CLONING AND SEQUENCING OF GC GENE OF BOVINE HERPESVIRUS 1 (BHV1) FROM BUFFALO BULL SEMEN

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ABSTRACT

The present study was aimed to characterize the local field bovine herpesvirus 1 (BHV1) by cloning and sequencing of the glycoprotein C (gC) gene segment of BHV1, amplified by the polymerase chain reaction (PCR). The PCR product of 173 bp of a representative positive semen sample was cloned using the pTZ57R/T vector supplied with the InsT/Aclone™ PCR product cloning kit. The cloned white colonies were confirmed by M13 PCR, which yielded an expected amplified product of 327 bp. The cloned PCR product of the field BHV1 gC gene was sequenced and then analysed by *in silico* analysis. The consensus sequence of 168 bp obtained was compared with the six published GenBank sequences, and it was found that the field BHV1 from Gujarat showed a range of homology (89% to 92%) with maximum identity (92%) with the BHV1 of three European and one North American isolates. The sequence obtained showed deletions at two positions i.e. 40 and 70 nucleotides and point mutations at seven different positions (2, 61, 116, 134, 143, 151 and 155) as compared to the six published sequences. Such alterations in the sequence indicated frameshift mutations in the gC gene region, with the corresponding alteration in amino acid (aa) sequence.

Keywords: buffalo bull, *Bubalus bubalis*, semen, cloning, bovine herpesvirus 1, BHV1

INTRODUCTION

Bovine herpesvirus 1 (BHV1) is associated with a variety of clinical diseases, including infectious bovine rhinotracheitis, conjunctivitis, reproductive disorders, encephalitis, and generalized systemic infections. BHV1 is a member of the genus *Varicellovirus* in the sub-family *Alphaherpesvirinae*, which belongs to the family *Herpesviridae*. It is an enveloped virus having an icosahedral nucleocapsid consisting of 162 capsomeres. The BHV1 genome is a single linear double stranded DNA having a total size of 135-140 kilobase pairs (kbp) (Mayfield *et al.*, 1983).

BHV1 can establish a latent infection like other alphaherpesviruses (Gibbs and Rweyemamu, 1977) in the nerve ganglia of infected but clinically normal animals after the primary infection despite the development of neutralizing antibody (Pastoret *et al.*, 1979). This kind of infection persists for the whole lifespan of an infected animal and therefore, such animals should be regarded as virus carriers and as a potential source of infection. Latent

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infections are especially important in bulls, because these animals shed the virus in semen during both clinical and sub-clinical infections. Use of BHV1 infected bulls or their semen for AI can transfer the infection to the female population.

Schroeder and Moys recognized the IBR disease in the United States during the 1950s for the first time. In India, the first case of BHV1 infection was reported by Mehrotra *et al.* (1976) from cases of keratoconjunctivitis amongst crossbred calves at an organized cattle herd in Uttar Pradesh. Various workers have characterized BHV1 by sequencing the targeted genes. Sequencing provides an authentic tool to genetically characterize the target genes and the virus, thus providing valuable information on comparative variation among the viruses. The various glycoproteins present on the envelope of BHV1 play important role in the viral pathogenesis and the immunity. Some of these like gB and gD, are essential for viral replication while others are non-essential. BHV-1 codes for four non essential genes, viz. gC, gG, gI and gE (Kamiyoshi *et al*., 2008). Glycoprotein C (gC) has been found important for the attachment of the virus (Liang *et al.,* 1991) and inducing neutralizing antibody response (Denis *et al.,* 1994) in the host. It has been targeted earlier for various studies (Fitzpatrick *et al.,* 1989; Brower *et al*., 2008; Anita *et al*. 2010; Lojkic *et al*., 2011). In the present study, the target gC gene was cloned and sequenced to characterize the Indian isolate of BHV1 from semen samples of breeding bulls.

MATERIALS AND METHODS

Cloning of gC gene in pTZ57R vector

The amplified PCR product (173 bp), using gC gene based primers described previously by Engelenburg *et al*. (1993), from a representative buffalo bull semen samples (of the 20 samples found positive by PCR out of the 52 buffalo semen samples processed) of an AI centre was purified using a Perfectprep® PCR cleanup kit (Eppendorf, Germany). The bulls kept on the AI centre had history of poor semen quality, and the females of the surrounding population had problem of repeat breeding and infertility. Purified PCR product was ligated in pTZ57R vector using an InsT/Aclone™ cloning kit (MBI Fermentas) and the vector containing the insert was propagated in *E.coli* hosts (DH5-α). The vector carries an ampicillin resistance gene (*bla*) as selectable marker for transformed cells. Thirty-five µl of 2% X-gal solution and eight µl of 20% IPTG solution were pipetted on to a pre-made 90-mm LB agar plates containing Ampicillin (50 µg/ml). The solutions were spread with the help of a sterile bent glass rod. The plates were incubated at 37°C until all the fluid disappeared. Each of the individual colonies obtained following the transformation procedure were picked up and streaked in a regular pattern on the X-gal/IPTG plate. The plates were incubated at 37°C for 12-15 h. Growth on those sectors showing white colonies was selected for further screening and confirmation of recombinant clones carrying the correct insert was done by M13 PCR using M13 primers. The amplified PCR products were checked on 2% agarose gel, and clones which showed the amplification of 327 bp DNA fragment were considered as positive clones carrying the desired insert of the gC gene. The recombinants obtained were selected and grown in large quantity for plasmid extraction using a Fastplasmid® Mini Kit (Eppendorf, Germany).

Sequencing of gC Gene Segment

The concentration of the purified gC gene

PCR product using plasmid DNA was determined and was subjected to automated DNA sequencing on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA). Sequencing was carried out using a BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) following the manufacturer's instructions. The sequences obtained in forward and reverse orientations were assembled by using BioEdit software. The consensus sequence of the gC gene obtained was aligned with known sequences of BHV1 available in GenBank using NCBI BLAST and CLUSTAL W (1.82) software. The PCR product used for sequencing was obtained directly from the field virus (named as Raj/ibr/Guj) without isolating the virus in cell culture.

RESULTS AND DISCUSSION

Cloning of gC gene of BHV1

Cloning of the targeted gene has been preferred prior to sequencing because cloning of the product facilitates sequencing allowing the use of primers in the vector, thus enabling us to determine the sequence of the whole insert without losing the sequence information of the initial stretch of the amplicon. Therefore, in this study, the PCR amplified product from a representative sample was purified and ligated to pTZ57R/T vector, having T overhang, supplied with the InsT/Aclone™ PCR product cloning kit. This vector has been reported to facilitate cloning of PCR product having A overhang (generated by Taq DNA polymerase). The ligated mixture was used to transform *E. Coli* (DH5-α) and the transformants were screened by blue white selection on X-gal/IPTG/LB agar plates containing ampicillin. After 12-15 h incubation, blue and white colonies were seen on the plates. All the white colonies which might contain the insert were streaked on a fresh plate for further analysis. The white colonies were screened for the presence of correct the PCR product by colony PCR using M13 primers. Colony PCR revealed the expected 327 bp product in the transformed colonies.

A similar approach had also been adapted by Rai *et al.* (2002) for cloning the PCR product of 520 bp sequence of gC gene of BHV1 using plasmid vector. Fuchs *et al.* (1999) cloned the PCR products obtained with strain LA of BHV1 as the template into the plasmid vector pCRII using the TA cloning kit.

Sequencing of gC gene of BHV1

The currated sequence obtained by using forward and reverse primers were assembled using the BioEdit software programme and a consensus sequence of 168 bp was obtained. This consensus sequence was then further used for alignment with the published sequence of BHV1 in GenBank using NCBI BLAST and CLUSTAL W (1.82) software.

Fuchs *et al.* (1999) used a similar approach for sequencing the gC gene of strain LA of BHV1 isolated from blood. They cloned the strain LA DNA and used the purified plasmid for DNA sequencing by automatic sequencing (model 377; Applied Biosystems, Weiterstadt, Germany) by using a dye terminator cycle sequencing kit (Applied Biosystems, Perkin-Elmer Corp., Weiterstadt, Germany). Sequence data were analyzed with the Wisconsin Package (version 9.1; Genetics Computer Group, Madison, Wis.) and BLAST.

Kholy and Abdelrahman (2006) directly sequenced the PCR amplicons of the Egyptian vaccinal Abu-Hammad strain of BHV1 gD gene in both directions with the same primers as those used to generate the PCR amplicons. Sequencing was carried out in an ABI PRISM system using

the dideoxy chain-termination method, which is based on the incorporation of fluorescent-labelled dideoxynucleotide terminators. The nucleotide sequences were aligned using the Clustal W (1.82) program from the European Bioinformatics Institute as done in the present study.

Brower *et al*. (2008) targeted and sequenced gC gene for the detection of BHV-1 in the brain tissues (an atypical manifestation of BHV-1) of aborted fetuses and differentiate it from BHV-5, which has 85% homology with BHV-1 throughout genome. But, these two differ considerably in some regions, viz. the thymidine kinase, gC and gE genes.

Lojkic *et al*. (2011) detected the BHV-1 infection in three dairy herds by gC gene based PCR and analysed the sequence of the gC gene of one isolate from each farm. On phylogenetic analysis based on gC gene sequence, the isolates were clustered with BHV-1.1.

The DNA sequence (168 bp) of the amplified gC region of BHV1 obtained in this study was deposited in GenBank under accession no. EU086706.

Sequence analysis

The nucleic acid sequences obtained were aligned with known sequences BHV1CGEN (Accesion no. AJ004801), BVH1LFT31 (Accession no. Z54206), HSBGPG3A (Accesion no. M27491), AY052397 (Accesion no. AY052397), AF135441 (Accesion no. AF135441) and BHV1GC (Accesion no. Z49223) of BHV1 published in GenBank. The sequence alignment of the field BHV1 (named Raj/ibr/Guj) sample with the published sequences is presented in Figure 1.

Nucleotide (nt) sequence alignment of the gC region of the field BHV1 and published sequences showed variable percentages of homology (89% to 92%) (Table 1). The three European BHV1 isolates (BHV1CGEN, BVHILFT31 and AF135441) and a North American isolate (HSBGPG3A) showed 92% homology with the field BHV1 (Raj/ibr/Guj), which was the maximum level of similarity. Lesser homology of 90% and 89% was observed with AY052397 (a South American isolate) and BHV1GC (a European isolate).

In contrast to present study, Fuchs *et al.* (1999) found homology of 100% between the amplified gC gene (LA strain) and the published sequence HSBGPG3A (accession no. M27491). Compared to the gC sequence of BHV1GC (accession no. Z49223), 10 base differences (leading to four different amino acids) accounted for 98.1% homology with the LA strain. Similarly, the homology was 89% due to 17 bp differences in the present study including deletions at two positions between the sequence obtained in the present study (Raj/ibr/Guj) and the BHV1GC (accession no. Z49223).

Comparison of the sequence of the amplified gC region of the field BHV1 with the published sequences showed deletions at 40 and 70 nucleotides, which indicate point deletion mutations at these places suggesting genetic variation in the gC gene of the field BHV1 of Gujarat. Out of the seven sequences subjected under multiple alignment, five sequences including the sequence (Raj/ibr/Guj) obtained in the present study [the other four being HSBGPG3A (Accesion no. M27491), AF135441 (Accesion no. AF135441), BHV1GC (Accesion no. Z49223) and AY052397 (Accesion no. AY052397)] revealed deletions from positions 90-93, 105-111 and 137-138. While these positions showed the properly aligned nucleotides in a conserved way among the two other sequences viz. BHV1CGEN (Accesion no. AJ004801) and BVH1LFT31 (Accession no. Z54206). Moreover,

the sequence obtained in the present study revealed point mutations at seven positions, i.e. at positions 2, 61, 116, 134, 143, 151 and 155, where A, C, C, C, G, T and C replaced G, A, A, G, C, G and A, which were found conserved in all the other six sequences. Such a situation of nt variations possibly indicates a frameshift mutation in the gene region, which can result in considerable amino acid (aa) variations.

The nt sequence obtained in the present study was translated into amino acid sequence by using Expasy software program. Out of three frames obtained, frame 1 was chosen for getting aa sequence as it yielded corresponding proper stretch of aa with proper alignment with the reference sequences, which was then further analyzed for alignment with the published aa sequences of BHV1 in GenBank using NCBI BLAST and CLUSTAL W (1.82) software.

The deduced aa sequence of the gC region of field BHV1 was compared with the published aa sequences obtained from the same six sequences BHV1CGEN (Accesion no. AJ004801), BVH1LFT31 (Accession no. Z54206), HSBGPG3A (Accesion no. M27491), AF135441 (Accesion no. AF135441), AY052397 (Accesion no. AY052397) and BHV1GC (Accesion no. Z49223). The reference sequences BHV1CGEN, BVH1LFT31 and AF135441 of the European region and HSBGPG3A of the North American region scored the highest gC sequence identity (79%) with the aa sequence of the gC region of field BHV1, followed by the European isolate BHV1GC (76%) and the South American isolate AY052397 (75%). The deduced aa sequence alignment with the published aa sequences is presented in Figure 2. In spite of high gC nt sequence identity (92%) between the field BHV1 and reference BHV1CGEN, BVH1LFT31, HSBGPG3A and AF135441 sequences, a lower deduced aa homology (79%) was observed. Similarly, lower aa homology percentages were recorded between the field BHV1 and the other reference sequences studied. This could be attributed to the occurrence of possible frameshift mutations as discussed earlier. Both nt and deduced aa sequence alignments revealed variable degrees of sequence homology between the field BHV1 and published sequences.

In a similar study carried out by Kholy and Abdelrahman (2006), frameshift mutations were observed at nt 509 and 615 of the Egyptian BHV1 gD gene. Both nt and aa sequence alignments revealed variable degrees of sequence similarity with other alphaherpesviruses. Thus, the genetic variations, in the field BHV1, resulted due to possible frameshift mutations in the gC gene regions indicate antigenic differences between the Indian field virus and BHV1strains from other parts of world.

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