AMPLIFICATION OF DNA OF BHV1 ISOLATED FROM SEMEN OF NATURALLY INFECTED BULLS

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The goal of the study was to compare two different methods of DNA isolation and to determine the usefulness of PCR assay for detection of bovine herpesvirus type 1 in bull semen. Virus isolation in cell culture was used as a reference test. Total number of 44 samples of bull semen was tested. In virus isolation test the presence of BHV1 was detected in 6 (13.6%) samples. PCR assays done with both standard phenol and QIAamp kit DNA extraction methods gave positive results in 8 (18.2%) and 5 (11.4%) samples, respectively.

Key words: BHV1, semen, PCR, DNA extraction, virus isolation.

Bovine herpesvirus type 1 (BHV1), can establish a latent infection like other \( \alpha \)-herpesviruses (1, 3, 4). This kind of infection persists for the whole lifespan of an infected animal therefore such animals should be regarded as virus carriers and as the potential source of infection. Transportation, cold weather, animal overcrowding, corticosteroid therapy, superinfection with other pathogens or other stress factors can reactivate latent infection which activates a complete replication cycle of the virus. Final outcome of this reactivation is the shedding of the virus. Shedding is irregular and usually asymptomatic. Therefore, latently infected animals look healthy, although they are the potential threat for other susceptible herdmates. Latent infections are especially important in bulls, because these animals shed virus in semen. Use of BHV1 infected bulls or their semen for artificial insemination can transfer infection to the female population. To eliminate this problem only BHV1-free semen should be used. Routine method for BHV1 detection in semen is virus isolation in cell culture. But this method is time consuming. Moreover semen toxicity for cell culture requires semen to be diluted before use. This can give false negative results when virus concentration in semen is low.

The aim of the study was to compare two different methods of DNA extraction from bull semen and to assess PCR usefulness for BHV1 detection in semen in comparison with virus isolation test.
Material and Methods

Semen of naturally infected bulls. In this study 44 samples of frozen semen were used. Seventeen samples were kindly provided by Dr Rijswijk from ID-DLO, Lelystad, the Netherlands. Thirteen of these samples were collected from bulls located in artificial insemination centres (AICs), in which infectious pustular vulvovaginitis (IPV) was diagnosed on the basis of clinical examination. Remaining 4 samples came from BHV1-free bulls from AICs. Additional 27 samples came from bulls owned by private farmers. Serological testing of these animals (owned by private breeders) for antibodies against BHV1 gave positive results.

Virus isolation test. Semen samples were thawed and diluted 1:10 in MEM supplemented with the following antibiotics: penicillin - 500 units, streptomycin - 500 µg and amphotericin B (250 µg/ml) - 10 µl/ml of medium. Diluted semen was incubated at room temperature for 30 min. Virus isolation test was done in bovine kidney cell line (MDBK) in 12-well plates with flat bottom. Briefly, 100 µl of diluted semen were added to each well, where cell culture monolayer was covered with maintaining medium (MEM without serum). Infected cell culture was checked every day for cytopathic effect for up to 5-7 d.

Phenol DNA extraction. Two hundred µl of semen plasma was used for DNA extraction with 2 µl of proteinase K (25 mg/ml) and 25 µl of 10% SDS. All the components were mixed thoroughly and incubated at 37°C for 60 min. Next, equal volume of phenol:chloroform:isoamyl alcohol mixture was added, mixed together and centrifuged at 12 000 x g for 5 min. Upper phase (water phase) was transferred to a new tube. 2.5 volumes of 96% ethanol and 1/10 volume of 3 M sodium acetate pH 5.2 was added. After mixing the tubes were incubated overnight at –20°C or for 60 min at –70°C. DNA was collected after centrifugation at 12 000 x g at 4°C for 15 min. DNA pellets were washed with 70% ethanol, dried, dissolved in 50 µl of redistilled water and kept at –20°C before PCR reaction.

QIAamp kit DNA extraction. DNA extraction was performed according to the manufacturer’s instructions (Qiagen). Briefly, to 200 µl of semen plasma in the Eppendorf tubes 25 µl of proteinase K with 200 µl of AL buffer were added, mixed thoroughly and incubated for 10 min at –70°C. Next, 210 µl of 96% ethanol was added, mixed again and transferred to a QIAamp column. Centrifugation was performed at 6000 x g for 1 min. The tube was discarded and the column was washed twice with 500 µl of AW buffer. DNA elution was done with 50 µl of AE buffer warmed up to 70°C. DNA collected after centrifugation was used directly for PCR.

DNA amplification. Primers for PCR were chosen from the glycoprotein gD coding region of BHV1 genome, based on the published nucleotide sequence of Cooper strain (7,9). Amplified product should have a size of 466 base pairs (bp). Amplification was done in 0.5 ml the Eppendorf tubes. Reaction mixture consisted of 5 µl of 10x buffer for thermostable DNA Prime Zyme™ polymerase, 8 µl of 1.25 mM dNTPs mixture, 1 µl of 5 mM dilution of each primer, 5 µl of DNA and 0.5 µl (2.5 units) of DNA Prime Zyme™ polymerase. To this mixture sterile water was added up to 50 µl. Samples were preheated for 5 min at 95°C followed by PCR amplification. Thirty-five cycles included: denaturation at 95°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min. Final elongation was done for 10 min. PCR was done in Personal Cycler (Biometra) with heated lid.
Analysis of amplification products. Ten µl of PCR products were analyzed in 2% agarose gel. Electrophoresis was run at 70V for 60 min in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). φX174/Hae III DNA ladder (Gibco BRL) was used as the molecular mass marker. After electrophoresis, gel was stained in ethidium bromide solution (1 µg/ml) and photographed under UV light. A positive result meant the presence of DNA band of expected size in the gel under UV light.

Results

Forty-four semen samples from bulls were tested in total. Six semen samples (13.6%) were positive for the presence of BHV1 in virus isolation test. In case of 5 samples positive results were obtained just in the first passage (Fig. 1, samples #1-5). The sixth sample gave positive result after the second passage (Fig. 1, sample #7). In remaining 38 samples, 3 consecutive blind passages gave negative results for BHV1. Results for PCR assays with phenol extracted DNA are presented in Fig. 1. Amplification products of expected size of 466 bp were visible in 8 semen samples (18.2%). Parallel to virus isolation test, 5 positive semen samples from the first passage were also positive in PCR test (Fig. 1, lanes 1-5). Additionally, 3 samples which have been negative in virus isolation test for 3 consecutive blind passages gave PCR positive results, (Fig. 1, lanes 6, 8, 10). Semen sample positive in the second passage in virus isolation test, was negative in PCR with selected primers (Fig. 1, lane 7). PCR with semen samples from bulls free from BHV1 infection and from bulls owned by private farmers also gave negative results (Fig. 1, lanes 14-17 and lanes 18-29 respectively).

Fig. 1. Electrophoresis of amplified DNA products from bull semen with phenol DNA extraction method. Lanes: M- marker, 1-17 - DNA isolated from semen samples from Dutch bulls, 18-29 – DNA isolated from semen samples from bulls owned by private farmers.
PCR results of the same semen samples, but using QIAamp extracted DNA are presented in Fig. 2. Positive reactions were observed only in 5 (11.4%) semen samples where the presence of BHV1 was detected in the first passage in virus isolation test (Fig. 2, lanes 1-5). Semen sample positive in the second passage in virus isolation test, was negative in PCR with selected primers. The rest of semen samples also gave negative results in PCR assay.

Discussion

Results of this study show that the highest detection level (sensitivity) of BHV1 in bull semen was achieved with PCR assay, where DNA was extracted with phenol method. Use of QIAamp kit for DNA extraction did not allow to detect viral DNA in PCR assay in all semen samples. One can suspect that differences in detection levels found in assays used in this study could be correlated with relatively low virus titre in semen samples. Sample #7 is the best example of this assumption. It was positive in virus isolation test starting from the second passage. Also samples #6, 8 and 10 were positive only in PCR when DNA was extracted with phenol method. This method of DNA extraction was succesfully employed by many researchers (5, 6, 8). Yason et al. (10) tested four different methods of DNA extraction and showed that the best results were obtained with Gene Releaser™ commercial kit. Use of phenol method with addition of proteinase K for DNA extraction gave a little bit worse PCR results. On the other hand Masri et al. (2), used NP-40 detergent and obtained the best results. According to them phenol extraction was useful for viral DNA extraction from cell culture. Also virus isolation test can fail to detect BHV1 antigen in some semen samples, especially when only one passage is done in cell culture. It can give false negative results which as a consequence means that the semen used for insemination...
might be contaminated with BHV1. Therefore it is recommended to perform 2-3 blind passages in cell culture when testing for BHV1. PCR assay with phenol extracted DNA detected BHV1 in bull semen in the highest number of samples tested in comparison with both PCR where DNA was extracted with commercial kit and the virus isolation test.

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References