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Culicoides Oxystoma a Potential Vector for Transmission of Bluetongue Virus 16 in Southern India

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Abstract

Bluetongue (BT) is a *Culicoides* vector borne disease of domestic and wild ruminants. It is caused by bluetongue virus (BTV). BT is infectious but non-contagious disease. The head–thorax region of *Culicoides Oxystoma* vector trapped on animal farm in TN was triturated and inoculated in BHK-21 cell culture. After few blind passages it showed cytopathic effect (CPE) in cell culture. After appearance of 75% cytopathic effect in BHK 21 cell culture, viral nucleic acid was extracted. The RNA-PAGE analysis showed BTV specific characteristics migration pattern of 3:3:3:1. Viral nucleic acid was allowed for cDNA synthesis followed by NS1 and VP2 gene based RT-PCR. The NS1 gene RT-PCR, CPE and specific migration pattern in RNA-PAGE confirmed the sample as BTV. The VP2 gene based serotype specific RT-PCR identified the isolate as BTV serotype 16. The study suggested that *Culicoides* oxystoma could be a potential vector for transmission of BTV in southern India.

Key words: Bluetongue virus 16, Serotype, VP2 gene, RT-PCR, Vector

Introduction

Bluetongue (BT) is *Culicoides* vector borne non-contagious but infectious viral disease of domestic and wild ruminants (MacLachlan, 1994). BT is caused by Bluetongue virus (BTV) of genus *Orbivirus* and family *Reoviridae*. BT is reported from several species of domestic and wild ruminants. Therefore, it is listed under multi species disease (OIE, 2013). The severe economic losses lead to prohibition of free movement of ruminant animals and their germplasm from BT endemic countries to BT free countries (Velthuis et al., 2009). BT is seen in severe form in white-tailed deer and sheep (Darpel *et al.,* 2007). Several animal species such as cattle, buffalo and goats may act as silent reservoirs and may transmit BT to other species of animals (Maclachlan *et al*., 2009).

BTV is an icosahedral virus having ten linear dsRNA genome segments. The genome of BT encodes 7 structural (VP1 to VP7) and 4 non-structural proteins (NS1, NS2, NS3/ NS3a and NS4). The outer capsid is made up of major and minor serotype specific VP2 and VP5 proteins . Non-structural proteins are produced in infected host cells and play major role in viral egression from infected host cell (Ratinier et al., 2011).

In India, 63 distinct *Culicoides* species are identified from different geographical regions .The highest *Culicoides* population and maximal annual BT outbreaks can be co-related during monsoon period.

Earlier reports have shown that *Culicoides oxystoma, Culicoides imicola* and *Culicoides peregrinus* are the predominant *Culicoides* sp. in Tamil Nadu (Ranjan et al., 2015). The peak activities of *Culicoides* during monsoon period in southern states such as Tamil Nadu, Karnataka and Andhra Pradesh have also been associated with severe BTV outbreak (Sreenivasulu et al., 2004). Isolation of BTV from *Culicoides* vector was also done in India (Dadawala et al., 2012).

Twenty seven distinct BTV serotypes have been reported worldwide so far (Jenckel et al., 2015). Serum neutralization and virus isolation in cell culture have proved the prevalence of 22 distinct BTV serotypes in India (Prasad et al., 2009; Susmitha et al., 2012). Several BTV serotypes such as 1-7, 11, 12, 14, 15, 16, 17, 19, 20 and 23 have been isolated from sheep and cattle in Tamil Nadu state (Prasad et al., 2009, Minakshi et al., 2012). In present study BTV serotype 16 was isolated from *Culicoides oxystoma* vector in Tamil Nadu state.

Materials and methods

Sample preparation

Culicoides oxystoma sample was obtained from Tamil Nadu state. For virus isolation head-thorax part having salivary gland of individual *Culicoides oxystoma* was cut and triturated in 1 mL of Phosphate buffer saline (PBS) containing Penicillin and Streptomycin antibiotic (Himedia, India). The resultant mixture was inoculated in day one old BHK 21 cell line. A total of 20 *Culicoides oxystoma* samples were inoculated in 24 well cell culture plate having day old BHK 21 cell monolayer. BHK 21 cells were maintained with 2 % minimum essential medium (Sigma, USA) till cell death (for 5 days). Afterwards blind passages were allowed to observe the cytopathic effect. In 9th passage one of the samples (CO/ABT/HSR) showed characteristics cytopathic effect such as cellular aggregation, foamy degeneration floating of the cells etc.

Viral nucleic acid extraction and RNA-PAGE

CO/ABT/HSR sample was allowed to grow in BHK 21 cells in 75 cm^2 of cell culture flask. Cells were harvested after appearance of about 75 % cytopathic effect (CPE). The harvested cell culture material was centrifuged at 2000 X g for 10 minutes (Remi, India). The cellular pellet was used for viral nucleic acid extraction using Guanidinium isothiocynate method (Chomoczynski and Sacchi, 1987). The nucleic acid was subjected to 8% RNA-PAGE analysis to visualize the viral RNA genome segments.

cDNA preparation and PCR

The cDNA was prepared from viral dsRNA using moloney murine leukemia (Mo-MuLV-RT) virus reverse transcriptase enzyme (Sibzyme, Russia) and random decamer primer (Ambion, USA) as per manufacturer's protocol in thermal cycler (Biorad i-Cycler, USA). The NS1 gene based group specific PCR was performed to confirm the samples as BTV. The group specific PCR was performed using primer pairs F (19-39 bp): 5' GTTGGCAACCACCAAACATGG 3' and R (384-361 bp): 5' TCCCACTTTTGCGCTAATCCTCAA 3' which produced an amplicon of 366 bp size in agarose gel electrophoresis (Kovi *et al.*, 2005).

The VP2 gene serotype specific PCR using primers specific to all the serotype was performed for serotyping of the virus. Individual serotype specific PCR was performed using serotype specific primers in a 20 µl reaction mixture having 20 µM of serotype specific primers, 2 µl cDNA, 0.4 µl of 10 mM dNTPs mix (Finnzyme, Finland), 3% DMSO, 4 µl 5X HF buffer and 0.4 U (2U/ µl) phusion high fidelity DNA polymerase (Finnzyme, Finland) in thermal cycler (Biorad iCycler, USA). The PCR amplification cycle was set as initial denaturation at 98°C for 3 minute, followed by 35 cycles of denaturation at 98°C for 15 second, annealing for 20 second at 54°C and primer extension at 72°C for 30 second. The final PCR extension was allowed at 72°C for 10 minute. The PCR products were visualized using gel documentation system (Biovis, USA) in 1% agarose gel (Sigma, USA) electrophoresis.

Results and Discussion

India is endemic for several BTV serotypes . Several BTV serotypes have been isolated from host animals in different geographical regions of India (Prasad et al., 2009; Minakshi et al., 2012; Ranjan et al., 2013, 2014, 2016). Being tropical climate, India is also endemic for *Culicoides* vectors. Recently, BTV1 was isolated from *Culicoides Oxystoma* in Gujarat state (Dadawala et al., 2012). In the present study, BTV isolate is isolated from *Culicoides Oxystoma* vector from Tamil Nadu. BTV isolate CO/ABT/HSR was isolated from head and thorax region of *Culicoides Oxystoma* vector. Head-thorax region was triturated and inoculated to BHK 21 cell culture. After 9 blind passages in BHK 21 cell line, it showed characteristics cytopathic effect such as aggregation and rounding of cells, vacuolation in cells, floating of dead cells in medium within 48 hours of inoculation (Figure 1).

Figures

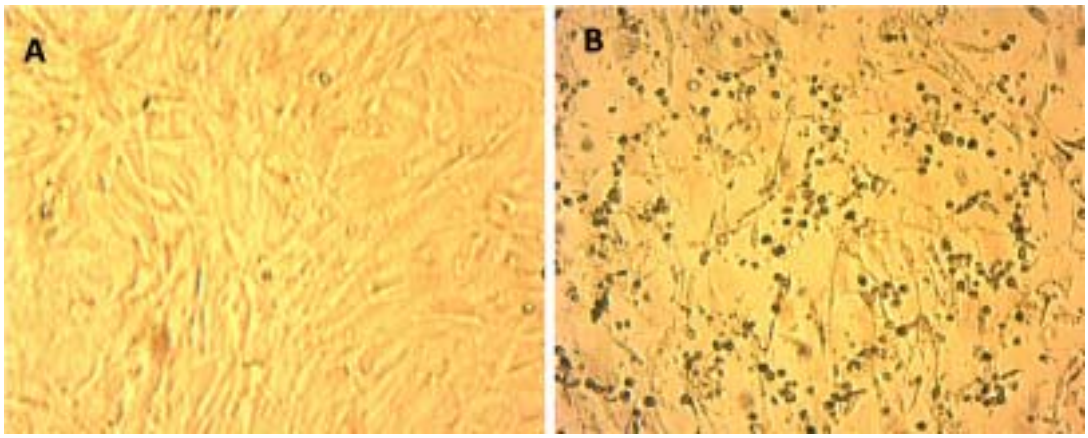

Figure 1: BHK-21 cell line after 48 hours of culture. (A): Uninfected BHK-21 cell monolayer (B): BTV infected BHK-21 cells (48 hours) showing characteristic cytopathic effect.

Figure 2: 8% RNA-PAGE analysis of CO/ABT/HSR isolate. The right side values indicate BTV segments.

The nucleic acid (dsRNA) was extracted from cell culture grown virus using Guanidinium isothiocynate method (Chomoczynski and Sacchi, 1987) and screened by RNA-PAGE and silver staining. The RNA-PAGE analysis showed BTV specific migration pattern (3:3:3:1) of viral dsRNA (Figure 2). The viral nucleic acid was allowed for cDNA synthesis using random decamer primer. The cDNA was allowed for BTV group specific NS1 gene based RT-PCR. The NS1 gene PCR amplicon showed 366 bp product size on 1% agarose gel electrophoresis (Figure 3). Thus, BTV specific cytopathic effect (CPE) in BHK-21 cell culture, specific migration pattern of viral nucleic acid (dsRNA) (3:3:3:1) in RNA-PAGE and 366 bp amplicon of NS1 gene RT-PCR confirmed the samples as BTV.

Further, the cDNA was allowed for VP2 gene serotype specific RT-PCR using serotype specific primers for all the available BTV serotypes. Upon agarose gel electrophoresis PCR amplicon visualized 768 bp amplicon on agarose gel electrophoresis, specific for BTV serotype 16 (Figure 4). However, the remaining serotype specific primers did not show any amplification. Thus CO/ABT/HSR isolate was serotyped as BTV16.

India is a tropical country which is suitable for *Culicoides* vector growth and multiplication. Globally more than 1400 different *Culicoides*

species have been reported. However, 63 are reported from different geographical locations of India (Ranjan et al., 2015). The persistence of large numbers of *Culicoides* vectors explains the prevalence of 22 different BTV serotypes in India (Susmitha et al., 2012). Tamil Nadu state is one of the major states infested with severe BT outbreak. Based on serum neutralization and virus isolation several BTV serotypes have been reported from Tamil Nadu (Prasad et al., 2009; Minakshi et al., 2012). BTV16 was also reported from sheep population in Tamil Nadu (isolate IND/Goat/2010/16/HSR) (Minakshi et al., 2012) and Andhra Pradesh (isolate, VJW66/IND, GNT-27/IND and MBN48/IND) (Minakshi et al., 2015).

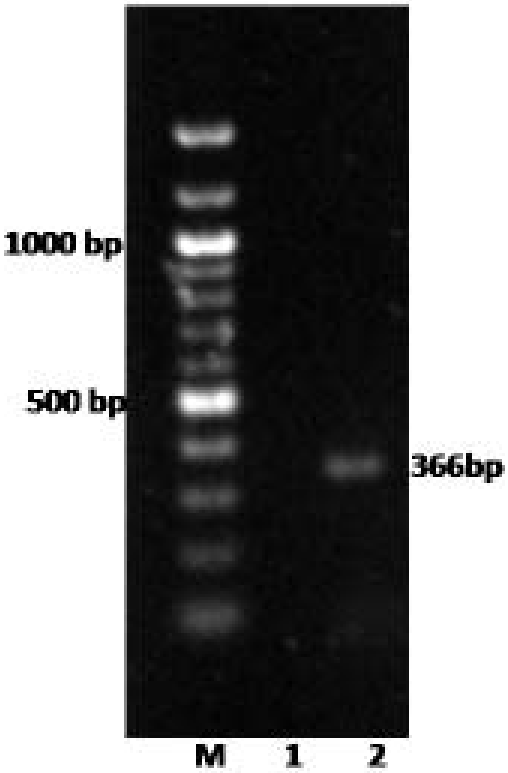

Figure 3: 1% agarose gel electrophoresis of NS1 gene RT-PCR of CO/ABT/HSR isolate. Lane M: Ladder 100bp, 1: Negative control (NFW), 2: CO/ABT/HSR. The left side numbers indicate DNA marker and right side PCR product size.

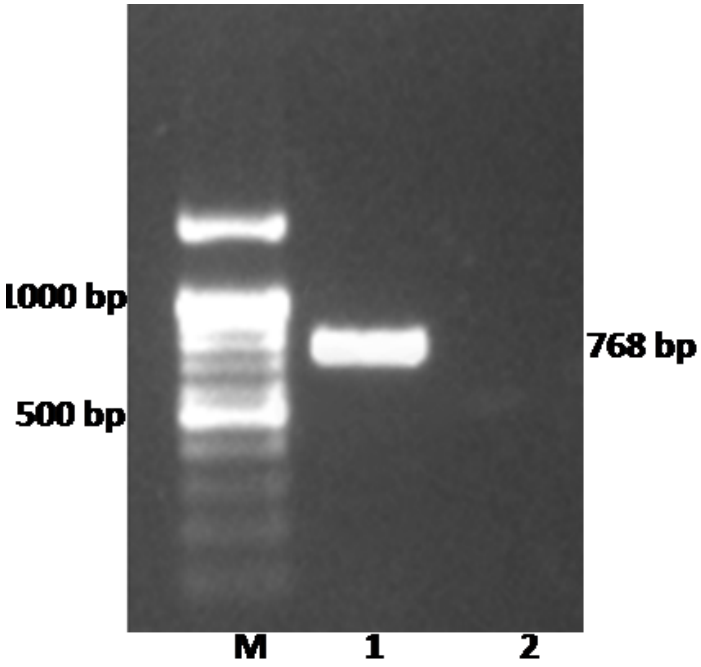

Figure 4: 1% agarose gel electrophoresis of BTV16 VP2 gene specific RT-PCR of CO/ABT/HSR isolate. Lane M: Ladder 100bp, 1: CO/ABT/HSR, 2: Negative control (NFW). The left side numbers indicate DNA marker and right side PCR product size.

Minakshi (2010) reported that the two neighbouring states Andhra Pradesh and Tamil Nadu are also endemic for a known BTV vector (*Culicoides oxystoma*). Thus, it may be assumed that BTV16 might be transmitted from Andhra Pradesh to Tamil Nadu or vice versa either through migrating sheep population, vectors or through wind velocity. For successful control of BTV, knowledge about the molecular epidemiology of all the BTV serotypes and vector potential of different *Culicoides* species is essential. The conventional serotyping assay along with molecular tests such as RT-PCR based serotyping can be used for BTV surveillance in India. It can also be supplemented with nucleic acid sequencing. The surveillance information can be incorporated in BTV vaccine development.

Conclusion

BTV 16 has been isolated from several host species such as cattle, sheep, goat in India. The BTV isolate (CO/ABT/HSR) was isolated form *Culicoides Oxystoma* vector from Tamil Nadu. The newly

identified isolate was allowed for group specific (NS1 gene) RT-PCR for confirmation of isolate as BTV and serotype specific (VP2 gene) RT-PCR for serotyping. The VP2 gene based RT-PCR confirmed the CO/ABT/HSR isolate as BTV serotype 16. To control BT in Indian states close surveillance regarding vector movement, import of live animal and its products should be practiced.

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Conflict of Interest: All authors declare no conflict of interest.

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