Isolation and Characterization of Bluetongue Virus Recovered from Blood Samples by Immunoaffinity Purification

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Abstract An immunoaffinity chromatography (IAC) method was optimized for the selective capture of bluetongue virus (BTV) from blood samples and isolation of the virus in cell culture. The antibody against BTV core particles (lacking the outer capsid proteins VP2 and VP5) was used for the optimization of IAC technique. The antibody against BTV core particle was conjugated with Protein A-virus complex and the complex was dissociated using elution buffer (4 M MgCl₂ with 75 mM HEPES, pH 6.5). The optimized IAC method specifically purified the BTV without capturing other commonly infecting small ruminant's viruses like goatpox virus (GTPV), sheepox virus (SPPV), Peste des petits ruminants virus (PPRV) and Foot and mouth disease virus (FMDV). The blood samples (n = 22), positive for BTV antigen in sandwich-ELISA were attempted for virus isolation in the BHK-21 cell using the optimized IAC method. A total of seven BTV were isolated by selective capturing of the virion particles. The isolated viruses were characterized by RNA-PAGE, sequence analysis and serum neutralization test (SNT). Electrophoretic analysis of viral dsRNA in the RNA-PAGE revealed the presence of ten dsRNA segments characteristic of BTV. Out of seven isolates, four isolates were identified as BTV-1 and three isolates were identified as BTV-16 based on nucleotide sequences of segment-2.

Phylogenetic analysis of segment-2 nucleotide sequence segregated BTV-1 and BTV-16 isolates to monophyletic cluster at close proximity to other eastern topotype. In SNT, hyperimmune serum (HIS) against BTV-1 neutralized the four BTV-1 isolates up to a titer > 256 and HIS against BTV-16 neutralized the three BTV-16 isolates up to a titer > 128. The IAC technique will be useful for the selective capture of BTV from mixed infection (BTV with other small ruminant's viruses) and isolation from blood sample having low viral load by enrichment.

Keywords Immunoaffinity chromatography · Anti-core antibody · Bluetongue virus · Virus isolation · Characterization

Introduction

Bluetongue is an infectious, insect-borne virus disease mainly of sheep but the bluetongue virus (BTV) can infect almost all domestic and wild ruminants [1, 2]. BTV is the prototype virus of the genus *Orbivirus* in the family *Reoviridae* [3]. The BTV genome consists of 10 linear double-stranded RNA (ds RNA) segments that code for seven structural viral proteins (VP1–VP7) and four non-structural (NS1, NS2, NS3/NS3a and NS4) proteins [4]. The virus is transmitted amongst the vertebrate hosts by certain species of *Culicoides* midges [5]. At present, 27 distinct BTV serotypes have been recognized worldwide [6]. Bluetongue is endemic in India and 26 serotypes of the virus have been reported at one time or another based on serology and virus isolation. So far, 13 BTV serotypes viz., BTV-1, 2, 3, 4, 6, 9, 10, 12, 16, 17, 18, 21 and 23 have been isolated from the country [7].

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Isolation of BTV is important for identification and further molecular epidemiological studies. BTV is commonly isolated in embryonated chicken egg (ECE) and certain mammalian cell lines, of which BHK-21 cell is one of the most sensitive systems [8]. BTV isolation by inoculation to embryonated chicken egg is very tedious and time-taking although it is the most sensitive system. Direct isolation of BTV from clinical samples on the BHK-21 cell is relatively easier. But mixed viral infections of samples often hinder BTV isolation due to the dominance of other virus (es) in cell culture. Mixed infections of small ruminants with *peste des petits ruminant virus* (PPRV), BTV, orf virus, capripox virus and pestivirus are occasionally reported in India and other countries [9–13]. From such mixed-infected blood samples, it becomes very difficult to isolate BTV. Small-genome and more cytotoxic viruses (e.g., border disease virus, Foot and mouth disease virus) complete their replication cycle much earlier than BTV, which generally takes 2–4 days. Upon concurrent infection of these viruses on a cell culture, BTV disappears slowly within a couple of passages. Chances of isolation of BTV on cell culture from such mixed infection may be increased by selective capture and enrichment of the samples by the immunoaffinity chromatography (IAC). Earlier, the waterborne enteric viruses was captured and purified by immunoaffinity chromatography and detected by reverse transcriptase PCR [14]. In previous report we described the purification of infective BTV by IAC using anti-core antibody [15]. Here, we describe the application of the IAC method for selective capture of BTV from blood samples and isolation of the virus in BHK-21 cell culture.

Materials and Methods

Optimization of Immunoaffinity Chromatography (IAC)

Cyanogens bromide activated Protein A-Sepharose CL-4B was coupled with purified antibody against core BTV particle (lacking the outer capsid proteins VP2 and VP5) as recommended by the manufacturer (Sigma, St. Louis, USA). Approximately 20 mg of cyanogens bromide activated Protein A-Sepharose CL-4B resin was conjugated with 500 µg of IgG (against BTV core particle) in binding buffer (0.02 M NaH₂PO₄, 0.15 M NaCl, pH 8.0) and incubated overnight at 4 °C. Columns were washed with 8–10 column volume of the same buffer. To the resin antibody complex, added 1 ml of unpurified BTV-1 in binding buffer and incubated overnight at 4 °C for binding of the virus with the antibody. After washing, virus and antibody complex was dissociated with elution buffer (4 M MgCl₂ with 75 mM HEPES, pH 6.5). The eluted fraction

was dialyzed against PBS using 300 kDa molecular weight cutoff (MWCO) column and concentrated by the same column. The eluted fraction was tested for the presence of BTV antigen by s-ELISA as per protocol described [16]. The eluted fraction was infected in BHK-21 cell line to assess its infectivity.

Isolation of BTV from Blood Samples by IAC Method

The blood samples collected in EDTA from cattle, sheep and goats from the different region of the country were initially screened by s-ELISA. The blood samples (n = 22), positive for BTV antigen were further confirmed by RT-PCR for the demonstration of viral nucleic acid [17]. The blood samples were processed by sonic vibration as described earlier [8]. The BTV were selectively captured and enriched from the processed blood samples by the IAC and the eluted virus was filtered through 0.22 µm membrane for inoculating the BHK-21 cells. The monolayer showing cytopathic effect (CPE) were harvested and kept at 4 °C for further identification and characterization.

Polyacrylamide Gel Electrophoresis (PAGE) of BTV dsRNA

The total RNA of isolated viruses was extracted by Tri-Reagent (Sigma, St. Louis, USA). BTV dsRNA was purified by sequential precipitation with lithium chloride (LiCl) [18]. The identity of BTV isolates were confirmed by PAGE of purified dsRNA as per method described earlier [19] with some modifications. The Resolved dsRNA segments were visualized by staining with silver nitrate as per Promega protocol [20].

RT-PCR, Cloning and Sequencing

RT-PCR of purified segment-2 viral dsRNA coding viral protein 2 (VP2) was carried out using primers (forward primer, 5'-GGG TTA AAA GAG TGT TCY AC-3'; reverse primer, 5'-GTA AGT GTA AGA AGG CCA CAG-3') corresponding to inter BTV serotype conserved to 5' and 3' terminal [21]. The gel purified PCR products were cloned into pGEM[®]-T Easy vector system-I (Promega, Madison, USA). The ligated product was transformed into bacterial host cells (*Escherichia coli* JM-109 strain). Restriction endonuclease (RE) digestion of purified plasmids was carried out using *EcoRI* for confirmation of desirable gene insert in the vector. The sequencing of the cloned gene in plasmid vector was carried out on the commercial basis. Data collection software v3.0 (Applied Biosystems, USA) was used for assimilation of graphic data into DNA sequence data.

Sequence and Phylogenetic Analysis

The sequence data generated were arranged by using DNASTAR (Lasergene Core suite v10, Lasergene®) software. The identity of the gene was checked by Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/>). The phylogenetic analysis of the nucleotide (nt) sequences of BTV isolates generated in this study and the nucleotide sequences of respective genes available in the public database (NCBI/GenBank) was carried out. Multiple alignment of sequences were performed using Clustal W algorithm. Pairwise distance calculations and phylogenetic tree constructions were done using MEGA 6 software using the p-distance parameter, neighbor-joining method and tested by bootstrapping 1000 replicates [22]. The nomenclature for the geographically based phylogenetic groups (topotypes) is done as described [23].

Serum Neutralization Test

Serum neutralization test (SNT) was performed on BHK-21 cells according to the method described [24] in 96-well tissue culture plate using 100 TCID₅₀ of viruses and two-fold serial dilution of individual HIS (available in the laboratory). Serum neutralization titer was calculated and the mean of anti-log values of three individual runs was considered as mean neutralization titer of the HIS against a particular BTV serotype.

Results

Isolation of BTV from Blood Samples

The elution buffer for optimization of IAC method was selected on the basis of its efficiency in elution, maintaining antigenicity and infectivity of the virus. The efficiency of 4 M MgCl₂ with 75 mM HEPES, pH 6.5, in dissociation of antigen- antibody complex was checked. After elution, using 4 M MgCl₂ with 75 mM HEPES, pH 6.5, elute was found positive for BTV antigen in s-ELISA and produced CPE in BHK-21 cell line. Therefore 4 M MgCl₂ with 75 mM HEPES, pH 6.5 has been used as an elution buffer for further isolation of BTV from blood samples. Before infection to BHK-21 cell line the eluted fraction was dialyzed using 300 kDa MWCO, to separate virus and antibody fraction. The diameter of BTV particle is 850 Å, it is safe enough to use dialysis with 300 kDa, which can effectively remove the entire left out molecule including antibody.

Seven number of the virus could be isolated in cell culture by IAC method from BTV positive blood samples. After passaging in the BHK-21 cell, the virus could be

isolated at different passage levels (i.e. between P: 3–5) (Table 1). The characteristic CPE produced by different isolates was evident at 24 h, characterized by rounding and ballooning of cells. At 48 h, degeneration and death of the cells observed resulting in detachment from the surface of the flask. The infected cell culture material was tested by s-ELISA for the presence of BTV antigen and OD₄₉₂ value of all the isolates was measured as 1.2 ± 0.2 (OD₄₉₂ value of positive and negative control were 1.5 and 0.2 respectively).

Identification of BTV and Analysis of Nucleotide Sequence Data

The migration pattern of segmented dsRNA genome was analyzed in the native RNA-PAGE stained with silver nitrate. Comparative migration of dsRNA segments in the electropherogram revealed 10 segments of dsRNA with characteristic migration pattern of BTV genome (Fig. 1). The sequence generated confirmed that they all were cognate with published sequences of BTV available in the database. On BLAST analysis, 4 isolates (JBP42/Ind, MUK126/Ind, DKL46/12/Ind and DPL36/12/Ind) were identified as BTV-1 and 3 isolates (MUK1/13/Ind, PDP5/14/Ind and PDP2/14/Ind) were identified as BTV-16 based on segment-2 gene.

Multiple alignment of full-length VP2 gene nucleotide and amino acid sequence using Clustal W algorithm revealed that BTV-1 isolates are more closely related to Australian BTV-1 isolates than the viruses of African and European origin. The close relationship of Indian BTV-1 isolates with the Australian BTV-1 isolates was evident by 85.7 to 85.8% and 89.2 to 89.8% identity at VP2 gene nucleotide and amino acid sequence level respectively. African and European BTV-1 isolates were found to be distantly related to Indian BTV-1 isolates where 31.1 to 31.8% and 25.8 to 27.1% divergence was observed at the nucleotide and amino acid sequence levels respectively. Multiple alignment of full-length VP2 gene sequence revealed that BTV-16 isolates are more closely related to Japanese, African, European and Australian origin. The close relationship of Indian BTV-16 isolates with the Japanese, Australian, African and European BTV-16 isolates was evident by 90.8 to 98.0% and 93.1 to 97.6% identity at the nucleotide and amino acid sequence levels respectively. There is a high degree of variation with one Nigerian isolate with 29.2 to 29.9% and 20.2 to 20.8% at the nucleotide and amino acid sequence level respectively.

Phylogenetic Analysis

Phylogenetic analysis segregated Indian isolates to monophyletic cluster at close proximity to other Eastern

Table 1 Bluetongue virus isolated in BHK-21 cell line by immunoaffinity chromatography (IAC)

S. no.	Virus isolates	Species	Source	Place	Passages no. (P) in BHK-21 cells	GenBank accession no.	SNT titre against type specific HIS (BTV type)	Serotype (topotype)
1.	BTV/JBP42/Ind	Cattle	Blood	Jabalpur, Madhaya Pradesh	P3	KP735614	256 (BTV-1)	BTV-1 (Eastern)
2.	BTV/MUK126/Ind	Goat	Blood	Mukteswar, Uttarakhand	P4	KY934047	256 (BTV-1)	BTV-1 (Eastern)
3.	BTV/DKL46/Ind	Sheep	Blood	Dhenkanal, Odisha	P4	KY934048	256 (BTV-1)	BTV-1 (Eastern)
4.	BTV/DPL36/Ind	Goat	Blood	Dhanapli, Odisha	P3	KY934049	256 (BTV-1)	BTV-1 (Eastern)
5.	BTV/MUK1/Ind	Goat	Blood	Mukteswar, Uttarakhand	P3	KY934050	128(BTV-16)	BTV-16 (Eastern)
6.	BTV/PDP5/Ind	Goat	Blood	Padampuri, Uttarakhand	P5	KY934052	128(BTV-16)	BTV-16 (Eastern)
7.	BTV/PDP2/Ind	Goat	Blood	Padampuri, Uttarakhand	P4	KY934051	128(BTV-16)	BTV-16 (Eastern)

Fig. 1 RNA-PAGE of genomic dsRNA segments of BTV isolates resolved in 10% polyacrylamide gel. The electropherograms showing 10 segments of dsRNA with characteristic migration pattern of BTV genome

topotype viruses. Australian BTV-1 isolates branched at close proximity to the monophyletic cluster of Indian BTV-1 isolates. African and European isolates segregated as a separate cluster in Western topotype divergent from Indian BTV-1 isolates (Fig. 2). Phylogenetic analysis of BTV-16 of Indian isolate and isolates from different geographical regions make two major Eastern and Western topotypic groups. Indian isolates make a monophyletic cluster with Eastern topotype and Western topotype (BTV-16/Nigeria-A) makes the separate cluster (Fig. 3).

Serum Neutralization Test

Serum neutralization test (SNT) was carried out following beta (β) method and characteristic CPE of BTV was observed in between 48 and 60 h of post infection. The HIS against BTV-1 neutralized the 4 isolates (JBP42/Ind, MUK126/Ind, DKL46/12/Ind and DPL36/12/Ind) up to a titer > 256. The HIS to BTV-16 neutralized the 3 isolates (MUK1/13/Ind, PDP5/14/Ind and PDP2/14/Ind) up to a higher titer > 128. There was no cross-neutralization between the BTV-1 and BTV-16 isolates.

Discussion

Affinity purification technique has been used to isolate, concentrate and purify the virus particles for subsequent detection of viruses in cell culture lysate, clinical and environmental sample by many workers [14, 25–27]. Zhen and coworkers [28] purified bluetongue virus from Vero cell culture by capturing Vero cell against Vero cell antibody. In that method, a biologically active BTV was purified from cell debris by co-immunoprecipitation with agarose Protein A. The current study was significantly different from the previous methods of BTV purification. In this method, an immunoaffinity chromatography has been demonstrated for the purification and isolation of BTV from the blood sample using antibody against core BTV particle.

Fig. 2 Neighbor-joining tree depicting phylogenetic relationship amongst BTV-1 isolates from India and other countries in their VP2 nucleotide sequence. Bootstrap consensus was inferred from 1000 replicates. Texas shows GenBank accession numbers and geographic origin of strains that were compared. BTV-1 isolates from different geographical regions make two major clusters of eastern and western topotype

The optimized immunoaffinity chromatography method specifically purified the BTV and could remove the other viruses like GTPV, SPPV, PPRV and FMDV after mixing with BTV [15]. After infection of the BHK-21 cells, only BTV could be propagated with characteristic CPE. Seven BTV were isolated at different passage levels from blood samples of subclinical bluetongue infection. No other viruses, other than BTV was isolated in cell culture by this method. However, there was no history of mixed infection in any of the animal from which blood samples were collected for the study. The identity of the BTV isolates was confirmed by PAGE showing a different electrophoretotypes pattern. Variation in electrophoretic migration of RNA segments may be due to the secondary structures of RNA and base composition of cognate genome segments [29].

Sequencing of Seg-2/VP2 provides a reliable method to identify the serotype of novel BTV isolates. Multiple alignments and phylogenetic analysis of VP2 gene nucleotide sequences suggest that, Indian BTV-1 isolates are more closely related to Australian BTV-1. The close relatedness of Indian and Australian BTV isolates have

been reported earlier which groups the viruses under ‘Eastern topotype’ of BTV [30, 31]. Indian BTV-16 isolate with isolates from different geographical regions makes two major Eastern and Western topotypic groups. Indian BTV-16 isolates are closely related with the Eastern strain from Japan, Australia and South Africa as reported earlier [32]. There is a high degree of variation between Eastern and Western topotype (BTV-16/Nigeria-A) as observed earlier [33].

Numerous studies demonstrate the VP2 gene of BTV to be serotype-specific and the most variable gene between serotypes [34, 35]. The HIS against BTV-1 neutralized the four BTV isolates and HIS to BTV-16 neutralize the three BTV isolates. The sequence analysis and neutralization behavior confirm the 4 isolates (JBP42/Ind, MUK126/Ind, DKL46/12/Ind and DPL36/12/Ind) as BTV-1 and the 3 isolates (MUK1/13/Ind, PDP5/14/Ind and PDP2/14/Ind) as BTV-16.

In conclusion, in the present study a simple and rapid method has been described for purification and isolation of infective BTV from blood samples by an immunoaffinity

Fig. 3 Neighbor-joining tree depicting phylogenetic relationship amongst BTV-16 isolates from India and other countries in their VP2 nucleotide sequence. Bootstrap consensus was inferred from 1000 replicates. Texas shows GenBank accession numbers and geographic origin of strains that were compared. BTV-16 isolates from different geographical regions make two major clusters of eastern and western topotype

chromatography technique. The molecular characterization of isolated virus confirms the present method specifically captures bluetongue virus. Using this technique, BTV can selectively be captured and purified for direct isolation on cell culture from a small amount of blood or tissue material co-infected with different other viruses.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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