Restriction analysis of conserved and variable regions of VP2 gene of Indian isolates of bluetongue virus serotype 1

Swati Dahiya, G Prasad*, Minakshi & Ramesh C Kovi

Department of Animal Biotechnology, College of Veterinary Sciences,
CCS Haryana Agricultural University, Hisar 125 004

Received 7 July 2004; revised 2 December 2004

Bluetongue virus (BTV) is a member of Orbivirus genus in family Reoviridae. The virus genome is composed of 10 double-stranded RNA segments. The RNA segment L2 encodes an outer capsid viral protein VP2, which is the main determinant of neutralization and serotype-specific immune response. BTV serotype 1 (BTV-1) specific novel primer pair was designed using VP2 gene sequences available in GenBank to amplify 1240-1844 bp region because two hypervariable and three conserved regions have been reported within these 694 nucleotides. This primer pair successfully amplified cell culture adapted six Indian isolates of BTV-1 from different geographical regions of the country. The 694 bp PCR product of VP2 gene of all six BTV-1 isolates yielded two fragments of 273 and 331 bp when digested with TaqI restriction enzyme. This indicated that there is only one TaqI site at 1513 bp (within 1240-1844 bp region) of VP2 gene of BTV-1 Indian isolates.

The in silico restriction analysis revealed that in BTV-1 South African isolate (BTV-1SA) there is no TaqI site while in BTV-1 Australian isolates (BTV-1AUS), there are two TaqI sites (at 1513 and 1567 bp) within 1240-1844 bp region of VP2 gene. The earlier reported VP2 gene based primer pair for BTV-1 was used in the present study to amplify 2242-2933 bp region of six BTV-1 Indian isolates as three conserved regions have been reported within these 691 nucleotides. The digestion of 691 bp PCR products with XmnI yielded three fragments of 364, 173 and 154 bp with all the six Indian isolates of BTV-1 suggesting that there are two XmnI sites within 2242-2933 bp region of VP2 gene. A single XmnI site was observed in silico in BTV-1AUS and BTV-1SA isolates at different positions within this region. The in vitro and in silico restriction profile analyses of partial VP2 gene sequences using TaqI and XmnI restriction enzymes indicated a close relationship of Indian isolates of BTV-1 with BTV-1AUS isolates but not with BTV-1SA isolate.

Keywords: Bluetongue virus, Orbivirus, PCR, Restriction analysis, Serotype specific VP2 Gene

Bluetongue (BT) is an arthropod-borne, infectious, non-contagious, economically important viral disease of domestic and wild ruminants. It is classified as ‘List A’ disease according to the Office International des Epizooties (OIE), Paris. It is thus considered to have the potential to spread rapidly, and have serious socio-economic consequences because of mandatory trade barrier on the movement of animals, their germplasm, embryos and other animal products from BT endemic to BT free countries.

Bluetongue virus, member of genus Orbivirus, family Reoviridae, is non-enveloped with double shelled structure enclosing the double stranded (ds) RNA genome having 10 discrete segments, each of which encodes atleast one protein. The virus structure consists of seven structural and four non-structural proteins. The BTV genome is highly conserved among the serotypes, with the exception of segment L2 and M5, which encode VP2 and VP5 proteins, respectively. VP2 is the most divergent and major serotype specific outer capsid protein, which induces protective immune response in the host.

Twenty-four serotypes of BTV have been defined, of which 21 have been identified in India. The BTVs are considered emerging infections due to their ability to undergo rapid evolutionary change through gene reassortments and mutations leading to emergence of new serotypes or antigenic variants of the same serotype. The higher levels of nucleotide sequence variation could be confined to the neutralization sites themselves, which have been observed in different hypervariable regions of the gene. These hypervariable regions may be important for the overall conformation of the protein or may serve as major neutralization epitopes, while the conserved amino acid sequences may serve critical function of
either epitope presentation, the overall folding of protein, or for protein-protein interaction in complete virion.

A combination of PCR, using well-characterized serogrouping and serotyping primers and restriction enzyme analyses of PCR products could allow rapid characterization of an unknown Orbivirus isolate. Also a different geographic origin for BTV isolates could be predicted based on different restriction fragment patterns of the PCR products following digestion with different restriction enzymes. Therefore, the aim of the present investigation is to establish the relationship of Indian isolates of BTV-1 with other BTV isolates reported from different parts of the world by restriction enzyme analyses of PCR products involving conserved and hypervariable regions of VP2 gene.

Materials and Methods

**Viruses**—Six Indian isolates of BTV-1: Avikanagar (A), Rajasthan; Hisar (H), Haryana; Chennai (C), Tamil Nadu; Sirsa 1 (S1), Sirsa 2 (S2), and Sirsa 3 (S3), Haryana (BT Lab isolates, Department of Animal Biotechnology, CCS HAU, Hisar) were propagated in baby hamster kidney (BHK-21) cell line. The reference strain of bovine rotavirus, UK strain was grown in African green monkey kidney cell line (MA104). After 36 hr of infection, 75% of the cells showed cytopathic effects of cell rounding, fusion and detachment of cells. The viruses were harvested by freezing and thawing and titrated.

**Extraction of viral RNA**—The viral RNA was extracted by guanidinium thiocyanate-phenol-chloroform method with minor modifications.

**Preparation of cDNA**—A 50 μl reaction mixture was prepared containing heat denatured 7 μg of viral RNA, 6% dimethyl sulfoxide (DMSO), 4 mM deoxynucleotide triphosphates (dNTPs), 50 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Stratagene) and 50 pmoles of each primer P1 and P2 and P3 and P4 (Table 1) in different reaction mixtures. In thermal cycler (Eppendorf master cycler gradient, Germany), the primers were allowed to anneal at 25°C for 10 min, then reverse transcription at 42°C for 60 min followed by heat inactivation at 90°C for 5 min.

**Polymerase chain reaction**—The cDNA synthesized by P1 and P2 and P3 and P4 primers were used for amplification of 604 bp and 691 bp PCR products, respectively of VP2 gene with all six BTV-1 isolates. PCR was carried out in 50 μl reaction mixture containing 5 μl cDNA, 5% DMSO, 50 pmol of each primer, 5 μl of 10X PCR buffer, 3 μl of 25 mM magnesium chloride, 1 μl of 10 mM dNTPs and 1.25 U of Taq polymerase (MBI Fermentas). For 604 bp PCR products, the cDNA was amplified for 30 cycles of 95°C for 3 min in first cycle followed by denaturation at 95°C for 1 min, primer annealing at 55°C for 100 sec, extension at 72°C for 1 min, with terminal extension at 72°C for 10 min. For 691 bp PCR products, the amplification programme was same as that for 604 bp product except for the annealing temperature and time. The P3 and P4 primers were standardized to anneal at 37°C for 90 sec. The amplified products were electrophoresed across a 1.8% agarose gel, stained with ethidium bromide and visualized under UV transilluminator (UVP GDS 5000).

**In vitro restriction enzyme digestion of PCR products**—The 604 bp and 691 bp nested PCR products of VP2 gene of all six BTV-1 Indian isolates were digested with TaqI (MBI Fermentas) and XmnI (Promega) restriction enzymes, respectively according to the manufacturer’s instructions. After mixing and incubating the reagents at 37°C for 1 hr, the digested PCR products were analyzed using 2% agarose gel electrophoresis.

**In silico restriction endonuclease profile analyses**—The in silico restriction analyses of 1240-1844 bp and 2242-2933 bp regions of VP2 gene sequences available in GenBank of all the BTV-1 isolates [two isolates of

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequences (5'-3')</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1240-1271</td>
<td>ATGGTGCGAGTTAACCTGGTTAATTACGTC</td>
<td>604</td>
</tr>
<tr>
<td>P2</td>
<td>1844-1813</td>
<td>AATTCCACGGTGTCGAAGAT</td>
<td>604</td>
</tr>
<tr>
<td>P3</td>
<td>2242-2256</td>
<td>TACCTCTTTCCTTTT</td>
<td>691</td>
</tr>
<tr>
<td>P4</td>
<td>2933-2884</td>
<td>TGATAGCGCGGCGACCCACCGGGTGTCATCTCGAGAG</td>
<td>691</td>
</tr>
</tbody>
</table>

Table 1—Serotype specific oligonucleotide primers for reverse transcription and amplification of RNA segment 2 (VP2 gene) of BTV-1 isolates
BTV-1 Australia (BTV-1AUS, Accession Nos X06464 and M21844) and one isolate of BTV-1 South Africa (BTV-1SA, Accession No. X55800) was done using Webcutter 2.0 software (© 1997 Max Heiman; Website: http://www.firstmarket.com/cutter/cut2.html) on Internet.

Results and Discussion

The nested RT-PCR targeting different BTV serotypes for amplification of serotype specific VP2 gene has been reported earlier. The complete RNA segment 2 and amino acid sequences of its encoded protein VP2 of different BTV serotypes have been compared by various workers. Five to six hypervariable regions and 7-11 conserved regions have been reported within these 691 nucleotides at 2283-2306, 2325-2357 and 2853-2900 bp. The nested RT-PCR assay was standardized using BTV-1 VP2 gene specific primers P3 and P4 yielding 691 bp expected product with all the six Indian isolates of BTV-1 (Fig. 2). No amplification was observed with BTV-18, and BTV-23 isolates (not shown in figure), heterologous rotavirus control, mock infected cell line and NFW controls. It was found that annealing and extension at 37 and 72°C, respectively, for 2 min each as reported earlier resulted in some non-specific amplifications, so the time was reduced to 90 sec at 37°C and for 1 min at 72°C. This suggested that the nested primers used along with standardized RT-PCR conditions were capable of amplifying the Indian BTV-1 isolates without any non-specific amplification.

The in vitro restriction digestion of 604 bp nested RT-PCR product of VP2 gene of all the six BTV-1 Indian isolates with TaqI yielded two fragments of 331 and 273 bp (Fig. 3). This clearly indicated that there is only one TaqI site i.e. TCGA within 1240-1844 bp region of Indian isolates of BTV-1. This was confirmed by in silico restriction analyses using BTV-1A, 1S3 and 1C (Accession Nos AY559058, AY559060 and AY559061, respectively) VP2 gene sequences (1240-1844 bp region) in which single TaqI site was observed at same position in all the three isolates i.e. at nucleotide 1513. The in silico restriction analyses of 1240-1844 bp region of VP2 gene of two isolates of BTV-1AUS (Accession Nos X06464 and M21844) revealed two TaqI sites, one at 1513 bp and another at 1567 bp described in Table 2. The second TaqI site in two BTV-1AUS isolates at 1567 bp did not exist in BTV-1 Indian isolates because of single base mutation from TCGA in BTV-1AUS to TTGA in BTV-1 Indian isolates. No TaqI site was observed in BTV-1SA (Accession No X55800) within these 604 bp DNA nucleotides.

![Fig. 1](image1.png)
Fig. 1—Nested 604 bp RT-PCR product for VP2 gene amplification using serotype 1 specific primers from the following Indian isolates of BTV-1, Lane 1: Hisar, 2: Avikanagar, 3: Sirsa 1, 4: Sirsa 2, 5: Sirsa 3, 6: Chennai, 7: BHK-21 cell control, 8: water control, M: 100 bp DNA ladder.

![Fig. 2](image2.png)
Fig. 2—Nested RT-PCR amplified 691 bp product of VP2 gene of BTV-1 Indian isolates. Lanes 1-6: BTV-1 Hisar, Avikanagar, Sirsa 1, Sirsa 2, Sirsa 3, and Chennai isolates, respectively; 7: Heterologous rotavirus; 8: BHK-21 cell control; 9: water control; M: 100 bp DNA ladder.
nucleotides. A synthetic peptide from amino acid 492-503 (nucleotides 1491-1526) has been used because of its conserved nature between serotypes to study the conformational structure of VP2 protein. The TaqI site at 1513 bp which was found to be conserved in all the Indian and Australian isolates of BTV-1 lie within this region, while there was no TaqI site in BTV-1SA at 1513 bp. Therefore, the closer relationship of BTV isolates could be predicted by restriction digestion of PCR products (obtained by using BTV-1 specific primer pair of VP2 gene) using TaqI restriction enzyme.

The digestion of 691 bp nested PCR products of VP2 gene by XmnI yielded three fragments of 364, 173 and 154 bp with all the six Indian isolates of BTV-1 (Fig. 4). This suggested that there are two XmnI sites i.e. GAANN|NNTTC, one at 2415 bp and the possibility of another site is at 2779 bp within 2242-2933 bp region of BTV-1 Indian isolates as shown in Table 3. The only XmnI recognition site (GAANN|NNTTC) within 2242-2933 bp region of VP2 gene of BTV-1AUS isolates was observed in silico at 2411-2420 nucleotides with digestion at 2415 bp. XmnI site was observed within these 691 nucleotides from 2652-2661 nucleotides with digestion at 2656 bp in VP2 gene of BTV-1SA isolate. The site from 2411-2420 bp in BTV-1 Indian isolates is

![Fig. 3 - Restriction profile of VP2 gene based 694 bp nested PCR product of BTV-1 Indian isolates with TaqI. Lane M: 100 bp DNA ladder; 1-6: BTV-1 H, A, S1, S2, S3 and C isolates, respectively; U: uncut BTV-1S2; L: 50 bp DNA ladder.](image)

![Fig. 4 - Restriction enzyme (XmnI) digestion of 691 bp PCR product of VP2 gene amplified from BTV-1 Indian isolates shown in Lanes 2-7: BTV-1 H, A, S1, S2, S3 and C isolates, respectively; 1: uncut BTV-1S3; M: 100 bp DNA ladder](image)

<p>| Table 2—In vitro and/or in silico restriction analyses of partial VP2 gene sequences (1240-1844 bp region) of BTV-1 isolates |</p>
<table>
<thead>
<tr>
<th>BTV-1 isolates</th>
<th>No. of TaqI sites</th>
<th>Position</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTV-1AUS</td>
<td>2</td>
<td>1513-1516</td>
<td>TL</td>
</tr>
<tr>
<td>BTV-1Hindia</td>
<td>1</td>
<td>1513-1516</td>
<td>TL</td>
</tr>
<tr>
<td>BTV-1SA</td>
<td>0</td>
<td>1513-1516*</td>
<td>TTGA</td>
</tr>
<tr>
<td>* No TaqI site because of single mutation</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| Table 3—In silico and/or in vitro restriction analyses of partial VP2 gene sequences (2242-2933 bp region) of BTV-1 isolates |</p>
<table>
<thead>
<tr>
<th>BTV-1 isolates</th>
<th>No. of XmnI sites</th>
<th>Position</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTV-1AUS</td>
<td>1</td>
<td>2411-2420</td>
<td>GAAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2652-2661*</td>
<td>GAGAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2775-2784*</td>
<td>GAAAGGATCG</td>
</tr>
<tr>
<td>BTV-1SA</td>
<td>1</td>
<td>2411-2420*</td>
<td>CAAGGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2652-2661</td>
<td>GAAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2775-2784*</td>
<td>GAGGGA</td>
</tr>
<tr>
<td>BTV-1 India</td>
<td>2</td>
<td>2411-2420</td>
<td>GAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2775-2784</td>
<td>GAA</td>
</tr>
<tr>
<td>* Not XmnI site</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N: A/G/T/C
matched with that of BTV-IAUS isolates (Table 3). Another XmnI site i.e. GAANN[N]NTTC in BTV-1 Indian isolates from 2775-2784 bp may have resulted by mutation (at last two bases) from GAAGGATCG in BTV-IAUS to GAANN[N]NTTC in BTV-1 Indian isolates. The XmnI site within these 691 nucleotides in BTV-1SA is observed at 2652-2661 nucleotides which matches neither with BTV-1AUS nor with BTV-1 Indian isolates. Both the XmnI sites in BTV-1 Indian isolates did not lie within the three conserved regions reported by Gould. Hence, there is every possibility of variation due to mutation of these restriction sites. Therefore, it is suggested that the relationship as well as the origin of BTV serotype 1 isolates could be predicted by variation due to mutation of these restriction sites.

Acknowledgement

Thanks are due to ICAR and Ministry of Environment and Forests for financial help. The infrastructural facilities provided by CCS Haryana Agricultural University, Hisar are duly acknowledged.

References


16 Wade-Evans A M & Mertens P P C, Expression of the outer capsid protein, VP2, from a full-length cDNA clone of genome segment 2 of bluetongue serotype 1 from South Africa, using both Sp6 and vaccinia expression systems and a comparison of the nucleic acid sequence of this segment with those of other serotypes, Virus Res, 15 (1990) 213.
