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Molecular Detection of *Culicoides* Midges Responsible of BTV Transmission

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Abstract

Bluetongue (BT) is an infectious, non-contagious arthropod borne viral disease of wild and domestic ruminants especially sheep which inflict major losses on subsistence of sheep farmers in southern India. Affected sheep may have erosions and ulcerations on the mucous membranes, dyspnea, lameness and inflammation of the coronary band. The disease is caused by bluetongue virus (BTV), the type species of the genus Orbivirus and family Reoviridae. BTV is transmitted between their ruminant hosts by certain species of haematophagous insects such as Culicoides (biting midge). BT is OIE list 'A' multispecies disease. Till date more than 27 serotypes have been reported. The present study was carried out with the objectives to identify and characterize the Culicodes species available in our laboratory. The Culicoides DNA samples extracted using a non-destructive DNA extraction method were amplified using mitochondrial cytochrome oxidase I (COI) gene specific primers. An amplicon of 523 bp was obtained in amplified sample. The samples were further morphologically confirmed as culicoides oxystoma by stereo microscope. In this study, 25 Culicoides samples were processed for molecular detection by PCR. Out of these 20 samples were identified as C. Oxystoma. Morphological study by stereo microscope also confirmed these 20 samples as C. Oxystoma.

Keywords: Bluetongue Virus; Non-Destructive DNA Extraction; *Culicoides Oxystoma*; PCR.

Introduction

Bluetongue (BT) disease is caused by Bluetongue virus (BTV). BTV is type species of genus *Orbivirus* within *Reoviridae* family. BT may cause infection in domestic and wild ruminants, camelids and occasionally in carnivores (Attoui *et al.*, 2009; Meyer *et al.*, 2009). BT is transmitted by biting of certain species of *Culicoides* (biting midges) vectors. BT causes huge economic losses to live stock sector and farming community. Globally, more than twenty seven distinct BTV serotypes have been recognized so far (Hofmann *et al.*, 2008; Maan *et al.*, 2011; Jenckel *et al.*, 2015). Including recent isolation of BTV5 and BTV24 a total of 24 different BTV serotypes have been reported from India (Prasad *et al.*, 2009; Krishnajyothi *et al.*, 2016; Hemadri *et al.*, 2016).

The BT virion particle is non-enveloped, icosahedral in structure. It has complex architecture with inner core and outer capsid. The inner core and outer capsid have diameter of 75 nm and 90 nm respectively (Grimes *et al.*, 1998; Nason *et al.*, 2004). BTV genome is made up of 10 segments

of dsRNA. The BTV genome segments encode 7 structural (VP1 to VP7) and 5 non-structural (NS1, NS2, NS3/NS3A, NS4 and NS5) proteins (Firth, 2014). The inner core of virus is composed of major (VP3 and VP7) and minor (VP1, VP4 and VP6) proteins which is surrounded by outer capsid, composed of VP2 and VP5 proteins. The virus specific non-structural proteins are produced only in infected host cells. The NS1 and NS2 proteins form tubules and inclusion bodies respectively in infected host cells.

India has tropical and rainy climate, which favour breeding of *Culicoides* vectors. Globally, more than 1400 different *Culicoides* species have been reported. Out of these, 63 are identified from different geographical regions of India such as Assam and West Bengal (Halder *et al*., 2013), Tamil Nadu (Ilango, 2006), Maharashtra (Narladakar *et al*., 1993), Andhra Pradesh (Reddy and Hafeez, 2008) and Karnataka (Archana *et al*., 2014). Very few species of *Culicoides* have been demonstrated as vectors for BTV transmission. BTV serotype 1 was isolated from *Culicoides oxystoma* vector in Gujarat state (Dadawala *et al*., 2012). Recently, BTV was also isolated from *Culicoides schultzei complex* from West Bengal state (Joardar *et al*., 2016). There is a critical need of study to analyze the vector-virus relationship. Several *Culicoides* species have been reported as putative BTV vector in India viz., *Culicoides actoni, Culicoides brevitarsis Kieffer, Culicoides dumdum, Culicoides fulvus, Culicoides imicola Kieffer, Culicoides oxystoma Kieffer* and *Culicoides peregrinus Kieffer* (Prasad *et al*., 2009). However, this implication is reported based on vector competence data collected and analyzed in other countries. Recently, *Culicoides oxystoma* and *Culicoides peregrinus* were reported as potent vector of BTV 16 and 23 in India (Ranjan et al., 2017a; 2017b). However, there is a scanty data available on vector competence of *Culicoides* to BTV in India.

Several varieties of ecological zones are found in the Indian subcontinent which harbors various *culicoides* species responsible for BTV transmission. However, the *Culicoides* species which transmit BTV in Indian livestock and wild-life are not fully characterized (Patel *et al*., 2007). Establishing a fundamental base is prerequisite for *culicoides* species identification in India for understanding BTV epidemiology correctly. Moreover, questions relating to *culicoides* populations phylogenetic and taxonomic relationships also exist within India with other global *culicoides* vector species. These questions may be answered through the morphological and sequence based molecular identification of *Culicoides* spp. from India. Thus keeping the above perspective in view, the present study was proposed for molecular and morphological detection of *culicoides* vector responsible for bluetongue virus infection.

Materials and Methods

Samples

A total of 25 *Culicoides* samples, available in our laboratory were used for molecular detection and morphological identification.

Culicoides *species identification*

The *Culicoides* species were detected using PCR and morphologically confirmed by stereo microscopy.

Extraction of DNA from ***Culicoides*** *samples*

The total *Culicoides* DNA was extracted using a non-destructive method standardised in lab and species identification was done through *cytochrome oxidase I* (*COI*) gene specific PCR.

Incubation of samples

Culicoides DNA extraction was done using non-destructive method standardised in laboratory. An aliquot of 200 µL of digest solution was taken in sterile and labelled 1.5 mL tubes. The *Culicoides* midges abdomen region were individually transferred to tube containing digest solution and incubated at 40^0C for 16 hours in a thermal-cycler (Quanta Biotech, USA). The *Culicoides* samples were removed from the tube and remaining mixture was further incubated at 70^0C for 15 minutes to inactivate the enzyme. Reaction tubes were removed from thermal-cycler and stored at 4^0C till further use.

Precipitation of DNA

In a 1.5 mL micro centrifuge tube, 20 µL Sodium Acetate, 600 µL ice cold 100% ethanol and 1µL glycogen were added sequentially. The DNA mixture stored at 4^0C was added to this tube. The resultant mixture was mixed and stored at -20^0C for 1 hours followed by centrifugation at 13,000 rpm (10,000Xg) at 4^0C from 30 minutes. Supernatant was discarded and resultant pellet was air dried and dissolved in 50 µL of elution buffer (10mM Tris HCL, pH 8.0) and used for PCR assay.

*Amplification of **Culicoides Cytochrome oxidase I** (COI) gene by PCR*

Culicoides spp. identification was done on the basis of mitochondrial COI gene sequences (Dallas *et al.*, 2003). The PCR assay was conducted in a 20 µL volume containing 5X Phusion HF Buffer 4 µL, DMSO 0.6 µL, 10mm dNTPs mix. 0.4 µL, Phusion polymerase enzyme 0.2 µL, 0.2 µL of 15 µM of each forward (C1-N-2191-mod: 5′ CCCGGTAAAATTAAAATATAAACTTC 3′) and reverse (C1-J-1718-mod: 5′ GGAGGATTTGGAAATTGATTAGTTCC 3′) primer (Dallas *et al.*, 2003), 2 µL of DNA template and 12.4 µL of nuclease free water. Thermal condition was set as initial denaturation at 98 °C for 2 minutes followed by 35 cycles of denaturation at 98^0C for 15 second, annealing at 56^0C for 20 second, elongation at 72^0C for 30 second. Final elongation was done at 72^0C for 10 minute.

Visualization of amplified PCR products by agarose gel electrophoresis

The amplified PCR products were analysed on 1.0% agarose gel containing 0.5 µg/mL of ethidium bromide. The gel was visualized under UV trans-illuminator (Biovis, USA).

Results and Discussion

*Molecular detection of Indian **Culicoides** species*

For confirmatory identification of various *Culicoides* species, molecular technique such as PCR based detection system is essential. DNA from a total number of 25 *Culicoides* samples was isolated using non-destructive method. Out of these, 20 were found PCR positive with C1-N-2191-mod and C1-J-1718-mod COI gene specific primers with the amplified region of 523 bp (Figure 1).

*Morphological identification of **culicoides** samples*

Further for the confirmation of species of *culicoides* vector morphological identification through wing pattern of *Culicoides* sp. was done under stereo microscope using standard illustration (Bellis, 2014). All the 20 samples showed morphological feature similar to *Culicoides oxystoma* of apical and basal pale markings in anal cell (bb), apical pale spot in cell m1 not reaching to margin of wing (bbb), and pale spots in anal cell (bbbb). Hence, all *culicoides* samples were found to be *culicoides oxystoma* (Figure 2).

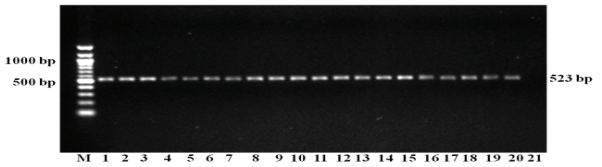

Fig. 1: Agarose gel electrophoresis of *Culicoides oxystoma* samples, showing 523 bp amplification of COI gene. Lane M: 100bp marker, Lanes 1-20: *Culicoides oxystoma* samples, Lane21: Nuclease free water negative control.

Discussion

BTV is transmitted by certain species of *Culicoides* vectors which is highly dependent on environmental changes (Wittmann *et al.*, 2001). Along with vector species, environmental changes can influence the incidence and evolution of vector transmitted infectious diseases such as BT (Jimenez-Clavero, 2012). Recent study by several researchers have shown the changes in global pattern of BTV serotype distribution with introduction of exotic BTV strains and serotypes in India, Europe, Australia, Korea, North America, Middle east and South America (Shimshony,

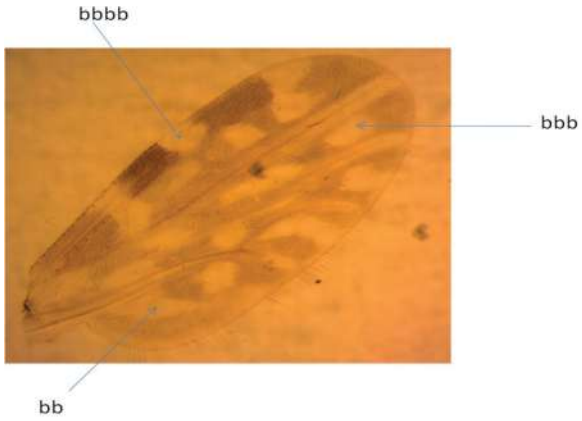

Fig. 2: Morphological identification of *Culicoides oxystoma* through wing pattern

2004; Brenner *et al.*, 2010; Maan *et al.*, 2012; Boyle *et al.*, 2012; Maclachlan *et al.*, 2013). This changed global pattern of BTV distribution can be linked to several factors such as climatic changes, increased travel and trade (Purse *et al.*, 2005). As far as BTV transmission through *Culicoides* vector in India is concerned, very limited information is available. In India, first evidence of involvement of *Culicoides* sp. in BTV transmission was reported in Haryana state (Jain *et al.*, 1988). Subsequently, *Culicoides oxystoma* was identified from animal farms where BTV sero-conversions were reported (Bhatnagar *et al.*, 1997).

The tropical humid climate favors the *Culicoides* vector breeding. About 63 different *Culicoides* species have been morphologically identified from different regions of India (Reddy and Hafeez, 2008; Halder *et al*., 2013; Archana *et al*., 2014). Molecular identification of *Culicoides* species has also started in India (Minakshi, 2014; Ranjan et al., 2017a; Ranjan et al., 2017b). For species confirmation of *Culicoides* through molecular technique total DNA was extracted from abdomen region of *Culicoides sp*. using non-destructive method and allowed for COI gene based PCR (Dallas *et al*., 2003) followed by nucleic acid sequencing. The abdomen region of *Culicoides* vector was also used earlier by researcher for DNA extraction followed by species confirmation using COI gene based PCR and nucleic acid sequencing (Foxi *et al*., 2016). The morphological identification is considered as robust technique for putative BTV vector (*Culicoides* species) identification, with no misidentifications of *Culicoides* species (Harrup *et al*., 2016).

The earlier researcher had also carried out the COI gene sequence and DNA barcoding based species confirmation of some of the *Culicoides* species such as *Culicoides Imicola, Culicoides actoni, Culicoides brevitarsis, Culicoides oxystoma, Culicoides peregrinus, Culicoides brevitarsis, Culicoides huffi, Culicoides innoxius, Culicoides kepongensis, Culicoides mesghalii, Culicoides peliliouensis, Culicoides peregrinus* and *Culicoides similis* in Southern India (Harrup et al., 2016). Seven new *culicoides* species has been reported for the Tunisian Funna and out of them five were suspected/confirmed for either BTV or EHDV vector (Sghaier et al. 2017). The subgenera contain *Culicoides, Monoculicoides*. *Hoffmania, Haematomyidium* and *Avaritia* (the main vectors for the bluetongue viral disease) are monophylatic, whereas *Oecacta*, the subgenus is paraphyletic (Augot et al. 2017). Thus, mitochondrial COI gene based molecular identification along with morphological study of wing pattern can be used for *Culicoides* species confirmation.

Conclusion

DNA was extracted using non-destructive method from abdomen of all *culicoides* samples. The extracted samples were invariably amplified by polymerase chain reaction to amplify the desired size of COI gene. Out of the 25 samples, 20 were found positive and amplified the product size of 523 bp. Morphological identification were found similar to the *C. oxystoma*.

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