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Full Length Research Paper

Seroepidemiology, molecular and entomological studies of bluetongue in sheep in Gujarat

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Seroepidemiology, molecular and entomological studies of bluetongue in sheep was carried out in Gujarat, India. Out of 980 sera sample screened, 361 (36.84 %) were found positive by c-enzyme-linked immunosorbent assay (c-ELISA). Out of the 103 blood samples tested for the presence of bluetongue virus (BTV) by reverse transcription-polymerase chain reaction (RT-PCR), 3 were found positive, producing 274 bp amplicons with NS1 gene specific primers. Collection and identification of *Culicoides* vectors from various farms suggest that the *Culicoides oxystoma* is the most prevalent species.

Key words: Bluetongue, sheep, c-Enzyme-linked Immunosorbent Assay (c-ELISA), NS1 *gene*, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), *Culicoides.*

INTRODUCTION

Bluetongue is an arthropod borne viral infection of domestic and wild ruminants. Although, all the ruminants are susceptible to bluetongue virus (BTV) infection, sheep is the most susceptible species to the clinical bluetongue disease. Bluetongue disease is a “List A” disease of the Office of International Epizootics (OIE). List A diseases are those diseases which can spread rapidly and the bluetongue is characterized by fever (42°C), hyperaemia, inflammation of the oral mucosa, tongue, coronaryband and occasionally the nasal mucosa, erosions and ulceration of the dermis and laminitis. Severe oedema of the tongue can result in restricted blood flow and cyanosis. The swollen tongue may protrude giving the appearance of a bluetongue. Sick animals may exhibit profuse salivation, depression, anorexia, weight loss due to muscle degeneration, stiffness of the limbs, lameness, and excessive nasal and ocular secretion. Death may occur in 8–10 days. The economic losses due to bluetongue is about 3 billion US$ per year in the world. The direct losses are death, abortions, weight loss and reduced milk and meat productions and indirect losses are export restrictions of live animals, semen and foetal calf serum (Bitew et al., 2013).

The causative agent, bluetongue virus (BTV) is a prototype virus of orbivirus of the family Reoviridae (Pringle, 1999). It is composed of 10 discrete segments of ds-RNA genome surrounded by two layers of protein capsid. Due to the presence of segmented RNA genome,

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the virus is prone to frequent mutations. This has led to emergence of genomically diverse serotypes/strains of the virus. There are 26 BTV serotypes currently identified by the specificity of interactions between the outer capsid (particularly protein VP2) and the neutralizing antibodies generated during infection of the host (Maan et al., 2011). Out of 26 serotypes distributed globally, 21 serotypes have been reported from India (Wilson and Mellor, 2009). BTV is transmitted biologically by hematophagous midges in the genus *Culicoides* and infection occurs throughout the tropical and temperate regions of the world and coincident with the distribution of competent *Culicoides* vectors (Tabachnick, 1996).

Thus, the present study was carried out to determine the prevalence and distribution of BTV antibodies by c-enzyme-linked immunosorbent assay (c-ELISA), detection of BTV using reverse transcription-polymerase chain reaction (RT-PCR) and collection and identification of *Culicoides* vectors in various organized sheep farms of Gujarat.

MATERIALS AND METHODS

A total of 980 sera samples were collected for detection of BTV group specific antibodies by using BTV c-ELISA kit (Veterinary Diagnostic Technology Incorporation, USA) from sheep belonging to rural areas and organized farms of different districts of Gujarat state during the year 2008-2009. The separated serum was collected in screw capped plastic vial and heat inactivated at 56°C for 30 min. The sera were held at -20°C temperature till further use. The c-ELISA test was performed as described by Afshar et al. (1987). The data were analyzed by the Chi-square and odds ratio. The level of significance was set at $P< 0.05$.

A total of 103 blood samples were collected separately in vacutainers (Becton, Dickinson and Company) from sheep stationed at Gujarat Sheep and Wool Development Corporation (GSWDC), Jasdan and Aseda, LRS, Sardarkrushinagar and SBF, Patan. The samples were collected from sheep showing initial rise in body temperature (104°F) and showing clinical signs resembling BT. The samples were also collected from apparently healthy sheep. The blood samples on arrival at the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, S.D. Agricultural University, Sardarkrushinagar were centrifuged at 800 *g* for 5 to 10 min in refrigerated centrifuge at 4°C. The blood cells were washed three times in equal volume of calcium magnesium free PBS. After the final wash, the equal volume of OPG medium was added in all the blood samples. The red blood cells were haemolysed as per the method described by Hosseini et al. (1998). After this, the blood samples were transferred in screw capped vials and stored at 4°C until further use. TRI RNA extraction method was used for total RNA isolation from blood samples as per the manufacturer's instruction (Molecular Research Centre, Inc., USA). The viral genomic RNA extracted by TRI reagent method was used as a template for cDNA synthesis by Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) enzyme (Bangalore Genei). The cDNA obtained was subjected to RT-PCR using partial length primer sequence for BTV group specific NS1 gene. The partial length PCR primers used were P1: 5'GTT CTC TAG TTG GCA ACC ACC3' and P2: 5' AAG CCA GAC TGT TTC CCG AT3' to generate an amplicon of 274 bp long. The NS1 gene based group specific PCR confirmed the samples as BTV. RT-PCR products where analyzed by agarose gel electrophoresis using 1.5% agarose gel prepared in 1X Tris-acetate-EDTA. The bands were visualized and photographed under Gel Documentation System (DNR Bio-imaging System) and photographed.

For the collections *Culicoides,* CDC miniature Insect Light Trap was employed close to animal sheds and operated from dusk to dawn (Figure 1). *Culicoides* were sorted out under a stereoscope and some *Culicoides* specimens were mounted and some were preserved in 70% alcohol for detailed identification. *Culicoides* collections were made from LRS, Sardarkrushinagar, GSWDC Sheep farm Jasdan and Aseda, SBF Patan. These way insects were collected, processed and finally sent to Centre for Animal Disease Research and Diagnosis, IVRI, Izatnagar for detailed identification.

RESULTS AND DISCUSSION

Presumptive diagnosis of BTV infection was made by serological test rather than by isolation and identification of the virus. To study the distribution and gravity of problem, seroepidemiology was considered as important tool and in the epidemiology of the bluetongue, and serological surveys are used to analyze the infection status of the ruminants in the area. The OIE manual of diagnostic tests and vaccines for terrestrial animals recommends AGID, c-ELISA and RT-PCR as prescribed tests for international trade. However, AGID is known for cross-reactions with other Orbiviruses such as epizootic haemorrhagicdisease virus (EDHV), thus its use has declined over time and replaced with the easier to use, rapid, highly sensitive and specific c-ELISA (Reddington et al., 1991).

Detection of virus specific antibodies in animals is an important criterion to study the epidemiology and provides an indirect evidence of virus circulation in a geographical area (Sreenivasulu and SubbaRao, 1999). Keeping the above facts in mind, a serological survey involving 980 sera sample from sheep was conducted using c-ELISA. Out of 980 sera sample screened, 361 (36.84%) were found to be positive (Table 1 and Figure 2). However, Sreenivasulu and SubbaRao (1999) reported higher rate of seroprevalence than this. Chandel et al. (2004) and Hinsu et al. (2000) recorded lower rate of 24.66 and 24.56% seroprevalence using Aar Gel Immuno-Diffusion (AGID) respectively, whereas Hinsu et al. (2000) reported a much higher rate of seroprevalence of 63.16% by c-ELISA from Gujarat State.

Out of 18 locations, the highest seroprevalence was recorded in GSWDC, Aseda while the lowest seroprevalence was recorded in Ahmedabad. The rate of seroprevalence in all these locations ranged between 27.59 and 44.29%. Univariate analysis showed that GSWDC, Aseda is more likely to be seropositive than Ahmedabad (OR=1.60), In contrast to the seroprevalence rate of 18.42% in Banaskantha, Chandel et al. (2004) reported 15.16% by BT-AGID and 32.00% in Kuchchh district; higher rate of seroprevalence, 59.64% by i-ELISA was reported by Bhalodiya and Jhala (2002). The reasons for this may be the variation in samples and place from where the sera were collected and tests

Figure 1. CDC miniature insect light trap employed at sheep farm.

Table 1. Seroprevalence of BTV antibodies in sheep.

Attribute	Number of samples tested	No. positive	Percent positive
Region			
North Gujarat	570	217	38.07
Kuchchh	50	16	32.00
Saurashtra	266	98	36.84
Central Gujarat	74	22	29.73
South Gujarat	20	08	40.00
Location			
Banaskantha	200	83	41.50
Mehsana	95	35	36.84
Sabarkantha	20	07	35.00
Ahmedabad	29	08	27.59
Gandhinagar	15	05	33.33
Porbandar	17	05	29.41
Bharuch	20	08	40.00
Bhavnagar	23	08	34.78
Patan	70	24	34.29
SBF, Patan	50	19	38.00
LRS, Sardarkrushinagar	65	18	27.69
Jamnagar	56	17	30.36
Surendranagar	50	18	36.00
Amreli	25	09	36.00
Panchmahal	30	09	30.00
GSWDC, Jasdan	95	41	43.16
GSWDC, Aseda	70	31	44.29
Kuchchh	50	16	32.00
Breed			
Patanwadi	530	186	35.09
Marwadi	255	69	27.05
Crossbred	190	102	53.68
Exotic	05	04	80.00
Overall	980	361	36.84

employed for detection of BTV antibodies.

Sera were tested from five different regions *viz.,* North Gujarat, Saurashtra, Kuchchh, Central Gujarat and South Gujarat. Out of these five regions, the highest positivity was 40% in South Gujarat followed by North Gujarat Saurashtra, Kuchchh and Central Gujarat regions. Univariate analysis showed that South Gujarat is more likely to be seropositive than Central Gujarat, Kuchchh, Saurashtra and north Gujarat (OR=1.57, 1.40, 1.13 and 1.08, respectively). In contrast to the present findings, Bhalodiya and Jhala (2002) reported 69.23% from Saurashtra, and 59.69% from Kuchchh region of Gujarat State.

In the present study, four breeds of sheep were included. The rate of seroprevalence was highest in exotic (80%), followed by crossbred (53.68%), Patanwadi (35.09%) and Marwari (27.05%). The present findings revealed that exotic breeds are more susceptible than crossbreds and the indigenous breeds. Univariate analysis of breeds showed that exotic is 6 times more likely to be seropositive than Patanwadi (OR=6.98), also, analysis showed that exoticbreeds are 2 times more likely to be seropositive than Crossbred (OR=2.00) but Marwadi breed showed non-significant Chi-square value (0.1127, p<00000). Similar findings have been reported (Prasad et al., 1987; Das et al., 1997). However, on the contrary, Hinsu et al. (2000) found native sheep more susceptible than crossbred sheep for BTV infection. Higher rate of seroprevalence in crossbred than indigenous has also been reported by Sreenivasulu and SubbaRao (1999). Within indigenous breeds, Patanwadi showed higher rate of seroprevalence than Marwari, which is in accordance with the observations of Chandel et al. (2004).

Primer-directed amplification of viral nucleic acid has revolutionized BT diagnosis. It is one of the prescribed tests by OIE for international trade of animal products. This PCR assay may be used, not only to detect the presence of viral nucleic acid, but also for sero group orbiviruses and provide information on the serotype and possible geographical source of BTV isolates within a few days of receipt of clinical samples such as infected sheep blood. In the present study, Out of 103 blood samples tested for the presence of BTV by RT-PCR, 3 were positive

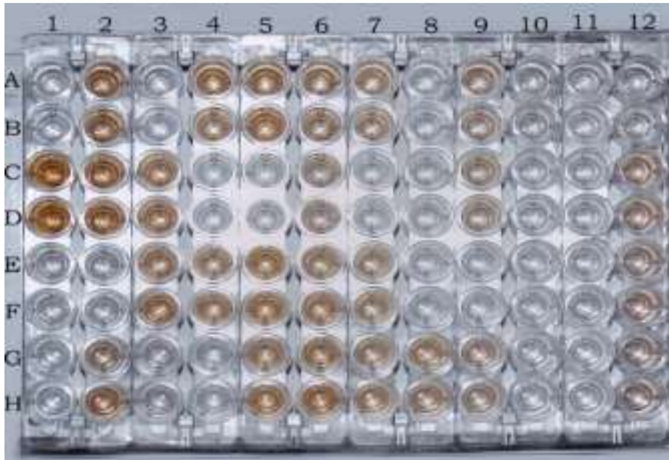

Figure 2. Microtitre ELISA plate showing results of c-ELISA. A1 and B1: Diluent control; C1 and D1: Negative control; E1 and F1: Weak positive control; G1 and H1: Positive control; A2 and B2; G12 and H12: Field sera.

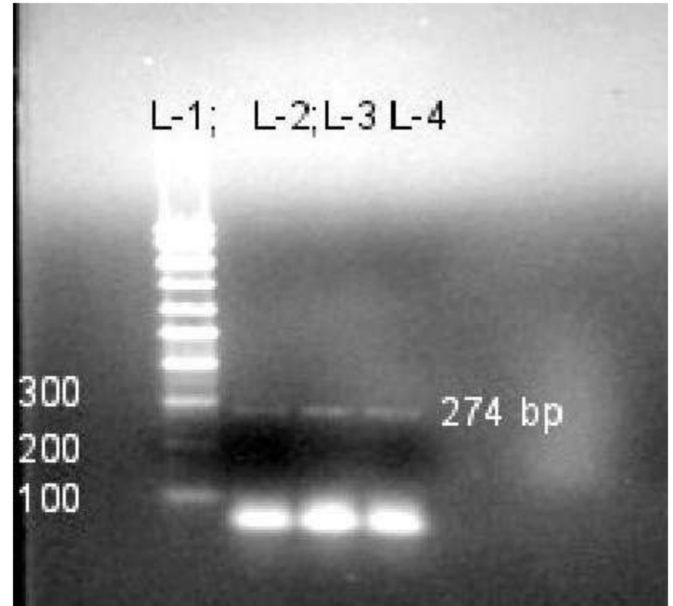

Figure 3. PCR product 274 bp by using BTV group specific nsl gene primer (L-1: 100 BP LADDER, Lane 2-4: Positive field samples).

for producing 274 bp amplicons with NS1 gene specific primers (Figure 3). Due to the conserved nature of NS1 gene, it was targeted for development of RT-PCR for detection of BTV. The NS1 gene primer sequence for partial length used in the study was the same as used by Katz et al. (1993). Similar, RT-PCR based amplification of 274 bp has also been carried out by Malik et al. (2001).

In the present study, collection and identification of *Culicoides* vectors from various farms suggest that the *C. oxystoma* is the most prevalent species and variable rate of seroprevalence has also been reported in these farms. As compared to the present study on collection and identification of Culicoides vectors, Patel et al. (2007) has also reported the prevalence of *Culicoides oxystoma* from LRS, Sardarkrushinagar; GSWDC, Jasdan; GSWDC, Aseda and LRS, Navsari which is in accordance with the results obtained in the present investigation. However, isolation of the virus from the trapped *Culicoides* and experimental transmission of the virus to the susceptible animals by vectors are required to confirm its role. Repeated trapping of a single species of *C. oxystoma* from sentinel herds/flocks, its close association with sheep and cattle and its continuous seroconversion in sentinel herds have been established in Punjab, Haryana, H.P. and Rajasthan (Kakkar et al., 2002). *Culicoides* are highly adapted to a wide range of temperature and moisture. In tropical areas, moisture is maintained by rain water, and in subtropical areas, it is through irrigation water. In South India, the monsoon season (June-December) with temperature ranging from 21.2 to 35.6°C appears to be favourable period for the multiplication of the vector resulting in more outbreaks. Bluetongue outbreaks in Karnataka, Tamil Nadu and Andhra Pradesh were associated with peak activity period of *Culicoides* spp.

Conclusion

In the present study, detection of BTV group specific antibodies and of BTV in blood samples by RT-PCR and identification of *C. oxystoma* from various farms indicate that the BTV is prevalent in Gujarat. Considering the immense economic importance of BTV and increasing trends of occurrence of BTV in India, potential vectors should be screened for identification, virus isolation and susceptibility of the infection and the virus transmission to vertebrate hosts. Forecasting of vector borne disease outbreaks through the development of appropriate models could be of great significance in controlling bluetongue disease.

Conflict of Interests

The authors have not declared any conflict of interests.

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