



Research Article

Prevalence of Midges: Potent Vectors for Bluetongue Virus Infection in West Bengal, India

Arkendu Halder, Siddhartha Narayan Joardar*, Panchanan Parui¹, Dhriti Banerjee¹, Vikas Kumar¹, Indranil Samanta, Chandan Lodh²

Department of Veterinary Microbiology; West Bengal University of Animal and Fishery Sciences; Post Office –Belgachia, Kolkata–700037; ¹Diptera Section, Zoological Survey of India, Post Office –New Alipore, Kolkata–700059, West Bengal, India; ²Department of Veterinary Medicine, Ethics and Jurisprudence, West Bengal University of Animal and Fishery Sciences; Post Office –Belgachia, Kolkata–700037.

*Corresponding author: joardar69@gmail.com

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ABSTRACT

With the aim of identifying the potent vector(s) of bluetongue virus (BTV) that is circulating in eastern Indian state, West Bengal, attempts were made to detect and isolate BTV from *Culicoides* midges, collected from localities having good number of sero-positive animals. Prevalence of various species of *Culicoides* in the state along with the dynamics of *Culicoides* population, their habitat and weather parameters were also studied to reveal their possible relation. *Culicoides* midges were collected from three different agro-climatic zones of West Bengal in different post monsoon months. Meteorological data of collection-months of that area were obtained and incorporated to investigate the environmental conditions at the collection time. Habitat and probable breeding areas of *Culicoides* sp. were also noted. For isolation of BTV, non-engorged midges were pooled to prepare sample and virus was isolated using embryonated chicken eggs, followed by isolation on cell culture (BHK–21). Confirmation of BTV was made by RNA–PAGE and RT–PCR. The present study reveals the vector competence of *Culicoides schultzei* that harbored BTV–16. Typical association between the dynamics of *Culicoides* population, their habitat and weather parameters were observed in the present study. It is concluded that West Bengal state possesses potent vector of bluetongue virus that warrants serious attention in near future.

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INTRODUCTION

The family Ceratopogonidae (Order Diptera) currently includes 6056 species; of which 1322 species belong to the genus *Culicoides* (Borkent, 2012). Most of the *Culicoides* midges are hematophagous, and are particularly important as a biting nuisance to humans and to livestock as vectors of several arboviruses of domestic and wild animals. Bluetongue Virus (BTV) and African Horse Sickness Virus (AHSV) are both *Culicoides*–transmitted and are formally classified by OIE (Office International des Epizooties). Bluetongue is an infectious, inoculable, viral disease of sheep. Cattle and goats may harbor the virus; however, they generally fail to show pronounced clinical symptoms. The BTV belongs to the genus *Orbivirus*, family *Reoviridae* (Borden et al., 1971). BTV is transmitted by biting of blood-feeding insect vectors of the genus *Culicoides* spp. (Diptera: Ceratopogonidae) (Mellor, 2000). Moreover, a new animal virus, not previously detected in Europe and initially named “Schmallenberg Virus (SVB)”, is a member of the Simbu serogroup within the genus *Orthobunyavirus* (family *Bunyaviridae*) that are predominantly transmitted by biting midges, mainly *Culicoides* spp., and mosquitoes (Hoffmann et al., 2011; Rasmussen et al., 2012). Out of > 1300 species present worldwide; at least 39 species have been reported in India. Very few species of *Culicoides* have been

demonstrated as vectors for the BTV, and the principal vectors varying geographically. Midges collected from Harayana, Punjab, Rajasthan and Himachal Pradesh was identified as *C. oxystoma* (Jain et al., 1988) and *C. imicola* and *C. oxystoma* were found to be prevalent in Tamil Nadu. A detailed description of vector–species responsible for transmission of BTV in India is not comprehensively studied and therefore requires future studies to investigate virus–vector relationships in India.

Unlike southern and western India, the BTV is not detected in the eastern part of the country. In fact, West Bengal state has not experienced any outbreak or active disease incidences so far. However, anti-bluetongue antibodies could be detected in sheep, goat and cattle population of eastern and north-eastern states of India, viz. West Bengal (Chakraborti et al., 2007, Biswas et al., 2009, Panda et al., 2011, Mandal et al., 2012), Assam (Joardar et al., 2013), Manipur (Joardar et al., 2012), thus might be considered as sub-clinically infected. Moreover, BTV was isolated from sheep of Kolkata Maidan pasture (in West Bengal) and serotyped as 15 and 21 (Joardar et al., 2009).

In general, developmental sites of *Culicoides* species are poorly known. They can breed in a wide range of soils, if they provide enough moisture and organic matter to allow the development of the larvae (Kettle, 1962). The large range of

breeding sites can be divided into three principal categories (Meiswinkel et al., 2004): (i) the water-saturated soil ecotone between aquatic and terrestrial habitats, (ii) dung pats (fresh dung), and (iii) moist, decaying organic matter (including manure). The immature stages of *Culicoides* spp. usually live in surface soil layers (0–5 cm depth), and rarely are found deeper than 8 cm (Uslu and Dik, 2006).

There are reports about significant correlations between rainfall and *Culicoides* activities in Ukraine (Zhadnova, 1975), Nigeria (Dipeolu, 1976), China (Chen and Lin, 1977), South Africa (Jupp et al., 1980) and Taiwan (Lien and Chen, 1981). Impact of thermo-aero-helio-hydrological weather parameters on population dynamics was also reported by Campbell and Kettle (1979), and Baylis et al. (1998).

In this context, it is quite expedient to explore the complex interaction of virus, vector (*Culicoides* midges) and environmental factors of this state (West Bengal) in a holistic manner for better understanding of the disease spectrum of bluetongue. Knowledge of suitable breeding sites of each species, particularly those implicated in the transmission of parasites or pathogens, is essential to evaluate the risk in an area and therefore to contribute in the development of integrated control strategies.

The state of West Bengal (latitude 21°38' N to 27°10' N, longitude 85°50' E to 89°50' E) has been divided into six agro-climatic zones, viz. Hill, Tarai, Old alluvial, New Alluvial, Red latterite and Coastal saline. The environmental parameters, viz.

rainfall, temperature and relative humidity being quite conducive for propagation of *Culicoides* sp. in West Bengal (Biswas et al., 2011), these midges breed profusely during the monsoons (May to September).

In order to identify the potent vector of BTV, circulating in West Bengal state and causing subclinical bluetongue, attempts were made to detect and isolate BTV from *Culicoides* midges that were collected from localities harboring good number of sero-positive animals.

MATERIALS AND METHODS

Sampling and Identification of Culicoides Midges

Culicoides midges were collected from three different agro-climatic zones of West Bengal (New Alluvial, Red Laterite and Coastal Saline) in different post monsoon months. The collection was performed by sweep netting in early morning, before sunrise or afternoon and just before the sunset. Meteorological data (temperature, relative humidity and rainfall) were obtained and incorporated to know the environmental condition at the collection time. The collected *Culicoides* midges were sent to Zoological Survey of India, Kolkata for species identification. The trapped insects were sorted under a light microscope and the identification was made based on the morphological characteristics of the midges (Sen and Dasgupta, 1959, Dasgupta, 1995).

Table I: Details of *Culicoides* collection vis-a-vis meteorological data in respect of collection months

Area of Collection	Agro-climatic zone	Month of collection	Temperature (°C)	Relative Humidity (%)	Rainfall (mm)
Khatra, Dist- Bankura	Red Latterite	April	Maximum-38 Minimum-24	73	55
Belgachia, Dist - Kolkata	New Alluvial	November	Maximum-30 Minimum-18	72	14
		February	Maximum-31 Minimum-18	72	13
Agarpara, Dist-North 24 Parganas	New Alluvial	December	Maximum -27 Minimum -15	75	04
Kamarhati, Dist-North 24 Parganas	New Alluvial	January	Maximum -26 Minimum -12	80	08
Panskura, Dist- Purba Medinipur	Coastal Saline	September	Maximum-30 Minimum-26	86	307
Nalpur, Dist -Howrah	New Alluvial	September	Maximum -30 Minimum -25	86	250
Patelnaga, Dist-Birbhum	Red Latterite	August	Maximum -32 Minimum -25	86	276
Hariharpara, Dist- Murshidabad	New Alluvial	November	Maximum -29 Minimum -18	78	05
Malda Town, Malda	New Alluvial	November	Maximum -28 Minimum -17	75	-
Sandeshkhali, Dist- North 24 Pgs	Coastal Saline	January	Maximum -25 Minimum -12	80	08

Habitat of Culicoides Midges

Detailed observations were made of the collection area of midges, as described below. Probable breeding area was also sought and noted.

Isolation and Identification of Bluetongue Virus from Culicoides Midges

Collection of Midges

Midges were collected in batches (n=6) from Agarpara region (22°67' N, 88°37' E) of 24-parganas district of West Bengal state. The area was chosen on the basis of presence of high percentage of sero-positive animals. Midges were collected from large

ruminant (buffalo) shed in the evening hours (4.30–6.30 pm) in the month of October using traditional sweep net.

Isolation of BTV from Midges

Each batch of non-engorged midges was pooled to prepare individual sample. The samples (n=3) each numbering up to 200 midges were ground in pestle and mortar with sterilized sand. After grinding, 10 ml of sterilized phosphate buffered saline (PBS, pH 7.2) was added into it and the cells were disrupted (at 0°C) using ultrasonicator (Hielscher Ultrasonics GmbH, Germany) titanium probe, with the peak-to-peak wavelength of 8µ to 10µ for 40 cycles of one minute's duration each, to

Table 2: List of *Culicoides* spp. identified from different Districts and Agro-climatic zones of West Bengal

Area of Collection	District	Agro-climatic zone	Species identified
Khatra	Bankura	Red Latterite	<i>Culicoides schultzei</i>
Belgachia	Kolkata	New Alluvial	<i>C. schultzei</i> , <i>C. palpifer</i> <i>C. definitus</i>
Agarpara	North 24 Parganas	New Alluvial	<i>C. schultzei</i>
Kamarhati	North 24 Parganas	New Alluvial	<i>C. schultzei</i>
Sandeshkhali,	North 24 Parganas	Coastal saline	<i>C. schultzei</i>
Nalpur	Howrah	New Alluvial	<i>Culicoides</i> sp.
Patelnagar	Birbhum	Red Latterite	<i>C. schultzei</i>
Hariharpara	Murshidabad	New Alluvial	<i>C. schultzei</i>
Malda Town	Malda	New Alluvial	<i>C. schultzei</i>
Panskura	Purba Medinipur	Coastal Saline	<i>Culicoides</i> sp.

release the virus. Intravenous inoculation of filtered aliquot (100µl) of the disrupted midge cell suspension into 10- to 12-day-old embryonated chicken eggs (ECE) was used to detect the presence of BTV (Foster and Luedke, 1968). For each sample, four ECE were used as test whereas two as control (where PBS, pH 7.2 was used instead of samples). The ECE were incubated at 33.5°C for seven days. Embryos that died within seven days of inoculation showing subcutaneous haemorrhages were considered suspected. Embryos that died within 24 hours of inoculation were not considered for subsequent processing. Three blind passages in embryonated eggs were carried out for all the samples. Selected embryonic tissues, viz. spleen, kidney, heart, liver and muscle were collected and emulsified, using a sterilized pestle and mortar, with PBS having antibiotics (100IU/ml benzylpenicillin and 100µg/ml streptomycin sulphate) to make a 10% (w/v) suspension. This suspension was clarified by centrifugation and inoculated on to baby hamster kidney (BHK-21) confluent cell monolayer (Figure 2a) in 25cm² tissue flask (Nunk) using Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with 10% fetal calf serum (Sigma, USA). Infected cells were observed under microscope for cytopathic effect (CPE) produced up to 72 hours post-infection (PI) and harvested for next cycle of infection. The harvested cells were filtered and 200 µl of inoculums were used to infect fresh monolayer. Thus three passages in BHK-21 cells were carried out for each case and observed for up to 72 hours PI for CPE.

RNA-PAGE

Viral RNA was extracted from the BHK-21 cells using TRIzol® (Sigma, USA) (Chomczynski and Sacchi, 1987) and RNA-PAGE was performed as described by Squire et al. (1983).

RT-PCR

To further confirm the positivity of cell culture sample for BTV, the RT-PCR was performed using primers specific to the BTV NS1 gene. These primers (primer 1: 5'- GTT CTC TAG TTG GCA ACC ACC- 3'; primer 2: 5'- AAG CCA GAC TGT TTC CCG AT- 3') (OIE, 2004), were used to amplify the partial sequence of NS1 gene.

RESULTS

Identification of *Culicoides* Midges

The midges collected in this study were identified as members of the *Culicoides schultzei* complex. The midges were medium sized flies with moderately hairy wings with numerous distinct pale spots including a pale spot over r-m cross-vein almost on the center of the vein. Paramere was broad at the base, curved and tapered towards the tip with apical hairs. These features are characteristics for *Culicoides schultzei*.

Habitat of *Culicoides* Midges

The principal habitat of different *Culicoides* spp. was mainly cattle or buffalo shed and decomposed manure. Besides, area-specific observation of the surroundings was noted during the study.

From Khatra (District- Bankura), midges were collected from cattle shed. The shed was situated just by the side of an open sewage disposal drain. In Belgachia (District Kolkata), *Culicoides* were found abundant in the cattle shed. Not only from cattle shed but also from nearby tree and bushes a huge number of midges was collected. It may be mentioned here that besides *Culicoides schultzei*, *C. palpifer* and *C. definitus* were also collected from Belgachia area. From Agarpara and Kamarhati (Dist - North 24 Parganas), *Culicoides* were collected from cattle and buffalo shed. The shed was surrounded by decomposed manure and straw. Open drain was present by the side of the shed and midges were present in both shed and decomposed manure. Sheep and goat sheds were the principal habitat of *Culicoides* in Sandeshkhali (District North 24- Parganas). Maximum animal sheds were situated by the side of a river. Saline soil was present in the area. From Patelnagar (District- Birbhum), *Culicoides* were collected from cattle shed. The shed was situated by the side of a biogas plant. The whole area was covered with trees and bushes. In Hariharpara (District- Murshidabad), large number of sheep and goat sheds was presented in densely populated and congested area. The sheds were surrounded by bushes. *Culicoides* were found present in both the sheds and nearby area. From Malda Town (District- Malda), *Culicoides* were collected from organised cattle farm. Though it was a clean area, but surrounding sewage canals were open. Cow dung was also present in the farm premises. From all the above places *Culicoides schultzei* was collected.

From Nalpur (District-Howrah), *Culicoides* midges were collected from buffalo sheds and nearby bushes. Surrounding area was muddy and with water logged condition. From Panskura (District- Purba Mednipur), *Culicoides* was collected from Buffalo shed. This was situated by the side of train tract. Surrounding muddy area is covered with bushes. More than one species of *Culicoides* were observed in these areas.

Isolation of BTV

Chicken embryos that died (in between 2 and 7 days of inoculation) in ECE showing subcutaneous haemorrhages (Figure 1) were suspected of having BTV. After processing, suspected samples (n=4) were inoculated in BHK-21 cell lines, one of the samples showed extensive CPE. Cellular destruction was evident as early as 24 hours, and the cells became enlarged, refractile and rounded (Figure 2b). After 48 hours inoculation, there was foamy degeneration, cell aggregation and vacuolation

inside the cells (Figure 3). CPEs were seen throughout the monolayer by 72 hours post infection (PI), clumps of cells were detached from the flask and floated in the medium. This BHK-21 cell lysate sample (CCS-1) was chosen for further processing.

RNA-PAGE

Polyacrylamide gel electrophoresis of extracted RNA of the suggestive sample (CCS-1) showed 10-segmented profile characteristic of BTV (Figure 4).

RT-PCR

A band corresponding to 274bp was obtained in agarose gel when RT-PCR was performed using NS1 specific primers (Figure 5).

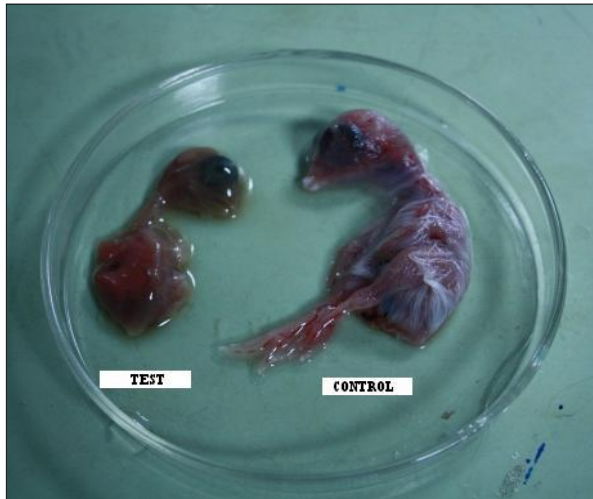


Figure 1: Pathognomonic lesion in chicken embryo after inoculating (I/V) suggestive sample of BTV from *Culicoides*

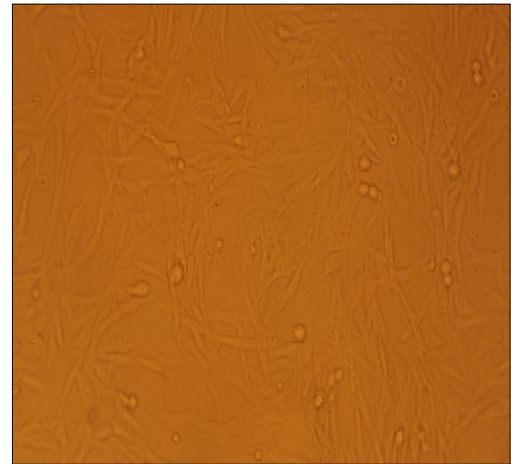


Figure 2a: Monolayer of BHK21 cell line

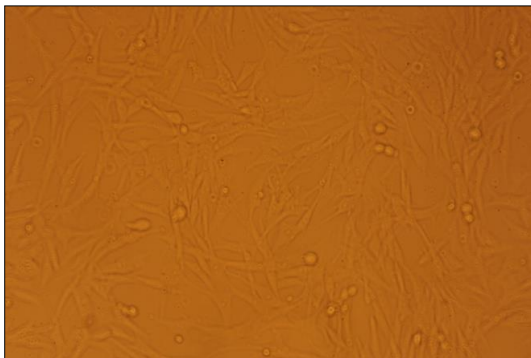


Figure 2b: CPE of BHK21 cell line after inoculation of suggestive BTV sample (at 24 hrs.)

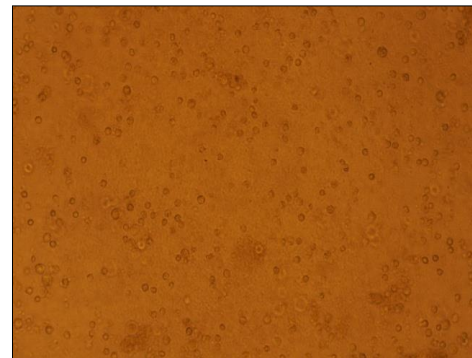


Figure 3: CPE of BHK21 cell line after inoculation of suggestive BTV sample (at 48 hrs.)

DISCUSSION

With the aim of exploring the bluetongue epidemiology, attempts were made to study the role of *Culicoides* midges in BT infection in ruminants of West Bengal. In this direction, *Culicoides* biology along with virus isolation from haematophagous midges was sought. *Culicoides* midges were collected from different agro-climatic zones of West Bengal and identification up to species level was carried out with the help of Zoological Survey of India (ZSI). *Culicoides* species identified from three agro-climatic zones (new alluvial, red latterite and coastal saline) were *Culicoides schultzei*, *Culicoides palpifer* and *Culicoides definitus*. In India, BTV was isolated first

time from *Culicoides* midges about 25 years ago. However, species was not identified (Prasad et al., 1988). Recently, from western state of India (Gujarat) BTV-1 was isolated from *Culicoides oxystoma* (Dadawala et al., 2011). In the present study, the BTV was isolated from *C. schultzei* collected from Agarpara area (North 24 District of West Bengal). The isolate was sent to AINP-BT (ICAR) typing centre (at Hissar) and found to be BTV-16. Previously, BTV-16 was isolated from sheep of Tamil Nadu, Maharashtra and Himachal Pradesh (Sreenivasulu et al., 2004). Recently, increasing trend for BTV-16 infection in different species of animals (sheep, goat and cattle) was observed from different states in India that suggest more studies on vector competence for transmission of infection

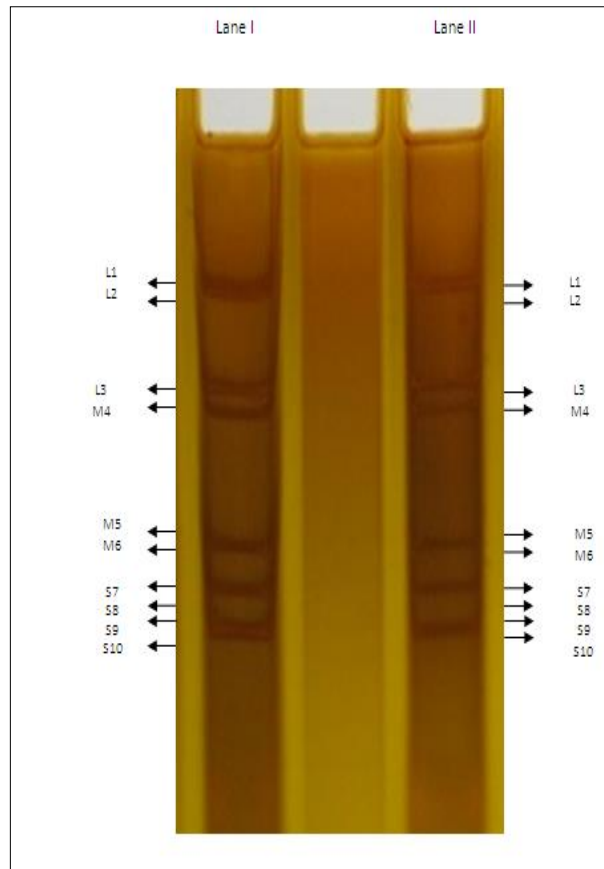


Figure 4: RNA-PAGE profile of suggestive sample of BTV; Lane -I, Lane-II; Suggestive sample-CCS-1

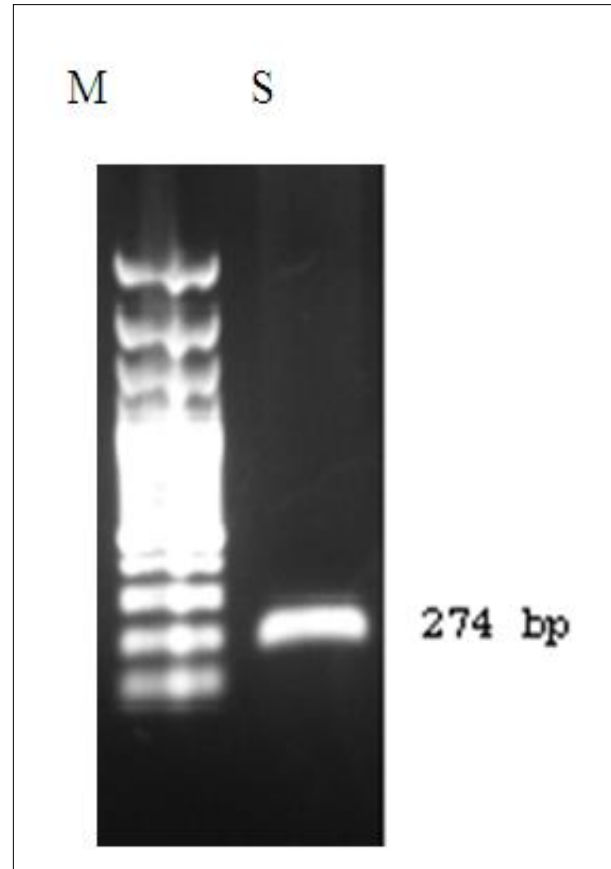


Figure 5: RT-PCR profile of suggestive sample in agarose gel; M- 100 bp DNA ladder; S- Sample (CCS-1)

(Anon, 2012). The present study confirms the vector competence of *C. schultzei* that harboured BTV-16. Recently, complete genome sequence of BTV-16 of goat origin from India was reported (Minakshi, 2012). Earlier, BTV-21 was isolated from *Culicoides palpifer* from Indonesia (Sendow et al., 1993). It may be mentioned here that *Culicoides brevitarsis* Keiffer and *C. imicola* Keiffer being proven vectors of BT virus, occur widely in India (Ilango, 2006). As virus transmitting species of *Culicoides* were observed in West Bengal, and high sero-positivity in ruminants were observed, the possibility of BTV circulation in West Bengal through vectors cannot be ruled out.

Prevalence of *Culicoides* midges and role in disease transmission were studied in Chittoor and Prakasam district of Andhra Pradesh during 2007 where disease was recorded previously during 2000 to 2003. A total of 1297 midges were collected using light trap method, out of which 982 (75.70%) were identified as female *Culicoides* and 315 (24.30%) as male species. *Culicoides actoni*, *C. anophelis*, *C. inoxius*, *C. oxystoma* and *C. perigrinus* were present in the catches (Reddy and Hafeez, 2008).

In the present study, habitat of collected midges was studied. The principal habitats were animal shed, surrounding manure and nearby bushes. Habitat, maximum and minimum temperature, rainfall and humidity of collection month were also observed in these districts from where midges were

collected. It was noticed that collection of midges was huge just after winter and just after a rainfall. Maximum collection was done just before evening. Collection was low during too hot or cold climatic condition.

The prevalence of midges was attributed to the availability of breeding sites such as decaying vegetable matter, swamps, edges of ponds, boggy and semi-water logged areas, tree holes, rotten cacti etc. that is quite similar to the present findings. Well lighted cattle shed is one of the attractive sources of the flies which then adapted to feed on the same animal host. The water level of ponds, lakes, swamps rises high after the monsoon which helps the larva to migrate from slow making water to tamped soil, mud and other water logged heads and wooden particles etc. (Reddy and Hafeez, 2008).

It was established earlier that the environmental condition around the animal house with the heaps of manure, slow moving water, dazzling of rain fall in low lying areas are ideal breeding sites for *Culicoides* midges (Sen and Dasgupta 1959, Dasgupta 1995) which corroborates with the present findings. Besides these, wallow tanks, muddy sites of ditch, drainage, pond margin with moderate level of slow moving water incorporated with decaying vegetables or organic manure are more suitable for breeding of majority of *Culicoides* spp. (Howarth, 1985).

Typical association between the dynamics of *Culicoides* population, their habitat and weather parameters were

observed in the present study. The seasonality in *Culicoides* population clearly indicated that monsoon season was most favorable for building up of population followed by winter. The sizeable population of *Culicoides* during winter season may act as a vector of BT disease which is of major concern in this area. These findings further reveal that the association of *Culicoides* with thermal and hygrological parameters of atmosphere and suggest that there are favourably good chances to control these parasites through improving the housing conditions of animals and regulation of their surrounding environment.

In short, it may be concluded that the eastern Indian state, West Bengal possesses potent vector of BTV that pose a menace and warrants control-based strategies.

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