DETECTION OF BOVINE HERPESVIRUS 1 (BHV-1) GENOMIC SEQUENCES IN BOVINE SEMEN INOCULATED WITH BHV-1 BY POLYMERASE CHAIN REACTION

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Summary. – Polymerase chain reaction (PCR) technique was applied to detect BHV-1 in bovine semen inoculated with BHV-1. The technique was found to be 10^6 times more sensitive than a non-isotopic dot-blot hybridization method in detecting viral genomic DNA. Of the three primer pairs used, the one chosen from glycoprotein gC appeared to be most sensitive as it could detect up to 0.01 $TCID_{50}$ of BHV-1 in the semen. The technique could be useful in screening breeding bulls or samples of frozen semen prior to use in artificial insemination.

Key words: bovine herpesvirus 1; PCR sensitivity; semen

Introduction

BHV-1 is the causative agent of a variety of disease syndromes in bovines including abortion and infertility (Gibbs and Rweyemamu, 1977). The danger of BHV-1 getting transmitted during artificial insemination through semen of clinically or subclinically infected pedigreed bulls has been classically reported by Kupferschmied *et al.* (1986) and Van der Oirschot *et al.* (1993). Routine screening of semen lots or donor bulls prior to insemination by conventional diagnostic methods is laborious and virus isolation may not be even sensitive enough to detect a very low level of infection (Bielanski *et al.* 1988). A PCR technique for amplification of BHV-1 genomic sequences for diagnostic purposes has been described by Vilcek (1993). The efficacy of PCR in detection of BHV-1 in naturally or artificially infected semen has been reported for amplification of specific conserved regions of glycoprotein gC gene (van Engelenburg *et al.,* 1993), gD gene (Weidmann *et al.,* 1993) or gB gene (Santurde *et al.,* 1996). We report here a detection of BHV-1 genomic sequences in bovine semen inoculated with BHV-1 by PCR and comparative efficacy of 3 different primer pairs derived from gB or gC gene.

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Abbreviations: BHV-1 = bovine herpesvirus 1; EDTA = ethylenediamine tetraacetate; PCR = polymerase chain reaction

Materials and Methods

Cells and virus. An Indian isolate of BHV-1 (Mehrotra *et al.,* 1976; Kataria and Rai, 1992) was propagated in MDBK cells (NFATTC, India) in the presence of Eagle's Minimum Essential Medium containing 2% foetal bovine serum.

Bovine semen was obtained from the Germ-Plasm Centre of this Institute in the form of a mixture with Tris buffer, glycerol and egg yolk, and was kept frozen.

Purification of virus and extraction of DNA. MDBK cell monolayers grown in 75 cm^2 flasks were infected with BHV-1 at a low multiplicity. The virus was harvested at 72 hrs post infection, frozen and thawed two times and clarified by a low speed centrifugation. The virus in the supernatant was pelleted by ultracentrifugation at 121,000 x g for 2 hrs in a Sorvall A641 rotor. The virus pellet was resuspended in TNE buffer (50 mmol/l Tris.HCl pH 7.6, 1 mmol/l ethylenediamine tetraacetate (EDTA) and 100 mmol/l NaCl) before purification by passing through a 30% sucrose cushion at 161,000 x g for 90 mins in a Sorvall TFT45.6 rotor. Viral DNA was extracted and purified by a standard method (Kataria

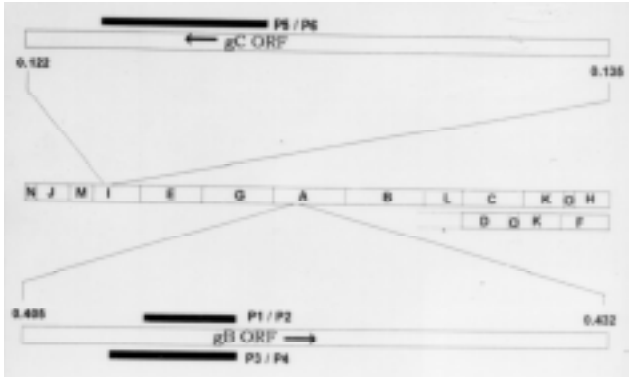

Fig. 1
Location of primers on *Hind*III restriction map of BHV-1 DNA
The Map was adapted from Mayfield *et al.* (1983). Solid bars show the anticipated PCR products on gB or gC genes.

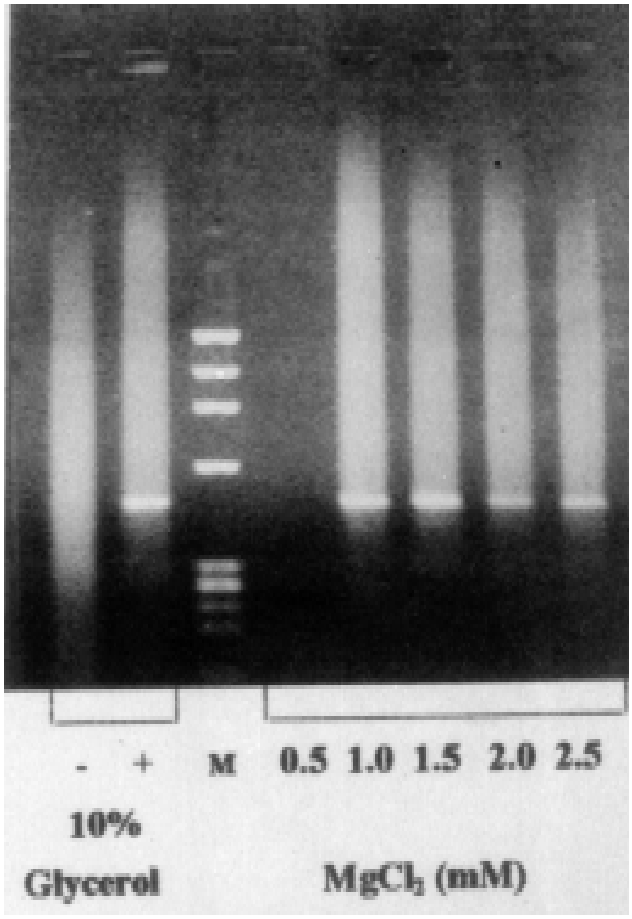

Fig. 2
Effect of 10% glycerol and various concentrations of $MgCl_2$ on PCR amplification of 468 bp product on gB gene of BHV-1
Glycerol was either added or not to the PCR reaction mixture to give a final concentration of 10%. Similarly, different concentrations of $MgCl_2$ were adjusted in the reaction mixture before PCR. Molecular size markers (lane M).

and Rai, 1992). This DNA was used for standardization of PCR, restriction analysis and confirmation of PCR products by Southern blot analysis.

Primers. Three different primer pairs were used for detection of BHV-1 genomic sequences, the details of which are given in Table 1 and Fig. 1. Of the three, pair No. 1 is that reported by Vilcek (1993).

Standardization of PCR technique was done using 50 pmoles of each primer of pair No. 1 (Table 1) in a 50 µl reaction mixture containing 0.2 mol/l dNTPs, glycerol, Taq polymerase buffer and approximately 25 ng of purified viral DNA. The reaction mixture was denatured by boiling in water bath for 5 mins and chilling in ice-cold water. Then, 3 U of Taq DNA polymerase was added and the mixture was overlaid with 35 µl of mineral oil. The reaction was carried out in a thermocycler for 35 cycles (95°C for 1 min, 56°C for 1 min and 72°C for 1 min). The final extension was carried out at 72°C for 5 mins. The cycles were the same also for primer pairs No. 2 and 3 except that the annealing temperatures were 60°C and 62°C, respectively.

Analysis of PCR products. PCR products were characterized by their size following electrophoresis in 1% agarose gel and by a Southern blot analysis by the technique of Bandyopadhyay *et al.* (1990). Digoxigenin-labelled hybridization probes were prepered with DIG-labelling and Detection Kit (Boehringer-Mannheim) following the manufacturer's instructions.

Extraction of total DNA from semen. To infect the semen artificially with BHV-1, 10 µl of each ten-fold dilution (10^{-1} – 10^{-8}) of BHV-1 was mixed with 90 µl of the bovine semen. A negative control contained 10 µl of TE buffer (10 mmol/l Tris.HCl and 1 mmol/l EDTA, pH 7.4) instead of virus dilution. Total DNA was extracted from samples by adding two volumes of lysis buffer (NaCl 0.15 mol/l, 0.75% sodium dodecyl sulfate (SDS), 1.5 mg/ml proteinase K, 10 µg/ml sheared salmon sperm DNA) and incubating at 60°C for 1 hr. The lysed material was treated with an equal volume of 6 mol/l sodium iodide at room temperature for 5 mins. After phenol: chloroform extraction, the DNA was precipitated by adding 0.6 volume of isopropanol. The DNA pellet was dissolved in 100 µl of TE buffer and re-extracted with 10 volumes of *n*-butanol before dissolving it finally in 50 µl of TE buffer. PCR was carried out as described earlier using 5 µl of this DNA template.

Results

Standardization and sensitivity of the PCR for detection of BHV-1 genomic DNA

The optimal parameters for PCR amplification were determined by using the primer pair No. 1 with BHV-1 genomic DNA, extracted from purified virions. Addition of glycerol (10% final concentration) was an absolute necessity and the optimal $MgCl_2$ concentration for obtaining a distinct 468 bp product was between 1.0 mmol/l and 2.5 mmol/l (Fig. 2). These parameters were considered optimal also for primer pairs No. 2 and 3. To confirm the amplicon, BHV-1

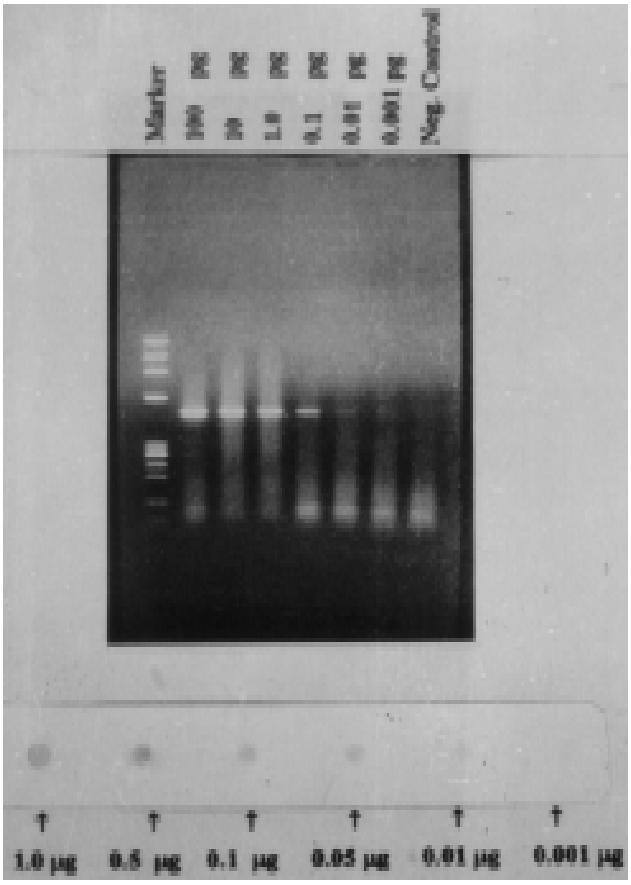

Fig. 3
Comparative sensitivity of PCR and dot-blot hybridization for detection of BHV-1 genomic DNA
Various amounts of target DNA extracted from purified BHV-1 virions were subjected to PCR amplification using primer pair No. 1 and to dot-blot hybridization. PCR products were analyzed by 1% agarose gel electrophoresis followed by ethidium bromide staining. The blot was hybridized with whole BHV-1 genomic probe labelled with digoxigenin.

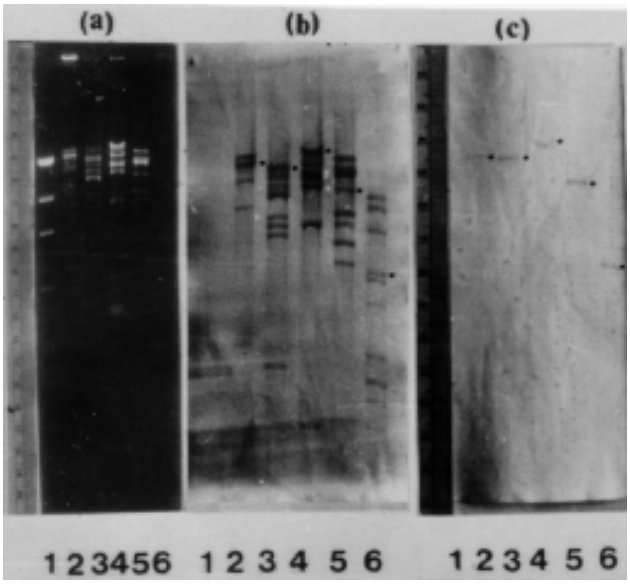

Fig. 4
Southern blot analysis of PCR product
(a) Agarose gel showing the restriction pattern of BHV-1 DNA before Southern transfer. Lane 1: molecular size markers, lane 2: *Hpa*I, lane 3: *Hind*III, lane 4: *Eco*RI, lane 5: *Bgl*II, lane 6: *Hinc*II.
(b) Hybridization of the blot with whole BHV-1 genomic probe labelled with digoxigenin.
(c) Hybridization of the same blot (after de-probing) with a probe prepared from the PCR product and labelled with digoxigenin. Note that the PCR probe hybridized only with restriction fragments containing the gB gene of BHV-1, e.g. *Hind*III A, *Eco*RI A and *Hpa*I C (marked by dots).

genomic DNA was digested with restriction endonucleases *Hind*III, *Eco*RI, *Hpa*I, *Bgl*II, and *Hinc*II. The restriction fragments were electrophoresed (Fig. 3a) and blotted onto a membrane. The membrane containing all the fragments was hybridized to a DIG-labelled whole BHV-1 genomic probe. Hybridization signals were obtained with all restriction fragments used (Fig. 3b). The same membrane was then deprobed and hybridized once again with a DIG-labelled probe prepared from the PCR amplified product. This time it hybridized only to restriction fragments containing the gB gene of BHV-1 (Fig. 3c).

The sensitivity of this PCR technique was determined by using different concentrations of target BHV-1 DNA in the PCR amplification reaction. Fig. 4 shows that up to 0.01 pg of genomic DNA could be effectively amplified, the product of which could be visualized following agarose gel electrophoresis and ethidium bromide staining. A non-isotopic DIG-labelled whole BHV-1 genomic probe, on the other hand, could detect only up to 0.01 µg of genomic BHV-1 DNA by dot-blot hybridization (Fig. 4).

Detection of BHV-1 in the semen

Freshly thawed bovine semen was inoculated with different doses of BHV-1. Total DNA was extracted from each sample and subjected to PCR amplification using the three different primer pairs (Table 1). Primer pair No. 1 (P1/P2) could amplify a specific product (468 bp) from semen containing at least 1.0 $TCID_{50}$ of BHV-1 (Fig. 5a). Primer pair No. 2 (P3/P4) derived from the same gB gene amplified the product of expected size, i.e., 637 bp, when 1.0 $TCID_{50}$ or more virus was present in the semen (Fig. 5b). However, primer pair No. 3 (P5/P6) derived from the gC gene could amplify the anticipated 520 bp product from at least 0.01 $TCID_{50}$ of BHV-1 in the semen.

Table 1. Different primer pairs used for amplification of BHV-1 genomic sequences

Primer pair No.	Primer designation	Sequence its location (nt)	Target gene and size (bp)	Expected product
1	P1	5'-CACGGACCTGGTGGACAAGAAG-3'	gB/624-645	
	P2	5'-CTACCGTCACGTGCTGTGTAC-3'	gB/1070-1091	468
2	P3	5'-GCATCGGCGTCATTTACAAG-3'	gB/470-489	
	P4	5'-GCACCCAGTCCCAGGCTACC-3'	gB/1087-1106	637
3	P5	5'-ACTGGTTCCGCAACGGCTAC-3'	gC/1070-1089	
	P6	5'-AGGACGGGGCTTCCGATTAG-3'	gC/1570-1589	520

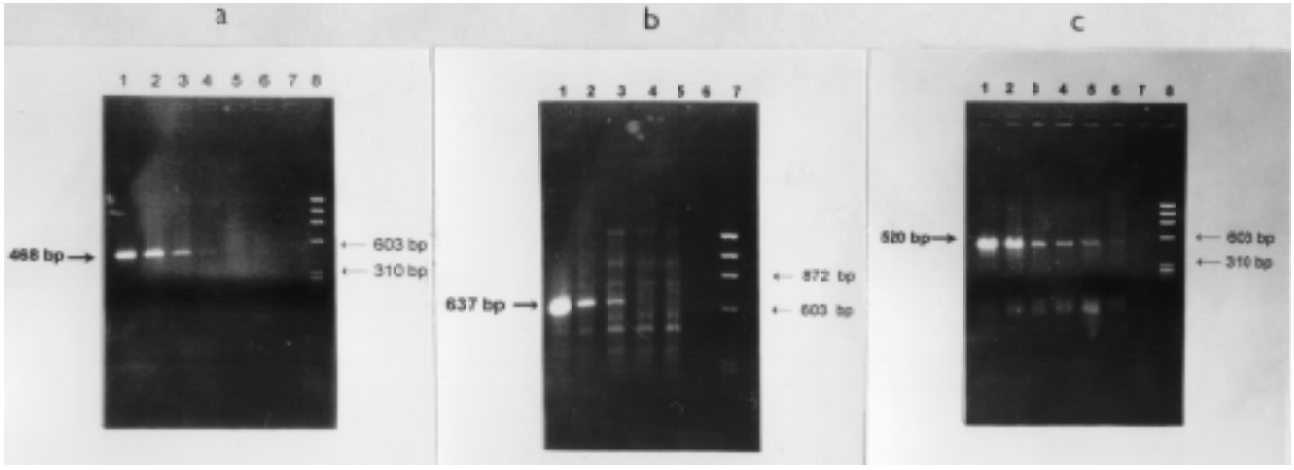

Fig. 5
Detection of BHV-1 genomic sequences in bovine semen by PCR
Bovine semen was inoculated with different doses of BHV-1. Total DNA was extracted and subjected to PCR amplification with three different primer pairs No. 1 (a), No. 2 (b) and No. 3 (c). Doses of BHV-1 (in $TCID_{50}$): 1000 (lane 1), 100 (lane 2), 10 (lane 3), 1.0 (lane 4), 0.1 (lane 5), 0.01 (lane 6). Uninoculated bovine semen (negative control) is in lane 7 in Figs. 5a and 5c and in lane 6 in Fig. 5b. Molecular size markers (the last lanes).

Discussion

Our results clearly show that PCR is an invaluable tool for detection of BHV-1, particularly when present in such biological fluids like semen, which otherwise poses a problem for conventional diagnostic techniques. Ever since Vilcek (1993) employed this technique for amplification of genomic DNA from purified BHV-1 virions, its utility in diagnosis of BHV-1 infection in general and for screening bovine semen for the presence of the virus has been demonstrated (Weidmann *et al.,* 1993; van Engelenburg *et al.,* 1993; Santurde *et al.,* 1996). The amplified regions chosen by all these authors were gB, gC or gD glycoprotein genes. We have compared efficacy of three different primer pairs in detection of BHV-1 in bovine semen inoculated with this virus. The use of one of these pairs (No. 1) with the sequence from gB gene was already reported by Vilcek (1993). The other two pairs were from gB or gC gene.

Optimal conditions for PCR including concentrations of glycerol and $MgCl_2$ were determined using the primer pair No. 1. This primer pair detected up to 0.01 pg of genomic DNA from purified BHV-1 as determined by visual observation of ethidium bromide-stained agarose gel. This sensitivity is ten-fold less than that reported by Santurade *et al.* (1996). However, compared to a non-isotopic dot-blot hybridization technique for detection of genomic BHV-1 DNA, this is approximately 10^6 times more sensitive. Although we have not used any internal oligonucleotides as a probe, the authenticity of the amplicon with primer pair No. 1 was established by hybridizing the labelled amplicon to a specific restriction fragment of BHV-1 genomic DNA. Similarly, the PCR products amplified with primer pairs No. 2 and No. 3 were confirmed by digestion with specific restriction enzymes (data not shown).

Among the three primer pairs used for the detection of BHV-1 in the semen, best results were obtained with primer

pair No. 3 amplifying a portion of gC gene. This primer pair detected up to 0.01 $TCID_{50}$ of BHV-1 in the semen compared to 1.0 $TCID_{50}$ with the other two primer pairs. This sensitivity of BHV-1 assay with primer pair No. 3 is higher than those with any other primer pairs reported till now. Both the forward and reverse primers for gC gene were chosen from regions less conserved but flanking a highly conserved region among alphaherpesviruses (Fitzpatrick *et al.,* 1989). On the other hand, both the primer pairs for gB gene were by and large from the conserved region only (Whitbeck *et al.,* 1988).

No special purification steps were taken for extraction of semen DNA except for the addition of sodium iodide and butanol extraction. Unlike the results reported by van Engelenburg *et al.* (1993) and Santurde *et al.* (1996), omission of such steps did not apparently affect our results. It is possible that the sensitivity of the test is not totally dependent on the removal of specific inhibitors from semen but on the primers chosen for amplification.

Although our experiments were limited to artificially "infected" semen only, their results reaffirm that PCR could be an effective tool for screening breeding bulls or semen banks for possible transmission of BHV-1 through artificial insemination, particularly in developing countries where intensive cross-breeding projects have been taken up to improve animal productivity. A latently infected pedigreed bull need not be condemned for the fear of shedding BHV-1 occasionally, as we now have a very sensitive method for detection of the virus in suspected semen samples.

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