Abortions in pregnant cows and deaths of young and adult animals were also observed.

Specimens. Twenty-five samples of blood serum, 25 nasal swabs, and tissue samples collected from dead cow and from dead 2 weeks old calf were tested. The nasal swabs were dipped in Eagle’s MEM containing antibiotics, thoroughly shaked and centrifuged at 1000 g for 10 min at 4°C. Tissue samples were homogenized with the addition of MEM to obtain 10% suspension. The supernatants from nasal swabs and specimen suspensions were used for virus isolation in the cell line and for viral DNA extraction.
Serological examination. BHV1 specific antibodies in sera were detected using HerdCheck IBRgB Ab ELISA kit (Idexx). This ELISA is specific for gB glycoprotein of BHV1. The test was performed according to manufacturer’s instructions.

Virological examination. Virus isolation test was performed in MDBK cell line grown in flat bottom 24-well plates (Constar). 2-3 days old monolayer of MDBK cells was inoculated with earlier obtained supernatant in the volume of 200 µl/well. The plates were incubated for 1 h at 37ºC and then the inoculum was replaced with maintenance medium (Eagle’s MEM with addition of 2% foetal calf serum) and incubated at 37ºC in 5% CO2 atmosphere. Infected MDBK cells were examined microscopically for the presence of cytopathic effect on the daily basis for the next 5-7 days. If CPE was found, the specificity of isolated strains was confirmed by indirect immunoflorescence assay (monoclonal antibodies against BHV1 gB glycoprotein, VMRD).

DNA extraction and PCR analysis. Total DNA was extracted from 500 µl of 10% suspension of homogenized tissue. Two microlitres of proteinase K (25 mg/ml) and 25 µl of 10% SDS was added, mixed and incubated at 50ºC for 2-3 h. Subsequently, equal volume of phenol:chloroform:isoamyl alcohol mixture (25:24:1, Sigma) was added, vigorously mixed and centrifuged at 12 000 g for 10 min. The supernatant was transferred into fresh microtube, supplemented with 3 M sodium acetate solution (Sigma) in volume of 1/10 and 2.5 volumes of 96% ethanol and stored at -70ºC for 1 h. Precipitated DNA was centrifuged at 12000 g for 15 min at 4ºC. The pellet was then washed with 70% ethanol, air dried, and resuspended in 50 µl of redistilled water.

Two sets of primers (external and internal) selected from the gene encoding gD glycoprotein of BHV1 were used in PCR (24). PCR mix consisted of 5 µl of 10x DNA polymerase buffer, 2 µl of 10 mM dNTP mix, 1µl of 5 mM solution of each primer and 2.5 U of thermo-stabile RED TaqTM DNA polymerase (Sigma). The mixture was supplemented with sterile water to a final volume of 50 µl. Five microlitres of extracted DNA was used in the reaction with first pair of external primers. PCR conditions for DNA amplification with each set of primers were identical. Each cycle consisted of the following steps: denaturation at 95ºC for 1 min, annealing at 60ºC for 1 min and elongation at 72ºC for 1 min. Thirty-five cycles were performed in total and the elongation step after last cycle was prolonged to 10 min. PCR products were analysed in 2% agarose gel (Invitrogen). The electrophoresis was run at constant voltage of 100 V in TAE buffer (0.004 M Tris acetate, 0.001 M EDTA) for 1 h. The PCR result was considered positive if the product of expected size (specific for primers used) was observed.

The specificity of PCR products amplified with external primers was confirmed by restriction enzyme analysis using following endonucleases: BglII, AluI, AvaI, BbvI (New England Biolabs), DraIII (Sigma) and MnlI (Fermentas) which were chosen according to restriction map of the amplified sequence (NEBCutter version 2.0). The length of expected restriction fragments and number of sites recognized by endonucleases used is presented in Table 1. Digestion products were separated in 12.5% polyacrylamide gel (ExcelGel, Amersham Biosciences) and silver stained (PlusOne Silver Staining Kit, Pharmacia Biotech) according to manufacturer’s instructions.

Results

The presence of BHV1 specific antibodies was found in serum samples collected from 24 animals. However, one sample from 2 weeks old calf gave doubtful result in ELISA.

The results of nasal swabs testing are presented in Table 1. The isolation test in MDBK cell line revealed the presence of BHV1 only in one sample taken from 2 weeks old calf which was doubtful in ELISA performed earlier. The remaining nasal swabs were negative. Specific PCR product with external primers was found in 11 samples collected from the nasal secretions. This group comprised samples from 2 weeks old calf, 3 imported heifers and 7 native cows. Twenty samples of nasal swabs were found positive in nPCR using the second pair of primers. All positive samples in the first PCR were also confirmed in nPCR.

Table 1
Detection of BHV1 in samples by virus isolation test and PCR assays

<table>
<thead>
<tr>
<th>Sample</th>
<th>Virus isolation test</th>
<th>PCR</th>
<th>nPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal swabs</td>
<td>1/25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11/25</td>
<td>20/25</td>
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<tr>
<td>Calf 2 weeks old</td>
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<tr>
<td>- lungs</td>
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<tr>
<td>- spleen</td>
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<td>- liver</td>
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<td>Cow</td>
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<sup>a</sup> positive result/number of samples tested, (+) - positive result, (-) – negative result.
Fig. 1. PCR results of BHV1 in tissue samples with external (top) and internal (bottom) primers for gD gene. Lanes M: DNA molecular mass marker, pUC Mix 8, (Fermentas); lanes 1 to 4: lungs, spleen, kidneys and liver of 2 weeks old calf; lanes 5 to 7: lungs, kidney and liver of adult cow; lane 8: BHV1 positive control, Cooper strain (subtype BHV1.1); lane 9: BHV1 positive control, K-22 strain (subtype BHV1.2); lane 10: reagent control.

Fig. 2. Restriction enzyme analysis of PCR product (468 bp). Lanes M: DNA molecular mass marker, pUC Mix 8, (Fermentas); lane 1: undigested BHV1 PCR product; lanes 2 to 7: BHV1 PCR products digested with BglII, AvaI, AluI, BbvI, DraIII and MnlI endonucleases, respectively.
Among all internal organ specimens examined, BHV1 was isolated only from the lungs collected from 2 weeks old calf (Table 1, Fig. 1). However, in PCR the presence of BHV1 antigen was confirmed in specimens derived from the lung and liver of this calf. Nested PCR revealed that almost all specimens tested contained viral DNA.

The specificity of PCR products was confirmed by digestion with selected restriction endonucleases (Fig. 2). The number and length of restriction fragments were in accordance with the restriction map of the amplified product.

**Discussion**

The results of laboratory testing confirmed the BHV1 to be the causative agent of respiratory tract disease observed in the cattle herd. The diagnosis was made upon the presence of specific antibodies in cattle sera as well as the presence of bovine herpesvirus 1 in nasal swabs and tissue samples collected postmortem. Introduction of new animals into the herd was probably the main factor of the activation of the disease outbreak. This statement is in agreement with other papers, where authors showed direct link between the purchasing of new animals and introduction of the virus into the herd or reactivation of a latent infection (22, 23). The examination also confirmed the usefulness of PCR technique in BHV1 laboratory diagnosis. The test was found to be more sensitive in comparison to virus isolation test. In virus isolation test BHV1 was present only in nasal swabs and lungs from 2 weeks old calf, whereas in PCR (especially in nPCR) most samples gave positive results. The specificity of PCR amplified products was confirmed by restriction enzyme analysis. PCR is becoming more and more frequently used diagnostic method for routine and confirmatory diagnosis of various infectious diseases. Among many advantages PCR enables analysis of samples where neutralizing antibodies are present or the quality of material tested is poor. In our study PCR was successfully used to detect the presence of BHV1 in semen samples of bulls (18).

The heterogeneity of clinical forms caused by BHV1 infection results in serious economic losses on cattle farms. Therefore, many countries introduced BHV1 eradication programmes. In Austria, Denmark, Finland, Sweden, and Switzerland such programmes were successful and these countries are considered BHV1 free (1). The method used was based on serological screening and elimination of seropositive animals. However, it should beunderlined that those countries had very good situation at the beginning of the programme since no vaccinations against BHV1 were done and the prevalence of infection was low. The same eradication programme used in Poland allowed to eliminate the BHV1 infection from bulls in semen collection centres. However, such programme was not introduced in dairy and beef cattle. On the basis of intensive animal trade and the absence of surveillance programme for BHV1 in Poland at the moment, it can be expected that more outbreaks similar to the described one will appear.

**References**


