Evaluation of Group specific Nested PCR for detection of Bluetongue virus

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Abstract

During the present study RT-PCR and nested PCR was evaluated for the detection of BTV in blood samples collected from suspected cases of bluetongue. Due to conserved nature, NS1 gene was targeted for the development of partial length and nested PCR assay. The partial length RT-PCR and nested PCR assays yielded a specific PCR product of expected 274 bp and 101 bp sizes, respectively. In, the present study out of 68 blood samples processed for BTV detection, 2 samples were found positive for BTV genome by RT-PCR as well as in nested PCR. The described BTV PCR based assay provides a valuable tool to study the epidemiology of BTV infection in susceptible domestic livestock.

Keywords: Ruminant, Mortality, Genome, PCR assays, Epidemiology, Economic.

Introduction

Bluetongue virus (BTV) is an arthropod transmitted virus infection of domestic and wild ruminants, and the causative agent of bluetongue disease of certain species of ruminants (MacLachlan, 1994). Bluetongue principally affects sheep and some wild ruminant species, in which it may cause a severe systemic disorder with moderate to high mortality. BTV is a prototype virus of orbivirus of the family Reoviridae (Pringle, 1999). It is composed of 10 discrete segments of ds-RNA genome surrounded by two layers of protein capsid. Three nonstructural and seven structural proteins are incorporated into the double layer protein coat (Roy, 1989). Bluetongue, though recognized more than a century ago, continues to be an economically very important disease affecting susceptible domestic and wild ruminants throughout the world. Early detection of infected animals could reduce drastically the consequences of the disease by reducing the virus pool and by containing the dissemination of the disease through export of potentially infectious animals. Since the conventional methods are time consuming and cumbersome, sensitive and reliable diagnostic tools are of paramount importance. It is, therefore, becoming increasingly obvious that the development of molecular assays for detection and differentiation of BTV and related viruses would be advantageous in a variety of circumstances including clinical disease investigations, vaccination programs, epizootiological studies and certification of animal for export and import purposes. Therefore, advent of molecular biological techniques have not only added to the knowledge on the virus but also paved the way for development of more sensitive, specific and accurate diagnostic techniques. In the present study, nested PCR assay was standardized using internal NS1 gene specific nested primers for the detection of BTV from cell culture adopted BTV-23 and blood samples collected from suspected sheep.

Materials and Methods

Clinical samples: A total of 68 blood samples were collected separately in vacuutainers (B.D.)from sheep stationed at Gujarat Sheep and Wool Development Corporation (GSWDC), Jasdan and Aseda. The samples were collected from sheep showing initial rise in body temperature (104^{o}F) and showing clinical signs resembling to BT. The samples were also collected from apparently healthy sheep. All the samples were processed for extraction of RNA and subsequent detection of BTV by RT-PCR and nested PCR.

Processing of blood samples: The blood samples on arrival at the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, S.D.Agricultural University, Sardarkrushinagar were centrifuged at 800 g for 5 to 10 minutes in refrigerated

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centrifuge at 4°C. The blood cells were washed three times in equal volume of calcium magnesium free PBS. After the final wash, the equal volume of OPG medium was added in all the blood samples. The red blood cells were haemolysed as per the method described by Hosseini *et al.* (1998). Thereafter, the blood samples were transferred in screw capped vials and stored at 4°C until further use.

BTV reference Virus: BTV serotype-23(Passage 53) was made available by the courtesy of Dr.R.K.Singh, Head, Division of virology, IVRI, Mukteswar and maintained in LN_2 container and further passaged up to P58 in BHK- 21 cell line.

Extraction of viral RNA by TRI reagent method TRI RNA extraction method was used for total RNA isolation as per the manufacturer's instruction (Molecular Research Centre, Inc., USA). In this method, in one ml of viral sample / blood sample equal volume of TRI reagent was added, vortexed and incubated for 5 min at 15-30°C to permit the complete dissociation of nucleoprotein complexes. In this 0.2 ml chloroform was added, vortexed and incubated at room temperature for 2-3 min. It was centrifuged at 12,000 rpm for 20 min at 4°C. Aqueous phase was transferred to fresh eppendorf tube and 0.5ml of isopropanol was added, and allowed sample to stand for 10 min at room temperature (No vortexing). This was centrifuge at 12,000 rpm for 15 min, the supernatant was discarded and two washing of 70% pre chilled ethanol were given to the palette. The RNA pellet was air dried and resuspended in 15ìl nuclease free water.

Reverse transcription-Polymerase chain reaction (RT-PCR) The reverse transcription-polymerase chain reaction (RT-PCR) assay for NS1 gene was standardized using cell culture grown BTV-23 and blood samples collected from suspected cases of bluetongue in sheep.

Group specific NS1 primers: The partial length and nested primer sequences for NS1 gene were used in the present study. The primers were got synthesized from Bangalore (Genei)The partial length PCR primers used were:

Primer 1: 5' to 3' GTT CTC TAG TTG GCA ACC ACC

Primer 2: 5' to 3' AAG CCA GAC TGT TTC CCG AT

The primer 1 extended from position 11 through 31. The primer 2 is located between positions 284 through 265 of the complementary strand. These primers produced 274 bp PCR products.

Nested primers: The nested primers sequences used were :

P1: 5' GCA GCA TTT TGA GAG AGC GA 3'

P2: 5' CCC GAT CAT ACA TTG CTT CCT 3'

The nested primer 1 extended from position 170 through 189 of the positive strand. The primer 2 is located between position 270 through 250 of the opposite strand of the complementary strand. These primers produced 101 bp PCR product.

RT-PCR with partial length primers: The viral genomic RNA extracted by TRI reagent method was used as a template for cDNA synthesis by Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) enzyme (Bangalore Genei) using M-MuLV RT-PCR kit was procured from Bangalore Genei.

cDNA synthesis Following reaction mixture for cDNA synthesis was prepared in a PCR tube with a final volume of 25ìl:

Viral ds RNA	2.0ìl
DMSO	1.5ìl
NS1 gene specific primer P1	0.5ìl
NS1 gene specific primer P2	0.5ìl
Nuclease free water	16.5ìl

The above mixture was boiled at 99°C for 5 min, snapped chilled on ice and than following reagents were added

10X RT buffer	2.5ìl
100mM dNTPs	1.0ìl
MMLV-RT	0.5ìl

After allowing the primer to anneal at 25°C for 10 min, reverse transcription was carried out at 42°C for 60 min in thermal cycler (Corbett Research, Australia). The cDNA is stored at -20°C till further use' for PCR amplification.

Polymerase chain reaction: The cDNA synthesized as above was used as template for amplification of desired gene.

Following reaction mixture for PCR was prepared in a PCR tube with a final volume of 25ìl

cDNA		5.0ìl
DMSO		1.25ìl
Forward primer		0.25ìl
Reverse primer		0.25ìl
NFW		13.5ìl
10X PCR buffer		2.5ìl
$MgCl_2$ 25mM		1.5ìl
10mm dNTPs	0.5ìl	
Taq polymerase (5U/ ìl)	0.25ìl	

The reaction was carried out in thermal cycler using the cyclic condition described below.

Step	Temperature	Time
Initial denaturation	95°C	2 min
3-step cycling for 30 cycles		
Denaturation	95°C	30 sec
Annealing	57°C	1 min
Extension	72°C	1 min
Final extension	72°C	10 min
End of the PCR cycling	4°C	8 min

Detection of PCR product by Agarose Gel electrophoresis: RT-PCR products where analysed by agarose gel electrophoresis using 1.5% agarose gel prepared in 1X Tris-acetate-EDTA. To the molten agarose, 0.5ìg/ml ethidium bromide was added just before pouring the agarose in to the gel tray. The amplified 5ìl of each PCR product and 100 kb ladder were mixed with 1ìl of 6X loading dye and loaded in to the wells of the gel. The electrophoresis was carried out at a constant voltage of 100 volts (Bangalore Genei) in 1X TAE running buffer till the dye reached 2/3rd of the gel. The bands were visualized and photographed under Gel Documentation System (DNR Bio-imaging System) and photographed.

Nested PCR: For the nested amplification of the 101bp PCR product, 5 ìl of the first amplification product (274 bp) were transferred to a PCR tube containing Nested primer$_1$: 5 ìl, nested primer$_2$: 5 ìl; 2X PCR master Mix (Bangalore Genei) 27 ìl and Nuclease free water 8 ìl. The PCR tubes were placed in the thermal cucler for another 40 cycles described below.

Step	Temperature	Time
Initial denaturation	95^{0}C	2 min
3-step cycling for 40 cycles		
Denaturation	95^{0}C	1 min
Annealing	55^{0}C	30 sec
Extension	72^{0}C	45 sec.
Final extension	60^{0}C	10 min
End of the PCR cycling	4^{0}C	**8**

Following amplification 30ul of PCR reaction containing amplified product were loaded in to 1.5% agarose gel and electrophoresed similar to the procedure shown in RT-PCR protocol. The bands were visualized and photographed under Gel Documentation System (DNR Bio-imaging System) and photographed.

Results

BTV-23 and 2 field blood samples produced approximately 274 bp amplicons with NS1 gene specific primers. Similarly, nested PCR yielded a DNA band of expected 101bp size in BTV-23 and 2 field samples.

Discussion

The economic importance of BTV infection is mainly attributed to clinical disease in sheep. Even in the absence of clinical disease , there is restriction on the international trade of livestock and associated germ plasm unless the animals are certified BTV free by conventional isolation and serology (Osburn et al, 1994). In addition, the pathological lesions caused by BTV in bovines are undistinguishable from those caused by EHDV, and hence these viral infections are of interest to veterinary diagnosticians (Aradaib et al, 1998). Moreover, conventional virus isolation and serology are time consuming and cumbersome (Pearson et al, 1992). It, is therefore, becoming increasingly obvious that the development of molecular diagnostic techniques for detection and differentiation of BTV and EHDV would be advantageous in a variety of circumstances including clinical disease investigation, vaccination programme and epidemiological studies (Pearson et al, 1992; Aradaib et al, 1998).

Polymerase Chain Reaction (PCR) procedure exploits in vitro DNA replication, producing large quantities of desired sequence of DNA from a complex mixture of heterogeneous sequences. PCR can amplify copies of 50 to several 1000 base pairs of a selected region into millions of copies. With RNA viruses, a complementary DNA (cDNA) copy of the RNA is first made using reverse transcriptase (RT), and then the PCR can be used for amplification.

Nucleic acid based assays have been proposed as suitable tools for detecting BTV directly in clinical specimens because of their exceptional sensitivity and specificity (Akita et al, 1992; Aradaib et al,1998).BTV sequencing and hybridization studies have suggested that the viral genome segment 6 encoding non structural protein (NS1) has 97-100 % nucleic acid sequence homology with other serotypes. Thus this gene segment is highly conserved (Gould et al, 1988), Aradaib et al, 1998). Therefore, it was suggested that a fragment of this genome could be targeted for detection of BTV in cell culture and clinical samples using a more sensitive nested PCR amplification technology.

Several RT-PCR assays have been described for the detection of BTV (Gould et al., 1988; Katz et al., 1993; Shad et al., 1997; Tiwari et al., 2000;). O.I.E. (2000) recommended PCR assay as one of the official tests for detection of BTV in animals for international trade. RT-PCR has a major advantage that this test is not dependent on the quality of the sample, which is a limiting factor for virus isolation studies. This makes this assay very attractive diagnostic approach particularly in tropical countries where samples are likely to be deteriorated during transportation due to loss of cold chain.

As compared to other genomic segments, genome segment 6 (NS1gene) was reported to be more specific to BTV serogroup and least likely to cross react with other Orbivirus members. Hwang et al. (1993) showed that the NS1 gene segment is most conserved among all the 10 dsRNA segments in BTV serogroup. Sequencing and hybridization studies have indicated that the viral genome segment 6 possesses the 97% sequence homology with other member of

BTV serogroup and thus is a highly conserved segment.

Due to the conserved nature of NS1 gene, it was targeted for development of RT-PCR for detection of BTV. The NS1 gene primer sequence for partial length used in the study was the same as used by Katz et al. (1993).

In the present study, partial length assay was developed and evaluated for detection of viral RNA extracted from cell culture grown BTV-23 and cell culture adopted field samples. Among these the partial length RT-PCR using NS1 gene specific partial length primers yielded a specific DNA band of expected 274 bp size in BTV-23 and 2 field samples adopted in BHK-21 cell lines. The primers were considered to be group specific and could amplify the BTV irrespective of the serotype. Similarly, RT-PCR based amplification of 274 bp has also been carried out by Malik et al (2001).

Nested PCR assay was standardized using internal NS1 gene specific nested primers. The nested PCR yielded a DNA band of expected 101 bp size. The NS1 gene primer sequences for partial length and nested PCR used in the study were the same as used earlier (Katz et al, 1993). The nested PCR assay provides greater specificity because it involves 2 rounds of amplification. In the first round, a pair of external primers used to get a larger fragment, which acts as a template for second round of amplification with second set of internal primers. This process provides an additional specificity to the reaction and greatly enhances the efficiency of amplification. A tentative diagnosis of BTV infection based on amplification of the specific 274 bp PCR product and a definitive diagnosis based on the amplification of the nested 101 bp PCR can be obtained within only 24 hours directly from the clinical samples. Similar results have also been reported by Malik (2000) and Aradaib et al, 1998).

Nested BTV PCR assay is simple and rapid and does not require hybridization confirmation which normally takes overnight. The rapidity, sensitivity and specificity of the nested PCR assay would greatly facilitate detection of BTV infection during an outbreak of the disease among susceptible ruminants (Aradaib et al, 1998). Shad et al, (1997) also developed a nested capture PCR assay and compared it with virus isolation and antigen capture ELISA. They reported that capture PCR assay is very rapid, easy and sensitive. It is worth mentioning that this PCR assay, for detection of BTV provides the basis for future diagnostic technique and could be used as valuable tool to study the epidemiology of BTV in susceptible domestic livestock.

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