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Dual infection with bluetongue virus serotypes and first time detection of serotype 5 in India.

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Running head: First time detection of BTV-5 in India

Summary

Bluetongue is endemic in India and has been reported from most Indian states. Of late, the clinical disease is most frequently seen in the southern states of Andhra Pradesh, Telangana (erstwhile Andhra Pradesh state), Tamil Nadu and Karnataka. Our analysis of diagnostic samples from BT outbreaks during 2010-11 from the state of Karnataka identified BTV serotype 5 (BTV-5) for the first time in India. One of the diagnostic samples (CH1) and subsequent virus isolate (IND2010/02) contained both BTV-2, as well as BTV-5. Seg-2 sequence data (400 bp: nt 2538 to 2921) for IND2010/02-BTV5, showed 94.3% nt identity to BTV-5 from South Africa (Accession no. AJ585126), confirming the virus serotype, and also indicating that Seg-2 was derived from a western topotype, which is in contrary to serotype 2, that belongs to eastern topotype. BTV-5 has been recently reported from Africa, China, French islands and the America. Although the exact source of the Indian BTV-5 isolate is still to be confirmed, recent identification of additional exotic serotypes in India is of real concern and might add to the severity of the disease seen in these outbreaks.

Keywords: Bluetongue, Serotype 5, India, Topotype

Introduction

Bluetongue (BT) is an economically important arthropod-borne viral disease that causes high case-fatality rates in naive sheep and white tailed deer. Bluetongue virus is classified as the prototype species of the genus *Orbivirus,* within the family *Reoviridae,* and includes a total of 27 distinct serotypes (Zientara et al., 2014), as well geographic groups and sub-groups (topotypes). Further, additional BTV strains have been identified that may represent new serotypes (Wright, 2013; Peter Mertens-unpublished results). The bluetongue virus (BTV) can infect all ruminants, including goats, cattle, buffaloes, camels and deer, as well as other wild artiodactyls, and can infect some carnivores via oral route (Alexander et al., 1994). BTV is transmitted primarily by adults of certain species of biting midges (*Culicoides* spp.) that are more abundant and active in hot and humid climates. It can also be transmitted vertically (Darpel et al., 2009), or orally, in its ruminant hosts. Consequently, BT is endemic in many countries around the world that lie between 35°S and 40°N latitudes, although in certain regions of North America, Europe and China, the disease has been detected up to 50°N (Gibbs and Griener, 1994; Tabachnick, 2004). In the countries where it is endemic, the disease can be cyclical, showing significant variations in both the size and severity of disease outbreaks (Prasad et al., 2009). In temperate regions, the abundance and activity of adult midges are seasonal (usually in summer and autumn), resulting in seasonal variations in the efficiency and frequency of BTV transmission, and BT may only occur as periodic outbreaks.

Bluetongue is endemic in India and has been reported from most Indian states; however, clinical disease is most frequent in the southern states of Andhra Pradesh, Telangana (former Andhra Pradesh state), Tamil Nadu and Karnataka (Prasad et al., 2009). Records available (1996-2016) both at the ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI) and The Animal Disease Surveillance Scheme (ADSS), show that BT outbreaks have occurred almost every year, barring the years 1997 & 2012, in the latter state, with 73 outbreaks occurring between July 2010 and January 2011. During this period, our analysis of diagnostic samples from BT outbreaks in Chitradurga and Chikkaballapura districts of Karntaka state have identified BTV serotype 5 (BTV-5) for the first time in India. One of the diagnostic samples and subsequent virus isolates contained both BTV-2 [which was already known to be present in India (Prasad et al., 2009)] as well as BTV-5. We report events leading to this initial isolation and characterization of BTV-5 in India.

Materials and methods

Outbreak data and collection of clinical samples

Bluetongue outbreak data were obtained from the ADSS, Department of Animal Husbandry and Veterinary Services, Government of Karnataka, for the years, 2010-11 and from the active surveillance work under the 'All India Network Programme on Bluetongue' (AINPBT). For the current study, heparinised blood was collected from suspect cases in the State along with detailed epidemiological information concerning outbreak dates, clinical signs, flock size, number of animals affected, number of fatalities, species and breed of animals affected and source of infection (if known). Geographic coordinates of the outbreak were determined using a global positioning system. Details of outbreaks attended by our team is given in Table 1.

Virus isolation

Heparinized blood samples were transferred on ice to the laboratory within 12 h of collection. Lysed blood cells were inoculated into 11-day-old embryonated chicken eggs (ECE), via an intravenous route (Goldsmit and Barzilai 1985; Clavijo 2000). Five embryonated chicken eggs were used for each clinical sample and observed daily for embryo mortality for about a week. After two blind passages in embryonated chicken eggs, clarified embryo homogenates were passaged three times in baby hamster kidney cells (BHK-21 cells) irrespective of whether cytopathic effects (CPE) were observed.

RT-PCR-based typing of virus isolates

Total RNA was extracted from infected BHK cells using Trizol Reagent (Invitrogen) (Attoui et al., 2000). The purified RNA was analysed by electrophoresis on 1% agarose gel to identify its RNA electropherotype, which is indicative of *Orbivirus* species. RT-PCR assays using group-specific segment 7 (Seg-7)-based primers were performed to confirm the isolates as BTV (Anthony et al., 2007). Genomic RNA was subjected to either serotype-specific conventional RT-PCR assays for 27 serotypes using SuperScript™ III one-step RT-PCR system (Invitrogen)(Maan et al., 2012), or by multiplex RT-PCR assays (Hemadri et al., unpublished data).

Sequencing and phylogenetic analysis

For confirmation of BTV type, Seg-2 amplicons of the expected size were used for direct sequencing using a 'Big dye cycle sequencing kit' with an ABI 3730 DNA sequence analyzer. Analyses and comparisons of the nucleotide sequence data were carried out using BLAST (NCBI), MEGA 6 (Tamura et al., 2013) and Lasergene software ver. 5.0.

Results and discussion

According to the data available with the Animal Disease Surveillance Scheme, Department of Animal Husbandry and Veterinary Services, Government of Karnataka, a total of 73 bluetongue outbreaks occurred in the state between July 2010 and January 2011. In November 2010, bluetongue outbreaks were reported from the southern districts of Chitradurga and Tumkur. Nineteen of these outbreaks involved four taluks (blocks or tehsils) of Chitradurga district, and while three were reported from Sira taluk of Tumkur district. The disease remained active in Chitradurga district in the month of December, and in addition, new cases were reported from Gauribidanur taluk of the neighbouring Chikkaballapur district. By January, the disease was in 'full flow' (15 outbreaks) in Chikkaballapur district, with outbreaks reported from two more taluks, viz., Siddlaghatta and Chintamani. In January, the number of outbreaks in Chitradurga (n=2) and Tumkur (n=1) declined.

Of these, our team attended outbreaks in Madakaripura, Dandina Kurubara Hatti, Hampayyana Malige Gollara Hatti, Belaghatta, Haikal, Bommakkanahalli, Bettada Nagenahalli villages (all in Chitradurga taluk) and the Govt Sheep Farm, Nayakana Hatti (Challakere taluk) of Chitradurga district in the month of December, 2010. In January 2011, suspected outbreaks were also attended in Gundugere, and Irgampalli villages of Siddlaghatta taluk, and Guttur Chindodpe village in Chintamani taluk of Chikkaballapura district. Details of these outbreaks are given in Table 1 and Fig. 1. Since exact starting date of outbreak was not available, a rough estimate of starting period was ascertained by interaction with the farmers and by examination of affected animals. For example, a farmer in Madakaripura village first noticed the disease in October 2010, and examination of the sheep flock (in December, 2010, during our visit) indicated that the disease had already passed off. However, examination of sheep flocks and interaction with farmers in other villages provided information that the disease had only started 15 days prior to our visit (last week of November, 2010). Detailed analysis of collected outbreak data indicated variations in the duration of disease outbreaks as well as case fatality rates (6.6% to 50%), suggesting the involvement of multiple BTV strains or serotypes. In order to confirm bluetongue disease and also to identify serotypes involved in these outbreaks, 2-3 blood samples were collected from suspected animals from each sheep flock within each village, in heparinized vacutainers, and transported on ice to the laboratory.

Serial passaging of 32 heparisinised blood samples in embryonated chicken egg (ECE) and BHK 21 cells yielded 3 isolates (IND2010/02, IND2010/03, IND2010/04), the details of which are given in the Table 2. After the initial identification of BTV by electropherotyping and Seg-7 RT-PCR, the isolates were subjected to serotype analysis by multiple-monoplex and/or multiplex RT-PCR assays (Maan et al., 2012;

Hemadri et al., unpublished results), which identified BTV-2 in all the three isolates; however, one of the isolates [IND2010/02], from Gundugere also contained BTV-5. Comparison of partial BTV-2 Seg-2 nucleotide sequences (nt positions 1079-1693 with reference to RSArrrr/02) from the above exclusively BTV-2 isolates (IND2010/03 and IND2010/04) showed that these were very closely related to each other (99.49% nt identity) and to the isolate from Gundugere (IND2010/02-BTV2; 99.49-99.83%).

It is important to note that in the village Dandina Kurubara Hatti, sheep in one of the flocks had been brought from Guttur Chindodpe village just prior to the outbreak (second week of November, 2010), , a village near Chintamani in Chikkaballapur District. Incidentally, these two villages reported the disease almost at the same time (December, 2010) and the high level of nt sequence identity is consistent with isolates derived from a single epizootic. Comparison of nt sequences of these three isolates with a strain isolated from Andhra Pradesh during 2010 (APKRM08/10) showed nt sequence identity of 98.99-99.67 %, while a nt identity of 96.99-97.71% was observed with 2007 BTV strains (Mahaboob Nagar, Tirupati) from the same state, indicating the sharing of recent common ancestry among the strains (Fig. 1). This provides further evidence of substantial interstate migration of sheep flocks that occurs in these regions, although movement of the insect midge or other subclinically infected ruminants cannot be ruled out.

The BTV-5 Seg-2 sequence data (400 bp: nt 2538 to 2921) for IND2010/02-BTV5, shows 94.3% nt identity to BTV-5 from South Africa (Accession no. AJ585126), confirming virus serotype, as well as indicating that Seg-2 was derived from a western topotype of BTV-5 (Fig. 2), which is in contrast to the serotype 2 strains studied here, which belong to eastern topotype. This level of sequence identity indicates that the BTV-5 isolate is derived from the introduction of an exotic virus strain (from outside India). At the time of writing of this article BTV-5 was isolated from the samples originating from Telangana state (Rao PP et al., unpublished results) The exact origin of this lineage of BTV-5 and its route of entry into sheep flocks in Gundugere and those from Telangana state will need further investigations, including the full genome sequence-characterisation.

BTV-5 was initially identified in South Africa (Howell, 1960), and has subsequently been isolated from Cameroon (Ekue et al., 1985), Nigeria (Mertens and Attoui, 2016; http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/BTV-Nos.htm#BTV-5), United States of America (Johnson et al., 2007), Israel (Brenner et al., 2010), Caribbean island of French Guadeloupe (Legisa et al., 2014), and very recently from China (Yang et al., 2016). Although there is previous serological evidence for antibodies to BTV-5 in the India (Prasad et al., 2009), this serotype was regarded as ‘exotic’ until its isolation, as reported here. Therefore, it is difficult to determine if this strain of BTV-5

is an introduction of a new strain, or represents the detection of an already existing strain which re-emerged recently.

The presence of mixed serotypes in individual diagnostic samples and cell culture recovered BTV isolates has previously been reported (Maan et al., 2015). The co-circulation of multiple serotypes in endemic areas inevitably leads to BT outbreaks involving more than one serotype (Brenner et al., 2010; Maan et al – unpublished data), and there is a report of involvement of three serotypes (4, 9 and 16) in an outbreak in Greece during 1999 (Oya Alpar et al., 2009).

The spread of exotic BTV serotypes to new geographical areas can occur through the movement of invertebrate and/or vertebrate hosts in addition to importation of live animals, embryos or semen, or through the illegal use of imported live-vaccines. Although the exact source of the Indian BTV-5 isolate is still to be confirmed, recent identification of additional exotic serotypes in India is of real concern and might add to the severity of the disease seen in these outbreaks.

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Table 1: Details of bluetongue outbreaks attended in Chitradurga and Chikkaballapur districts of Karnataka State during 2010-2011

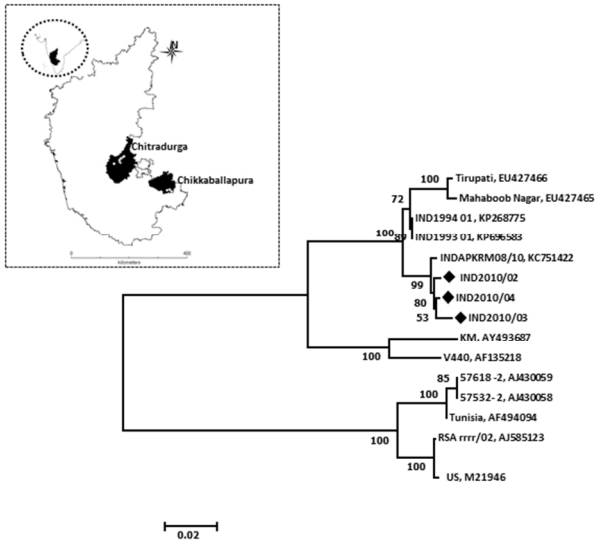
S. No.	Village Name	District	Month/Year of Outbreak	Number of animals affected	Number of animals died	Number of susceptible animals in flock	Case fatility rate (CFR)
1	Madakaripura	Chitradurga	Oct-Nov/2010	30	15	100	50%
2	Dandina Kurubara Hatti	Chitradurga	Nov-Dec/2010	20	04	95	20%
3	Hampayyanamalige Gollara Hatti	Chitradurga	Nov-Dec/2010	300	100	800	33.3%
4	Belaghatta	Chitradurga	Nov-Dec/2010	18	6	100	33.3%
5	Haikal	Chitradurga	Nov-Dec/2010	100	50	400	50%
6	Bommakanahalli	Chitradurga	Nov-Dec/2010	40	6	300	15%
7	Nayakana Hatti	Chitradurga	Nov-Dec/2010	60	10	400	16.6%
8	Bettada nagenhalli	Chitradurga	Nov-Dec/2010	60	4	400	6.6%
9	Gundugere	Chikkaballapura	Jan 2011	10	5	80	50%
10	Guttur Chindodpe	Chikkaballapura	Jan 2011	5	1	20	20%
11	Iragampalli	Chikkaballapura	Jan 2011	10	2	100	20%

Table 2: Details of BTV isolates recovered in cell culture

S.No.	Village Name	District	Original Sample ID	Accession number	No. of passages in ECE/BHK
1	Gundugere	Chikkaballapura	CH1	IND2010/02*	2/3
2	Dandina Kurubara Hatti	Chitradurga	C-12	IND2010/03	2/3
3	Guttur Chindodpe	Chikkaballapura	CH5	IND2010/04	2/3

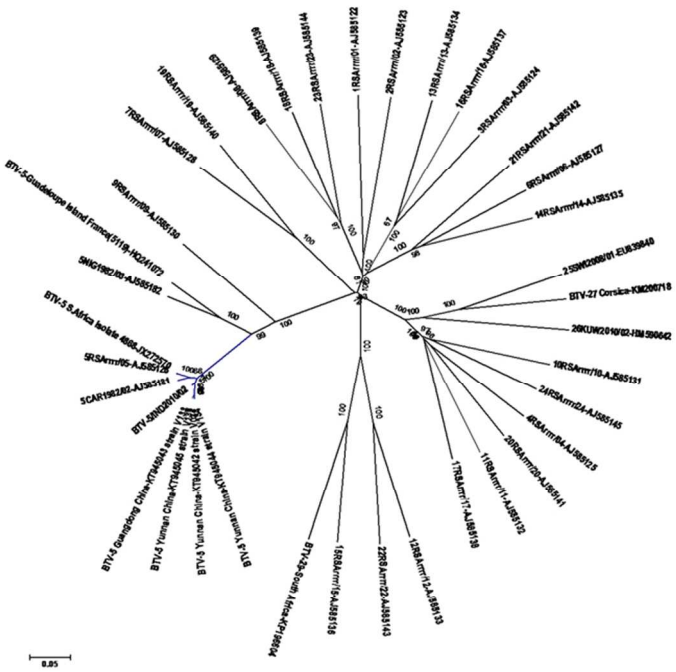
* Original isolate, IND2010/02, has been designated as IND2010/02-BTV5 and IND2010/02-BTV2 depending on the serotype in the manuscript

Fig. 1.



Neighbour joining tree showing the grouping of Karnataka BTV-2 isolates (filled diamonds) with others in the genomic segment-2 region. The optimal tree with the sum of branch length = 0.37262761 is shown. The bootstrap values (1000 replicates) are shown as percentage at the nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Map showing the locations of bluetongue outbreaks attended in Chitradurga and Chikkaballapur district are shown in the inset.
254x190mm (96 x 96 DPI)

Fig. 2.



Phylogenetic analysis based on Seg-2/VP2 gene of Indian isolate of BTV-5 with other global isolates of BTV-5. Phylogenetic relationship of partial Seg-2 nucleotide sequences was inferred in MEGA 6 using neighbour-joining method and tested by bootstrapping 1000 replicates. Indian isolate of BTV-5 (IND2010/02) is depicted with filled diamond. The node labels in each figure refer to bootstrap confidence values.
254x190mm (96 x 96 DPI)