Detection of Different Iranian Isolates of Bovine Herpes Virus Type-1 (BHV-1) Using Polymerase Chain Reaction

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Summary

Based on primers from gI glycoprotein, a polymerase chain reaction (PCR) assay was optimized for detection of bovine herpes virus type 1 (BHV-1). A 468bp DNA fragment of nine isolates was amplified. The PCR products were detected by ethidium bromide staining after gel electrophoresis and confirmed by sequencing. The nucleotide sequence alignments revealed a highly conserved region in gI gene within the isolates. Results suggest that PCR can be used as a reliable diagnostic tool for rapid detection of BHV-1 infections.

Key words: bovine herpesvirus 1, gI gene, polymerase chain reaction, Iran

Introduction

Infectious bovine rhinotracheitis (IBR) virus or bovine herpes virus type 1 (BHV-1) is a member of the family *Herpesviridae* and the subfamily α-herpesvirinae. BHV-1

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is an economically important pathogen of cattle and causes a variety of disease syndromes including respiratory tract disease and reproductive problems (Miller 1991, Donskersgoed & Babiuk 1991). It has a double–stranded DNA genome of about 137-139kbp (Wyler *et al* 1989), which consists of unique long (100kb) and short (13kb) regions. The latter is bounded by inverted (terminal and internal) repeat sequences (11kb each) (Mayfield *et al* 1983) and therefore belongs to group-D herpes virus (Roizman 1991). On the basis of clinical manifestations it is possible to differentiate BHV–1 into three subtypes: BHV-1-1, BHV-1-2 and BHV-1-3, which are associated with IBR, infectious pustular vulvovaginitis and encephalitis, respectively (Miller 1991). Detection of BHV-1 in diagnostic virology laboratories is based on virus isolation, examination of tissues by fluorescent antibody technique and serologic tests i.e., serum neutralization and ELISA on paired and single-serum samples. The polymerase chain reaction (PCR) has become an important diagnostic tool for veterinary laboratories, which allows several viral diseases of animals to be diagnosed rapidly and accurately (Vilcek *et al* 1994). The objective of this study was to use PCR for detection of different BHV-1 isolates from different geographical regions in Iran in order to better understand their epidemiology and taxonomic status and to develop an important diagnostic tool for veterinary laboratories.

Materials and Methods

Virus and cell culture. The nine local bovine herpes virus isolated from respiratory tract and abortion episodes in cattle were used. The Madin Dorby bovine kidney (MDBK) cell line was used for replication of the viruses. Cells were cultivated in 175ml DMEM medium containing 5 or 10%fetal calf serum and penicillin (120Iu/ml)-streptomycin (120µg/ml). Once the cells were 70% confluent they were inoculated with 0.5-1ml of homogenate tissue sample, dissolved in 10ml DMEM medium and incubated for 2h at 37°C. The supernatant was removed and 30-40ml DMEM medium was added. Cell cultures were monitored up to 48-72h

postinoculation until 90% CPE was observed. Virus stocks were collected and tittered in cell culture microplates. The tissue culture infective dose ($TCID_{50}$) was calculated for each sample and recorded (Table 1).

Table1. *Characteristics of nine local bovine herpes virus isolate from cattle*

Virus	Virus titer	Year of Isolation	Source	Tissue
1	$10^{7.79}$	1991	Tehran	Aborted fetus
2	$10^{7.16}$	1993	Tehran	Respiratory tract, Nose
3	$10^{7.03}$	1998	Khorasan	Ocular form, Nose
4	$10^{6.54}$	1998	Tehran	Aborted fetus
5	$10^{6.91}$	1999	Fars	Respiratory tract, Ocular form, Nose
6	$10^{7.12}$	1999	Qazvin	Ocular form, Nose
7	$10^{6.5}$	2000	Tehran	Ocular form, Nose
8	$10^{5.94}$	2000	Khorasan	Nose
9	$10^{5.83}$	2001	Qazvin	Ocular form, Nose

Total DNA extraction. Viral DNA was extracted from cell culture supernatant by a procedure, which is developed in National Research Center for Genetic Engineering and Biotechnology (NRCGEB). Briefly in a 1.5ml tube, 200µl of cell culture supernatant was added to 900µl of extracted solution. The sample was vortexed and 400µl chloroform was added. After centrifugation at 12000*g* for 15min, the aqueous phase was transferred to other tube and equal volume of cold isopropanol was added. The sample was incubated for 15min on ice and DNA was precipitated by centrifugation at 12000*g*. The pellet was washed with 70% ethanol, dried in air and resuspended in 20µl dH_2O_2. The DNA concentration and purity was measured by spectrophotometer.

Primers. Based on Vilcek (1993) study PCR primers from gI glycoprotein gene were selected to enable detection of various BHV-1 subtypes. The sequences and nucleotide positions or primers are as follows:

P1-51 5'-CACGGACCTGGTGGACAAGAAG-3' (Positions 624-645) and P2-51 5'-CTACCGTCACGTGCTGTGTACG-3' (Position 1070-1091).

Conditions of PCR. PCR was performed in a total volume of 50µl containing 5µl of DNA template (0.5-2µg), 1.5mM $MgCl_2$, 250ng of each primer. 5µl of 10X PCR reaction buffer, 0.02mM dNTPs and 0.2µl of Taq DNA polymerase. The following cycling program was performed in a thermocycler: 94°C for 3min (one cycle), 94°C/30 seconds for denaturation, 52°C/30 seconds for primer annealing and 72°C/30 seconds for amplicon extension (30 cycles) and 72°C/10 min for final extension. Optimization of PCR was carried out using positive and negative controls. Nine virus isolate were also tested in PCR.

Analysis and detection of amplified DNA. Typically, 10µl of amplified product was analyzed by electrophoresis using a 1% agars gel (100V for 45min). Gels were stained with ethidium bromide and amplified DNA was visualized by a UV transluminator.

Sequencing. The PCR products were purified using High pure PCR product purification kit (Roche, Germany) and sequenced. The nucleotide sequences were aligned using clustral method of DNASTAR software (DNA STAR Inc., Madison, Wis.).

Results

Amplification of DNA sequences. A correct size DNA fragment (468bp) was observed in positive control containing BHV-1 while no DNA amplified in MDBK cell line as negative control (Figure 1). A 468bp DNA fragment was also amplified when nine virus isolates were tested in PCR using primers selected from the BHV-1 gI gene sequences (Figure 2).

1 2 3

Figure 1. *Optimization of PCR conditions using positive and negative controls. A 468bp fragment was observed in a 1% agarose gel. Lane 1: DNA size marker (Ladder 100), lane 2: BHV-1 as positive control, lane 3: uninoculated MDBK cell culture as negative control*

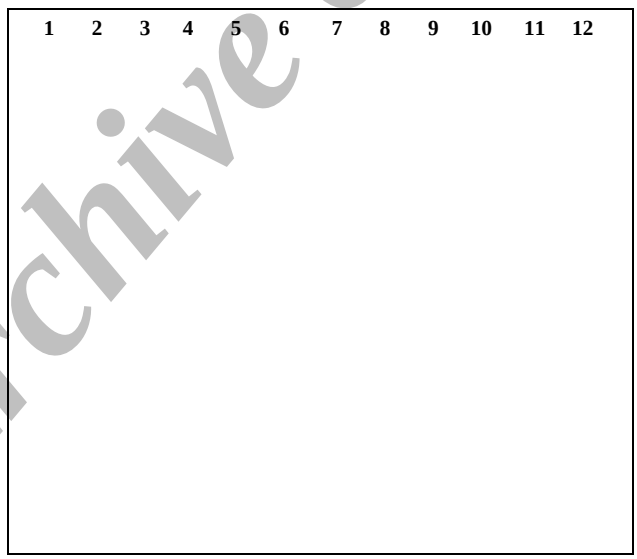

Figure 2. *Amplification of a 468bp DND fragment from BHV-1 isolates in 1% agarose gel. Lane 1 and 12: DNA size marker (ladder 100), lane 2-10: BHV-1 isolates, lane 11: uninoculated MDBK cell culture (negative control)*

Sequencing analysis. In this study a part of the gI gene from BHV-1 was sequenced and aligned with each other. Comparison of sequenced data with GenBank revealed the specificity of the results. Sequence alignments of the gI gene showed a close relationship of all the studied isolates.

Discussion

Bovine herpes virus type 1 is an important pathogen for ruminants and is classified into three subtypes according to the major clinical manifestations (Wyler *et al* 1989). The availability of reliable diagnostic test is important for successful control programs. Serological tests like ELISA is usually available for routine diagnosis. However, differences in the sensitivities of commercially available BHV-1 ELISAs can lead to various results in different laboratories and this can be become a major concern in control programs. Virus isolation is a sensitive method but requires cell culture facilities and is time consuming. The PCR represents an excellent tool for the fast and very sensitive detection of viral genomes in biological and clinical specimens. Major problems with amplification of DNA from certain herpes viruses arise, probably due to their GC rich segments. The BHV-1 genome is known to be more than 70% GC rich (Leung-tack & Riviere 1994) that increases the chances of nonspecific products (Innis *et al* 1990). In order to avoid the amplification of such nonspecific products the selection of a region with lower GC content was strategically important to successfully amplify the BHV-1 DNA fragment. The aim of the present study was to develop a specific diagnostic method for the detection and identification of BHV-1 for improvement the control of IBR and other diseases that caused by this virus. In this study nine BHV-1 viruses isolated from clinical samples were used to develop a PCR assay for rapidly detection BHV-1 viruses. The successful detection of BHV-1 isolates and specificity of the products were confirmed by sequencing PCR amplicons. Although the genome sequence of BHV-1 is available today, the related BHV have been poorly characterized so far,

hampering the development of a specific diagnostic system for this group of related viruses. In this study, comparison of the sequences, revealed a highly conserved gI gene among tested BHV-1 isolates. In addition results revealed a high identity in the analyzed region of the gI from all BHV-1. The results indicate that the gI gene is a valuable target for diagnostic PCR because the highly conserved sequences allow the amplification of geographically and chronologically distant isolates. The selected primers and PCR conditions for BHV-1 (gI gene) detection indicate that PCR has a good applicability in the diagnosis of BHV-1 infection in cattle, aborted fetuses and early identification of viral isolates in tissue culture. While virus isolation in conjunction with neutralization test require several days, the PCR can be completed within 2h. The results presented here demonstrate that PCR assay represent excellent alternative or additional tools for the detection of BHV-1 isolates.

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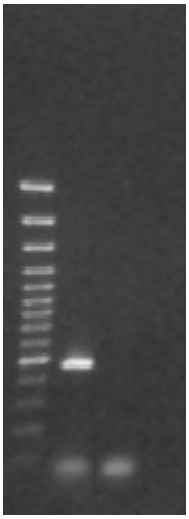

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