ABSTRACT

A polymerase chain reaction (PCR) has been developed for rapid and sensitive detection of bovine herpesvirus-1 (BoHV-1) in semen samples using primers targeting gene coding for glycoprotein I. The PCR reaction was optimized with DNA extracted from BoHV-1 infected cell culture supernatant to amplify a specific 468 bp amplicon. To improve the efficiency of PCR, different concentrations of DMSO, DTT and glycerol were used. The best result was obtained using either 4% DMSO or 2% DTT or 5% glycerol for specific amplification of BoHV-1 DNA. The PCR result was further substantiated by digesting the amplicons with Dde I restriction enzyme. By using the same method, 406 semen samples from 2008-1010 were tested and only 14 were found positive.

Keywords: BoHV-1, DMSO, DTT, glycerol, polymerase chain reaction

INTRODUCTION

Bovine herpesvirus-1 (BoHV-1) is considered to be the most common viral pathogen found in bovine semen. BoHV-1 is responsible for a variety of clinical conditions in cattle and buffaloes, viz., infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), infectious pustular balanoposthitis (IPB), mastitis, abortion, infertility, encephalitis and fatal disease in newborn calves, and thus causes great economic losses to the livestock industry (Nandi et al., 2009). The virus is primarily transmitted via the respiratory route. However, the virus can also be transmitted venerally and through semen (Van Oirschot et al., 1993). Bulls may shed the virus in semen during both clinical and subclinical infections (Gibbs and Rweymamu, 1977). The virus also induces latency in cattle and buffaloes and the trigeminal ganglion and the sacral ganglion harbour the virus in the respiratory form and the genital form of the disease respectively (Van Oirschot, 1995). Shedding of virus reoccurs during periodic reactivation of virus generally thought to be induced by natural or artificial stress (Gibbs and Rweymamu, 1977). So it is necessary to screen semen samples for BoHV-1 infection before being used for breeding purposes to prevent spread of virus through natural or artificial insemination. The polymerase chain reaction has been shown to be sensitive and is one of the commonly used methods for detection of BoHV-1 in tissue samples as well as in semen samples (Deka et al., 2005; Nandi et al., 2008).
The G+C content of the BoHV-1 genome ranges from 71-72%. It is difficult to amplify sequences having high G+C contents because of the low efficiency of template dissociation due to alteration in the melting point of the DNA template (Ros and Belak, 1999). To overcome this problem the addition of substances like glycerol, dithiothreitol (DDT), dimethylsulfoxide (DMSO) etc. have been recommended to enhance the specificity and/or the yield of the PCR by facilitating DNA denaturation and proper primer annealing (Ros and Belak, 1999; Gupta et al., 2006). The present study was undertaken in order to evaluate the effect of glycerol, DDT and DMSO on PCR results for proper diagnosis of BoHV-1 infection.

MATERIALS AND METHODS

Virus: The cell culture adapted BoHV-1 maintained in the virus laboratory of the CADRAD, IVRI, Izatnagar, U.P. was used to infect the MDBK cell line grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 2 mM L-glutamine, 1% tryptose phosphate broth and 10% heat inactivated fetal calf serum (Gibco).

Genomic DNA of BoHV-1: Semen from a healthy bull spiked with 50 μl of MDBK cells propagated BoHV-1 having a titre of $10^{5.5}$ TCID50/ml was used as known positive control. Semen samples were taken in 1.5 ml eppendorf tubes, centrifuged at 5,000 rpm for 5 minutes and 200 μl of supernatant was collected from each sample. Proteinase K and SDS were added to a final concentration of 250 μg/ml and 1%, respectively and incubated for 1 h at 56°C in a water bath. Finally, DNA extraction was done by the phenol-chloroform method and dissolved in nuclease free water and stored at -20°C until used.

PCR assay: The PCR was performed in 200 μl thin layered PCR tubes (Axygen) with a reaction volume of 50 μl. The forward (5' - CAC GGA CCT GGT GGA CAA GAA G - 3') and reverse (5' - CTA CCG TCA CGT GAG T GG TAC G - 3') primers (Imperial Biomedics) were used to amplify the part of the gene coding for gI glycoprotein of BoHV-1 to yield a product of 468 bp (Rocha et al., 1998). The reaction mixture contained 200 μM dNTPs, 10 pmol of each primer, 5 μl of 10X PCR buffer, 3 μl of 15 mM MgCl2, 5 μl of known BoHV-1 Genomic DNA, 1 μl of Taq DNA polymerase (1 μl/U) and remaining nuclease free water. Further, the effect of different chemicals, viz., dimethyl-sulphoxide (DMSO), glycerol and dithiothreitol (DTT), which are helpful in decreasing the annealing temperature and secondary structure in the genomic DNA of BoHV-1, were studied. The PCR was carried out using different concentrations of the above chemicals, namely, 2%, 4% and 6% DTT (0.2 M) and 5%, 10% and 15% glycerol and 1%, 2% and 4% for DMSO.

Amplification was performed in a thermocycler (Applied Biosystems). The cyclic condition included initial denaturation at 95°C for 5 minutes followed by 30 cycles of 95°C for 1 minute, 56°C for 1 minute and 72°C for 1 minute. The final extension was given at 72°C for 10 minutes. After PCR, the amplified products were analyzed on 1.0% agarose gel containing ethidium bromide to a final concentration of 0.5 μg/ml. Then, 10 μl of amplified product was mixed with 2 μl of bromophenol (6X) dye and loaded into the well and run along with 100 bp DNA ladder in 1X TAE electrophoresis buffer at 5 volts/cm² and the progress of mobility was monitored by migration of dye. At the end of the electrophoresis, the gel was visualized under a UV transilluminator (Nandi et al., 2008).
Restriction endonuclease (RE) analysis: The PCR products were purified from gel using QIAquick gel extraction kit (Qiagen Inc. Valencia, USA) as per the manufacturer’s protocol. To 4 μl of PCR product, 1 μl of restriction enzyme Dde I (10 U/μl), 2 μl of 10X RE buffer and 13 μl nuclease free water were added followed by incubation at 37ºC for 4 h. The enzyme activity was stopped by freezing the content at -20ºC. The RE digest thus obtained was electrophoresed in 1% agarose gel at 80 volts for 1 h and visualized under U.V. transilluminator/Gel documentation system.

Testing of semen samples: By using the same method as described above, 406 semen samples were tested. The details of samples are given in the Table 1.

RESULTS AND DISCUSSION

In this study, the primer pair used to amplify part of the gI glycoprotein had average G+C content for the BoHV-1 genome, considering that a high GC content promotes secondary structures which might affect both primer annealing and extension. DMSO and DTT play a role in the loosening up of complex secondary structures and have been found to improve the efficiency of amplification reaction. The optimum concentration of DMSO for amplification was found at 4% level and the results are in accordance with Ros and Belak, (1999) (Figure 1). The most appropriate level of DTT for PCR amplification was found to be at 2% level (Figure 1). Glycerol acts as a cooling agent and may have an effect on decreasing the effective annealing temperature, and it also decreases the tendency of secondary structure formation. The glycerol concentrations tested ranged from 5 to 15% and substantial amplifications were observed both at 5% as well as 10% (Figure 1). The results are in accordance with the findings of Van Engelenburg et al. (1993) and Gupta et al. (2006) who reported 5% and 10% glycerol, respectively. In the control reaction that lacked any of the three, there was little amplification of DNA template. From the above results, it can be inferred that the most efficient amplification was observed using DMSO for PCR based detection of BoHV-1 genomic DNA. In the

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Origin of samples</th>
<th>Number of Samples</th>
<th>PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Frozen Semen Bank, Bassi, Jaipur</td>
<td>74</td>
<td>- 7 4</td>
</tr>
<tr>
<td>2</td>
<td>Project Directorate on Cattle, Meerut</td>
<td>188</td>
<td>1 187</td>
</tr>
<tr>
<td>3</td>
<td>Cross Breeding Project, Dalpatpur, Moradabad, U.P.</td>
<td>41</td>
<td>12 29</td>
</tr>
<tr>
<td>4</td>
<td>Regional Disease Diagnostic Lab., Jalandhar</td>
<td>101</td>
<td>1 100</td>
</tr>
<tr>
<td>5</td>
<td>Deep Frozen Semen Production Centre, Pashulok, Rishikesh</td>
<td>2</td>
<td>- 2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>406</td>
<td>14 (3.44%) 392</td>
</tr>
</tbody>
</table>

Table 1. Details of the semen samples tested by PCR.
Figure 1. Agarose gel showing the amplification of genomic DNA and effect of different concentration of DTT, DMSO and Glycerol.

Group 1: Effect of different concentration of DTT.
Lane M: 100 bp ladder.
Lane 1: PCR amplification at concentration of DTT 2%.
Lane 2: PCR amplification at concentration of DTT 4%.
Lane 3: PCR amplification at concentration of DTT 6%.

Group 2: Effect of different concentration of DMSO.
Lane 1: PCR amplification at concentration of DMSO 1%.
Lane 2: PCR amplification at concentration of DMSO 2%.
Lane 3: PCR amplification at concentration of DMSO 4%.

Group 3: Effect of different concentration of Glycerol.
Lane M: 100 bp ladder.
Lane 1: PCR amplification at concentration of Glycerol 5%.
Lane 2: PCR amplification at concentration of Glycerol 10%.
Lane 3: PCR amplification at concentration of Glycerol 15%.
Lane 4: Negative control.
Figure 2. Agarose gel showing the RE digestion of 468 bp PCR product with Dde I enzyme.
Lane M: 100 bp ladder.
Lane 1: RE product of 340 bp and 128 bp.

semen samples tested for presence of BoHV-1, only 14 (3.44%) samples were found positive. In the RE analysis of PCR products by restriction enzyme, Dde I, it was found that specific cleavage of the PCR yielded fragments of desirable sizes of 340 bp and 128 bp indicating the specificity of the PCR product (Figure 2). The results were in accordance with the results reported by Rocha et al. (1998).

BoHV-1 is one of the most common viral pathogen secreted in the semen of bovines. Its transmission through artificial insemination may cause serious complications in the inseminated cows. Molecular techniques such as PCR targeting TK, gB, gC, gD and gE genes have been widely used for the detection of the genomic DNA of BoHV-1 in various body fluids (Rocha et al., 1998; Ros and Belak, 1999; Deka et al., 2005; Gupta et al., 2006; Nandi et al., 2008). PCR is very rapid, and sensitive and can provide results within 6 h compared to virus isolation and virus neutralization test, where more than 3 days are needed. PCR has more advantages in comparison to virus isolation because of being 5 times more sensitive and can detect DNA in inactivated virus particles and in latently infected sensory ganglia (Van Engelenburg et al., 1993). But, due to the high G+C content of BoHV-1 genome, it is difficult to amplify because of alteration in melting point of DNA template and secondary structure formation (Ros and Belak, 1999).

Further, the disadvantage of the PCR technique is that it is prone to carry over contamination yielding false-positive results. So, the PCR product was characterized by RE analysis using Dde I enzyme to confirm the specificity of PCR amplification. It was found that specific cleavage of the PCR products by the restriction enzyme, Dde I, yielded fragments of desirable sizes of 340 bp and 128 bp. So, various substances should be used to improve the efficiency of PCR for sensitive and rapid detection of BoHV-1 genomic DNA, which, in turn, helps in proper utilization of healthy semen in artificial insemination purposes and rejection of BoHV-1 positive semen samples. Also, the PCR amplification followed by RE analysis would be extremely helpful in obtaining the accurate and unambiguous result about the status of BoHV-1 in semen samples.
REFERENCES


