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THE APPLICATION OF POLYMERASE CHAIN REACTION IN DETECTION OF BOVINE HERPESVIRUS 1 IN CLINICAL SAMPLES

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The objective of this study was the application of polymerase chain reaction in detection of bovine herpesvirus 1 in clinical samples. Twenty samples of bovine nasal swabs were separately inoculated in Vero cells. Laboratory strain TN 41 of bovine herpesvirus 1 served as a control in the experiment. The cytopathic effects were recovered in the cell line previously inoculated with a sample of bovine nasal swab, as well as in cells inoculated with the laboratory strain TN 41 of the bovine herpesvirus 1 after a period of incubation of 24h, 36h and 48h. Identification of the isolated strains of the virus was done by the virus - neutralization test with specific immune sera against BHV 1, with a titre of 1:16. Comparative analysis of DNA fragments of the laboratory strain of BHV1 and isolated strain obtained by polymerase chain reaction with primers for viral gB glycoprotein and thymidine-kinase coding region, confirmed that the isolated strain of the virus belongs to bovine herpesvirus 1 (BHV 1).

Key words: bovine herpesvirus 1 (BHV 1), Vero cells, virus - neutralization test (VN-test), virus isolation, polymerase chain reaction (PCR)

INTRODUCTION

Bovine herpesvirus 1 (BHV 1) is a member of the *Varicellovirus genus*, *Alphaherpesvirinae* subfamily and *Herpesviridae* family with positive sense double – stranded DNA genome. This virus is a causative agent of infections in ruminants such as bovine rhinotracheitis (IBR), pustular vulvovaginitis (IPV) and balanoposthitis in bulls, conjuctivitis, encephalitis, abortions and fatal multi – systemic infections. Bovine herpesvirus 1 can establish latent infections in cattle and this animals becomes a constant source of infection during the periods of viral reactivation and excretion of the infected viral particules from the infected organisms. The major economic losses in the cattle industry occur due to respiratory and reproductive disease caused by the virus (Albayrak *et al*., 2007). Diagnosis of virus infection is usually done by virus isolation or detection of viral antigens by using the ELISA or immunofluorescence technique. There have been

numerous reports of attempts to use nucleic acid hybridisation techniques to detect viral DNA (Vilcek *et al*., 1994). Polymerase chain reaction (PCR) is one of the DNA manipulation techniques that has stimulated a strong interest in this area. Polymerase chain reaction is a nucleic acid – based detection technology where in a selected piece of DNA is amplified to a million fold in few hours thus making it detectable visually in ethidium bromide stained gel. This is a sensitive detection test which is rapid, economical and can be used on a wide variety of specimens such as nasal swabs, tissue, secretion and excretion products, body fluids and tissue culture materials containing viable and nonviable virus particules (Carmencita *et al*., 1995).

The objective of this study was the application of two sensitive PCR assays specific for viral glycoprotein B and thymidine kinase coding region for the identification of bovine herpesvirus 1 in clinical samples. It was shown that PCR assays represent an excellent tool for a fast and very sensitive detection of viral genomes in examined clinical specimens.

MATERIAL AND METHODS

Clinical samples of nasal swabs

Twenty samples of nasal swabs were taken from the cattle raised in different areas of Republic of Serbia and were examined on the presence of bovine herpesvirus 1. All samples were submitted to the laboratory in viral transport medium – Eagle MEM with 2% of fetal bovine sera and frozen at -20^{o}C.

Reference strain of bovine herpesvirus 1

Reference strain TN 41 (lot 971119, American Bio Research, Sevierville Tennesse, USA) of bovine herpesvirus 1 (BHV1) with a titre of $10^{8,1}$ $TCID_{50}$/0.1 mL, served as a control during the examination. The strain TN 41 of BHV1 was propagated in Madin Darby Bovine Kidney (MDBK) cells and Vero cells.

Cell culture

Isolation of bovine herpesvirus 1 from the nasal swabs, virus – neutralization test, and the preparation of the samples for polymerase chain reaction were performed on Vero cells.

Specific immune sera

Virus – neutralization test (VN test) was done by using specific immune sera against bovine herpesvirus 1 with a titre of 1:16.

Polymerase chain reaction (PCR)

Reagents for PCR assays

a) DNA extraction from the samples prepared after inoculation of reference and isolated strain of BHV1 in Vero cells were done by using QIAmp DNA mini kit (50) for 50 DNK extraction from tissue, QIAGEN Inc., Valencia, CA, USA;

b) QIAGEN Taq PCR Master Mix Kit (QIAGEN Inc., Valencia, CA, USA);

c) Water - Molecular Biology Grade H_2O (Eppendorf, Hamburg, Germany);

d) Primers selected from the sequence of the thymidine kinase and glycoprotein B gene region, Operon Biotechnologies GmbH, Cologne, Germany.

Reagents for horizontal gel electrophoresis

e) SERVA DNA Standard pBR328 Mix, lyophilized – DNA Ladder consist of 12 fragments from 154 Bp to 2176 Bp (154, 220, 234, 298, 394, 453, 517, 653, 1033, 1230, 1766 i 2176Bp.), SERVA, GmbH, Heidelberg, Germany;

f) SERVA DNA Standard pBR322 x Hae III lyophilized – DNA Ladder consist of 22 fragments from 8 Bp to 587 Bp (8,11, 18, 21, 51, 57, 64, 80, 89, 104, 123, 124, 184, 192, 213, 234, 267, 434, 458, 502, 540 i 587 Bp), SERVA, GmbH, Heidelberg, Germany;

g) Agarose (Serva Electrophoresis GmbH, Heidelberg, Germany);

h) Buffer -1x Tris-acetate-EDTA (TAE); (Serva Electrophoresis GmbH, Heidelberg, Germany;

i) 10 mg/mL ethidium bromide (Serva Electrophoresis GmbH, Heidelberg, Germany);

j) 5x loading buffer.

Isolation of bovine herpesvirus 1 from nasal swabs in Vero cells

All samples of the nasal swabs were centrifuged at 2000 rpm at 4°C for 15 min and 0.5 mL of the obtained supernatant fluids were used for virus isolation in Vero cells. After incubation of all inoculated Vero cells for 60 minutes at 37°C, 9.5 mL of Eagle – MEM with 2% fetal bovine sera was added and cultures were further incubated at 37°C for 5 days with daily microscopic examination on the presence of the cytopathic effects (CPE).

Vero cells

Vero cells were grown in minimum essential medium Eagle MEM containing 100 U of penicillin/mL, 100 μg streptomicin/mL, 25 μg of fungizone and 10% fetal bovine serum. The cell lines were maintained in 75 cm^2 flasks in minimum essential medium with the same concentration of antibiotics and the fetal bovine serum in the concentration of 2% and incubated at 37°C with a 5%CO_2 air atmosphere. The cell monolayers were individually inoculated the next day with the samples of the referent viral strain and with samples of the supernatant fluids obtained from the cattle nasal swabs.

Virus – neutralization test (VN - test)

Twofold serial dilutions of heat – inactivated specific immune serum against bovine herpesvirus 1 (BHV 1) were incubated with equal volumes of the supernatants from inoculated Vero cells with cytopathic effects (CPE) in the wells of microplates. The samples than were incubated for 1h at 37°C. Reference strain TN 41 of BHV1 served as a control in the assay. After the end of the incubation period, the aliquotes prepared from the mixture of supernatants and specific immune sera and the mixture of reference strain TN 41 of BHV 1 and immune sera, were individually inoculated in Vero cells and incubated for three days at 37°C.

Preparation of the samples with the reference and isolated strain of bovine herpesvirus 1, individually inoculated in Vero cells for the extraction of viral DNA

The samples of viral suspensions of reference and isolated strain of bovine herpesvirus 1, 0.5 mL, were individually inoculated in Vero cells and incubated for 1h at 37°C. After incubation of all inoculated Vero cells for 60 minutes at 37°C, 9.5 mL of Eagle – MEM with 2% fetal bovine sera was aded, and cultures were further incubated at 37°C and observed daily for cytopathic effect. When cytopathic effects were observed in 60% of Vero cells, the samples were centrifugated at 1500 rpm at 4°C for 15 min. Supernatants (viral suspensions) were frozen at -20°C and the sediments of infected Vero cells were used for the extraction of viral DNA.

Extraction of DNA from the infected Vero cells, inoculated with the reference and isolated strain of bovine herpesvirus 1

1. Add 20 μL QIAGEN Protease (or proteinase K) into the bottom of a 1.5 mL microcentrifuge tube.

2. Add 200 μL sample to the microcentrifuge tube. Use up to 200 μL of the sample. If the sample volume is less than 200 μL, add the appropriate volume of PBS.

3. Add 200 μL buffer AL to the sample. Mix by pulse – vortexing for 15s. Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

4. Incubate at 56°C fro 10 min.

5. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.

6. Add 200 μL ethanol (96%-100%) to the sample and mix again by pulse – vortexing for 15s. After mixing, briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.

7. Carefully apply the mixture from step 6. to the QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at 6000xg (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube, and discard the tube containing the filtrate.

8. Carefully open the QIAamp Mini Spin column and add 500 μL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000xg (8000 rpm) for 1 min. Place the QIAamp Mini Spin column in a clean 2 mL collection tube, and discard the collection tube containing the filtrate.

9. Carefully open the QIAamp Mini Spin column and add 500 μL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20000xg); 14000 rpm) for 3 min.

10. Recomended: Place the QIAamp Mini Spin column in a new 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed fro 1 min.

11. Place the QIAamp Mini Spin column in a clean 1.5 μL microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini Spin column and add 200 μL Buffer AE or distilled water. Incubate at

room temperature (15-25°C) for 1 min and then centrifuge at 6000xg (8000 rpm) for 1 min.

PCR assays

The PCR assays used primers from the sequence of thymidine kinase and glycoprotein B gene region which amplify 183 bp (thymidine kinase) and 478 bp (glycoprotein B) fragments. The nucleotide sequences of the primers follows: TK1 5' - AGA CCC CAG TTG TGA TGA ATG C - 3' and TK2 5' - ACA CGT CCA GCA CGA ACA CC - 3' and gB1 5' - TAC GAC TCG TTC GCG CTC TC - 3' and gB2 5' - GGT ACG TCT CCA AGC TGC CC - 3'. The cycling conditions consisted of a preliminary denaturation step of 95°C for 1 min, 34 cycles of denaturation at 95°C for 1 min, primer annealing at 61°C for 1min and extension at 72°C for 1 min and a final cycle of 95°C for 1 min, 61°C for 1 min and 72°C for 5 min.

Analysis and detection of amplified DNA

Typically, 10 μL of amplified product was analysed by electrophoresis using a 1% agarose gel (105 V for 60 min) with 40 mM Tris – acetate, 1 mM EDTA pH 7.5 as a running buffer. Gels were stained with ethidium bromide and the size of DNA bands calculated by comparison with two DNA ladder (SERVA DNA Standard pBR328 Mix and SERVA DNA Standard pBR322 x Hae III).

RESULTS

Virus isolation from nasal swabs

Twenty samples of bovine nasal swabs were separately inoculated in the Vero cells and daily observed for cytopathic effects (Figure 1). Laboratory strains TN 41 of bovine herpesvirus 1 served as a control. First cytopathic effects were observed after 24h in the form of the characteristic syncytium in Vero cells separately inoculated with one sample of cattle nasal swab and a laboratory strain of bovine herpesvirus 1. The cytopathic effects recovered in Vero cells inoculated with samples of a nasal swabs and the laboratory strain of bovine herpesvirus 1 were more intensive after 36h and 48h of incubation (Figure 2 and 3).

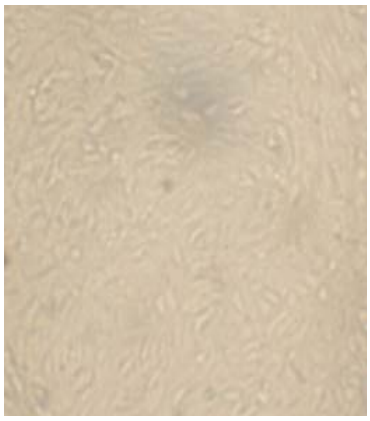

Figure 1. Non – inoculated Vero cells

Virus - neutralization test (VNT)

Identification of one isolated viral strain was done by virus–neutralization test with specific immune sera against BHV1 with a titre of 1:16. By using the abovementioned test, it was confirmed that the isolated strain belongs to bovine herpesvirus 1 (BHV 1). Reference strain TN 41 of bovine herpesvirus 1 served as a test control.

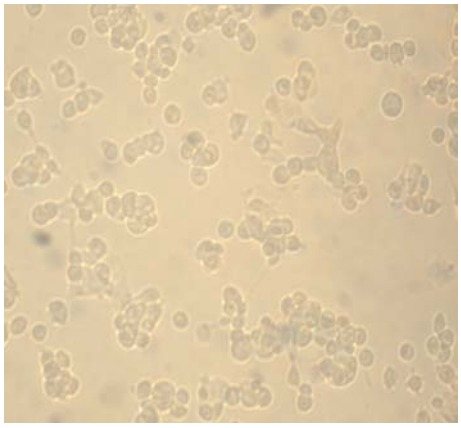

Figure 2. Cytopathic effect recovered in the Vero cells inoculated with laboratory strain TN 41 of the bovine herpesvirus 1

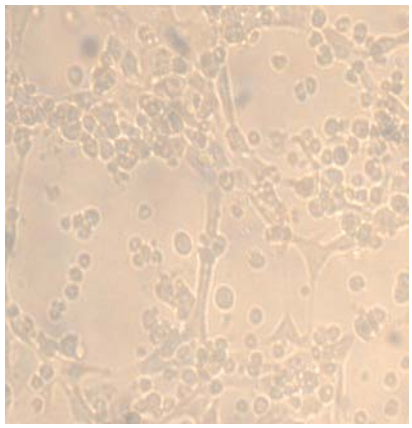

Figure 3. Cytopathic effect recovered in the Vero cells inoculated with one sample of bovine nasal swab

PCR amplification of BHV 1 thymidine kinase and glycoprotein B gene

PCR using primers for glycoprotein B gene region gave a PCR product of 478 bp (Figure 4) and for thymidine kinase gene region a PCR product of 183 bp (Figure 5). Comparative analysis of DNA fragments of the laboratory strain of BHV1 and of the isolated strain obtained by polymerase chain reaction with primers for viral gB glycoprotein gene and thymidine-kinase coding region, confirmed that the isolated strain of the virus belongs to BHV 1.

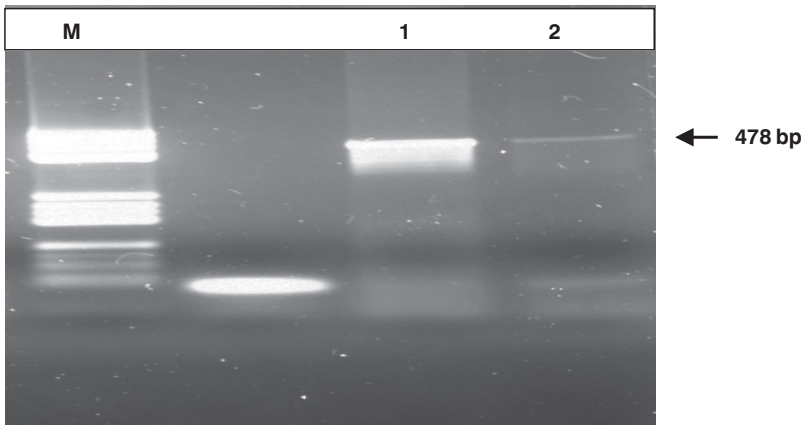

Figure 4. PCR amplification of a 478 bp DNA sequence from the glycoprotein B gene of BHV 1. lane M: DNA ladder standard; lane 1 – DNA fragment of laboratory strain TN 41 of bovine herpesvirus 1; lane 2 – DNA fragment of isolated strain of bovine herpesvirus 1

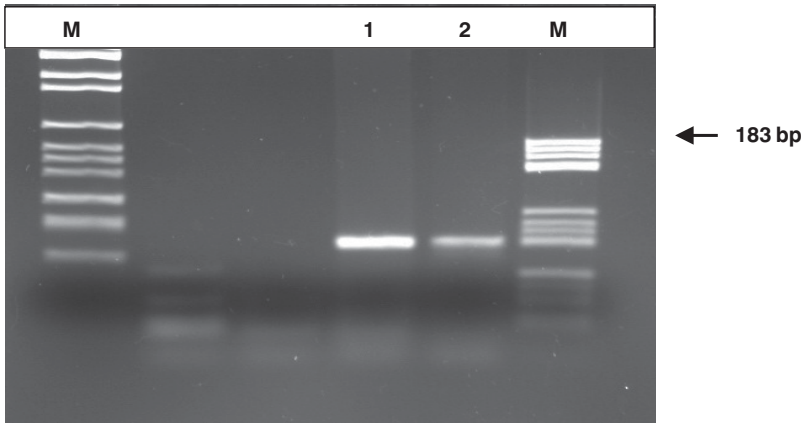

Figure 5. PCR amplification of a 183 bp DNA sequence from the thymidine kinase gene of BHV 1. lane M: DNA ladder standards; lane 1 – DNA fragment of laboratory strain TN 41 of bovine herpesvirus 1; lane 2 – DNA fragment of isolated strain of bovine herpesvirus 1

DISCUSSION

The aim of this study was the application of polymerase chain reaction in the detection of bovine herpesvirus 1 in clinical samples. Laboratory strain TN 41 of bovine herpesvirus 1 and twenty samples of cattle nasal swabs were individually inoculated in Vero cells. Inoculated Vero cells were daily observed on the presence of cytopathic effects. The characteristic syncitial cytopathic effects in Vero cells separately inoculated with one sample of nasal swab and with the sample of laboratory strain TN 41 of bovine herpesvirus 1 were recovered after 24h. The cytopathic effects in inoculated cells became more intensive after 36h and 48h from inoculation. Identification of the isolated strain was done by the virus - neutralization test with specific immune sera against BHV1 with a titre of 1:16. By using the two abovementioned methods i.e. the virus isolation and virus – neutralization test, it was confirmed that the isolated viral strain belongs to bovine herpesvirus 1. Santrude *et al*. (1996) intranasally inoculated a 3 – week old BHV 1 – seronegative calf with 2 mL of BHV 1 per nostril with an infective dose of $3.2x10^7$ $TCID_{50}$/mL. The calf was sacrificed 11 days after infection and samples were taken from the lung, trachea, spleen, kidney, brian, adrenal glands, tonsils, mediastinic, thoracic, scapular and inguinal lymph nodes. For swabbing the nasal and ocular cavity, cotton swabs were used daily up the 11th post – inoculative day. Swabs were immediately immersed in 2 mL of RPMI 1640 and frozen at -80°C until analysis. All samples were centrifuged at 3000xg at 4°C for 15 minutes and 500 μL of supernatant was used for virus isolation in MDBK cells. Bovine herpesvirus 1 was isolated from nasal and ocular swabs, as well as from lung, trachea and tonsils samples. Identification of the isolated strains of bovine herpesvirus was done by using the virus – neutralization test.

Further identification of the isolated strain of bovine herpesvirus 1 was done by using the polymerase chain reaction (PCR) assay based on two pairs of primers selected from the sequence of gB glycoprotein gene which amplify a 478 bp fragment and thymidine kinase gene which amplify a 183bp fragment. Ros *et al*. (1999) showed that the glycoprotein B (gB) and D (D) genes from five ruminant alpha-herpesviruses, bovine herpesvirus 1, bovine herpesvirus 5, caprine herpesvirus 1, cervine herpesvirus 1 and rangiferine herpesvirus 1 were partially sequenced. The nucleotide sequence aligments revealed a highly conserved gB gene with homologies ranging between 87.2 and 99.6%. The results indicate that the gB gene is a valuable target for diagnostic PCR because the highly conserved sequences allow the amplification of geographically and chronologically distant isolates. Our results showed that PCR using primers for glycoprotein B gene region gave a PCR product of 478bp. Comparative analysis of DNA fragments of laboratory strain of BHV1 and isolated strain obtain by polymerase chain reaction with primers for viral gB glycoprotein gene coding region, confirmed that the isolated strain belongs to bovine herpesvirus 1. Identification of isolated strains of bovine herpesvirus 1 was done by using polymerase chain reaction based on primers selected from the sequence of thymidine kinase gene which amplify a 183 bp fragment, also. According to previous work of Moore *et al*. (2000), we chose to target the BHV 1 thymidine kinase coding gene for PCR amplification because thymidine kinase has been shown to play a role in viral pathogenicity and part of tk gene is deleted in BHV5 thus offering the potential to discriminate between this virus and BHV 1. Our results showed that PCR using primers for thymidine kinase gene region gave a PCR product of 183bp. Comparative analysis of DNA fragments of laboratory strain of BHV1 and isolated strain obtained by polymerase chain reaction with primers for viral thymidine kinase gene coding region, confirmed that the isolated strain belongs to bovine herpesvirus 1. Carmencita *et al*. (1995) established conditions for the detection of bovine herpesvirus 1 by polymerase chain reaction using primers in the thymidine kinase region. Polymerase chain reaction (PCR) for detection of bovine herpesvirus 1 was developed and optimized using 22bp sense and 20bp antisense primers in the thymidine kinase coding region. The amplification product was 183bp. Their results compared with the traditional methods of BHV 1 detection such as virus isolation in tissue cultures and the fluorescent antibody technique showed that the PCR represents a sensitive, rapid and economical assay for the detection of live and inactivated viruses and that the BHV 1tk PCR can be potentially used for identification of BHV 1 in the diagnostic virology laboratory.

On the basis of these results, it can be concluded that the described methods, particulary polymerase chain reaction, can be succesfully used for the detection and identification of bovine herpesvirus 1 in clinical samples.

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in detection of bovine herpesvirus 1 in clinical samples

PRIMENA LANČANE REAKCIJE POLIMERAZE U DOKAZIVANJU PRISUSTVA GOVEĐEG HERPESVIRUSA 1 U KLINIČKIM UZORCIMA MATERIJALA

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SADRŽAJ

Cilj nàših istraživanja je bio primena lančane reakcije polimeraze u dokazivanju prisustva goveđeg herpesvirusa 1 u kliničkim uzorcima materijala. Dvadeset uzoraka nosnih briseva goveda pojedinačno je inokulisano u ćelijsku liniju Vero. Referentni soj TN 41 virusa BHV 1 služio je kao pozitivna kontrola u ogledu. Posle 24h, 36h i 48h od inokulacije, utvrđena je pojava citopatogenog efekta u ćelijskoj liniji inokulisanoj uzorkom poreklom od jednog nosnog brisa goveda. Iste promene su ustanovljene i posle inokulacije referentnog soja TN 41 virusa BHV 1 u ćelijsku liniju Vero. Identifikacija izolovanog soja virusa poreklom iz uzorka nosnog brisa goveda, vršena je primenom testa virus – neutralizacije uz korišćenje specifičnog imunog seruma protiv virusa BHV 1, titra od 1:16. Uporednom analizom DNK fragmenata referentnog soja virusa BHV 1 i izolovanog soja virusa, dobijenih primenom lančane reakcije polimeraze uz korišćenje dva para prajmera koji su amplifikovali gene na molekulu DNK koji kodiraju sintezu glikoproteina B spoljašnjeg omotača virusa BHV 1 i timidin – kinaze, potvrđena je pripadnost izolovanog soja virusa goveđem herpesvirusu 1.