Sero-epidemiology and molecular detection of Bluetongue virus in Indian ruminants

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Keywords
Bluetongue virus, Competitive enzyme-linked immunosorbent assay, India, Polymerase chain reaction, Ruminants, Seroprevalence.

Summary
Bluetongue (BT) is a non-contagious arthropod-borne viral disease of domestic and wild ruminants. It is endemic to India and clinical outbreaks of disease have been reported mainly in sheep, although BT is often asymptomatic in other ruminant species. In the present serological survey, a total of 576 serum samples, comprising of 416 cattle and 160 sheep, covering different agro-climatic zones of Rajasthan, Uttar Pradesh, and Karnataka states, were screened for the presence of Bluetongue virus (BTV) specific antibodies using competitive enzyme-linked immunosorbent assay (c-ELISA). Overall 73.08% (304/416) of the cattle and 53.30% (87/160) of the sheep serum samples were positive for BTV antibodies. The prevalence of BTV antibodies in cattle in different agro-climatic zones ranged between 60-80% in Rajasthan and 66-70% in Uttar Pradesh. During the study, a nested polymerase chain reaction (PCR) based on the BTV NS1 gene (genome segment 5) was optimized for detection of BTV's nucleic acid from a cell adapted strain of BTV-23, and field derived clinical blood samples. In the present study, 19/70 of cattle and 9/30 of sheep blood samples tested positive for BTV RNA by the nested PCR, which amplified specific products of 274 bp and 101 bp sizes, respectively. From this study, it can be concluded that cattle showed higher percentage of sero-positivity in comparison to sheep. The improved sero-surveillance system for BTV in endemic areas will be of great help to understand the epidemiology of BTV and to formulate effective control and preventive strategies.

Virus della Bluetongue nei ruminanti in India

Parole chiave
Bluetongue, ELISA competitiva, India, Reazione a catena della polimerasi, Ruminanti, Sieroprevalenza, Virus della Bluetongue.

Riassunto
La Bluetongue (BT) è una malattia virale non contagiosa trasmessa da artropodi a ruminanti domestici e selvatici. È endemica in India, dove epidemie sono state segnalate soprattutto negli ovini. La malattia è spesso asintomatica in altre specie di ruminanti. In questo studio sono stati esaminati 576 campioni di siero, 416 campioni di bovini e 160 di ovini, per la presenza di anticorpi specifici contro il virus della Bluetongue (BTV) mediante ELISA competitiva (c-ELISA). I campioni sono stati prelevati in diverse zone agro-climatiche indiane comprese negli stati di Rajasthan, Uttar Pradesh e Karnataka. Il 73,08% (314/416) dei campioni bovini e il 53,3% (86/160) dei campioni ovini sono risultati positivi per gli anticorpi anti-BTV. La prevalenza di anticorpi contro il virus nei bovini è risultata oscillare tra 60-80% in Rajasthan e 66-70% in Uttar Pradesh. Nell'indagine, la tecnica *nested polymerase chain reaction* (PCR) basata sul gene BTV NS1 (segmento genomico 5) è stata ottimizzata per l'individuazione dell'acido nucleico del BTV sul ceppo BTV 23 adattato su culture cellulari e su campioni di sangue di animali risultati positivi all'ELISA competitiva. Diciannove dei 70 campioni di sangue bovino e 9 dei 30 campioni di sangue ovino sono risultati positivi per BTV RNA alla *nested PCR*. Questa tecnica ha permesso l'amplificazione di prodotti specifici rispettivamente

di 274 bp e 101 bp. I risultati ottenuti hanno evidenziato prevalenze di infezione più elevate nei bovini rispetto agli ovini. Il miglioramento del sistema di siero-sorveglianza per BTV in aree endemiche potrebbe essere di grande aiuto per comprendere l'epidemiologia della malattia e per formulare efficaci strategie preventive e di controllo.

Introduction

Bluetongue (BT), which is mainly a disease of sheep, is regarded as a globally emerging disease and is endemic in India. Cattle and goats are considered as reservoir hosts for Bluetongue virus (BTV) and infection is generally subclinical in these animal species. The virus contains 27 recognised serotypes, which include 3 recently characterized novel serotypes, like BTV-25 Toggenburg orbivirus (TOV) in sheep from Switzerland (Hofmann *et al.* 2008), BTV-26 from goats in Kuwait (Maan *et al.* 2011), and BTV-27 from goats in Corsica, France (Jenckel *et al.* 2015). Recently, 2 more novel BTV serotypes have been reported: BTV-28 from capripox vaccine preparation in the Middle East (Kyriaki Nomikou: personal communication, Maan *et al.* 2015b) and BTV-29 isolated from a lama in South Africa (Wright 2013).

Bluetongue was first reported in India in 1961 in the state of Maharashtra (Sapre 1964). Since then outbreaks of clinical BT have been reported from most of the Northern and Southern states of India. In India, 23 out of 27 BTV serotypes (except 19, 22, 25, and 26) have been found to be prevalent (Ranjan *et al.* 2015, Rao *et al.* 2016, Krishnajyothi *et al.* 2016). Bluetongue is a 'notifiable' disease by the World Organization for Animal Health (OIE) due to its major effects on animal health and economic impact (OIE 2010). Bluetongue virus is a non-enveloped virus, with a triple-layered icosahedral protein capsid and has a double-stranded, RNA genome composed of 10 discrete segments, identified as segments 1 to 10, which encode 12 distinct virus proteins. Seven of these (VP1 to VP7) are structural components of BTV, while 5 (NS1 to NS5) are non-structural proteins (Mertens and Diprose 2004, Kar *et al.* 2007, Roy 2008, Maan *et al.* 2015a). The outer-capsid layer consists of 2 major proteins, VP2 and VP5, of which VP2 is the major determinant of BTV serotype and elicits serotype-specific immunity (Mertens *et al.* 1989, Nason *et al.* 2004, Roy and Noad 2006). The middle 'core-surface' layer is formed by VP7, which as the main immuno-dominant protein contains serogroup specific epitopes, which can be targeted in ELISA for detection of antibodies against BTV (Oldfield *et al.* 1990, Anthony *et al.* 2007). The innermost 'sub-core' capsid layer is composed of VP3, which determines the overall size and structural organisation of the virus particle, as well as surrounding the 10 viral genome segments and the VP1, VP4, and VP6 proteins which form the viral transcriptase-complexes.

Early diagnosis, rapid serotyping and characterization of BTV are essential steps for the identification of the origin of the virus, its prevention, and control. Serologic diagnosis of BTV by monoclonal antibody-based competitive enzyme-linked immunosorbent assay (c-ELISA) targeting VP7 has been validated and found to be highly specific and sensitive for detection of anti-BTV (serogroup) antibodies in ruminants (Reddington *et al.* 1991). The c-ELISA is an OIE approved diagnostic technique for detection of BTV infection to support the international trade of livestock.

Nucleic acid based diagnosis for BTV has revolutionized diagnostic procedures by being quick, sensitive, and serogroup/virus-species (or serotype) specific. The NS1 gene (genome-segment 5) is highly conserved between different BTV serotypes (Huismans and Cloete 1987) and was recommended as a 'group-specific' reverse transcription polymerase chain reaction (RT-PCR) target by OIE. In the present study, an NS1-gene based RT-PCR assay was tested using BTV RNA isolated from a cell culture adapted strain of BTV-23 and field derived blood samples, which were positive for BTV-specific antibodies by c-ELISA.

Materials and methods

BTV reference virus

The Indian strain of BTV serotype 23 was isolated from a sheep during a severe BT outbreak in Rahuri of Maharashtra State in 1988 and maintained in the virus laboratory of the Center for Animal Disease Research and Diagnosis (CADRAD), Indian Veterinary Research Institute (IVRI), India. Full genome sequence analysis of this strain revealed that most of the genome segments belong to the major "Eastern" BTV topotype. However, genome segment 5 belongs to the major "Western" BTV topotype, thus demonstrating that the BTV-23 used in the present study is a reassortant virus (Maan *et al.* 2012).

Collection of clinical samples

In the present sero-epidemiological study, a total of 576 'whole blood in EDTA' and serum samples, were collected from cattle (416) and sheep (160) in different agro-climatic zones of India. About 5 ml of blood, mixed with anticoagulant (EDTA - 1.5 mg/ml), was used for BTV nucleic acid detection by NS1 based RT-PCR, and 5 ml of blood were used for serum separation for BTV antibody detection by c-ELISA (BTV antibody test kit, c-ELISA, VMRD, Pullman, Washington, USA). The separated serum samples were stored at -20°C prior to analysis.

Samples were collected from Tharparkar, Kankrej as well as from crossbred adult cattle in Suratgarh, Jaipur, and Bikaner districts of Rajasthan, in Jhansi and on the Indian Veterinary Research Institute (IVRI) experimental farm in the Izatnagar region of Uttar Pradesh, India. Samples were also collected from Chokla, Garol, and Malpura breeds of sheep from the Central Sheep and Wool Research Institute (CSWRI), Avikanagar district of Rajasthan. Sample collection also included Bellary, Decani, and Kanguri breeds of sheep from the Chitradurga district of Karnataka State, India.

Competitive enzyme-linked immunosorbent assay (c-ELISA)

All of the serum samples were screened for the presence of BTV group-specific antibodies using the Bluetongue Antibody Test Kit, c-ELISA (VMRD, Pullman, Washington, USA) which is based on recombinant expressed VP7 protein. The test was performed as per the manufacturer's protocol. Briefly, a volume of 25 µl of undiluted test serum was dispensed along with positive and negative controls in their respective wells of an antigen coated ELISA plate and incubated at room temperature for 15 minutes. About 25 µl of antibody-peroxidase conjugate were added to all the wells and incubated at room temperature for 15 minutes. After washing the plate 3 times with 1x washing buffer, 50 µl of chromogen-substrate solution were added to all the wells, and the plate was kept at room temperature for 15 minutes until blue colour was developed. At this point, 50 µl of stop solution were added to all wells of the plate to stop the reaction.

The optical density (OD) of wells was measured at 620 nm wavelength on an ELISA plate reader. The mean of negative controls producing an optical density (OD) values greater than 0.30 and less than 2.00, and similarly positive controls producing an OD values less than or equal to 50% of the mean of negative controls were validated and noted. The sample was considered positive for presence of BTV antibodies if the optical density values were less than 50% of the mean of negative control. The samples with optical density values greater than or equal to 50% of the mean of the negative controls were considered negative.

Viral dsRNA extraction and c-DNA preparation

The RT-PCR assay for the NS1 gene was optimized using a cell culture adapted strain of BTV-23 and blood samples collected from suspected cases of BT in sheep. The blood samples of animals showing high titers of BTV antibodies by c-ELISA and BTV-23 reference virus maintained and passaged in the BHK-21 cell line at CADRAD virus laboratory were processed for extraction of total RNA using TRIZOL® reagent (Life Technologies, Waltham, Massachusetts, USA) method as per the manufacturer's instruction.

Briefly, 1 ml of blood sample or cell culture supernatant fluid was added to an equal volume of TRI reagent, vortexed, and incubated for 5 minutes at room temperature for the complete lysis of cells and dissociation of nucleoprotein complexes. After lysis, 0.2 ml of chloroform were added, mixed vigorously for 15 to 20 seconds and incubated at -20°C for 10 minutes and then centrifuged at 10,000 g for 15 minutes at 4°C for phase separation.

The colourless upper aqueous phase was transferred to fresh RNAse free 1.5 ml eppendorf tubes. Then, RNA was precipitated by adding 0.5 ml of chilled isopropyl alcohol and the sample kept at -20°C for 20 minutes. Later, the RNA was pelleted by centrifuging at 13,000 g for 10 minutes at 4°C. The RNA pellet was washed twice with 1 ml of 75% pre chilled ethanol by centrifugation at 7,500 g for 5 minutes at 4°C. The supernatant was removed and the RNA pellet was briefly air dried for 5 minutes then re-suspended in 30 µl of nuclease free water and stored at -80°C until further use. Approximately 2 µg total RNA was reverse transcribed using random hexamer primers by avian myeloblastosis virus (AMV) Reverse Transcriptase enzyme (Reverse Transcription System, Promega, Madison, Wisconsin, USA) according to manufacturer's protocols. All c-DNA samples were then stored at -80°C until further use.

PCR amplification of genome segment 5 (NS-1 gene) using gene specific primers

The c-DNA prepared as above was used as template for amplification of the BTV NS-1 gene using gene specific primers and DreamTaq Green PCR Master Mix (Fermentas, Waltham, Massachusetts, USA) following the manufacturer's instruction. The reaction mixture for PCR was prepared in a PCR tube with a final

Table I. *Oligonucleotide primer sequences and optimal amplification conditions for the Bluetongue virus (BTV) reverse transcription-polymerase chain reaction (RT-PCR) assay.*

Gene	Primers sequence (5'-3')	Nucleotide position	Primer (bp)	Product size (bp)	Annealing temperature (°C)
BTV-seg-5/F-NS1	GTTCTCTAGTTGGCAACCACC	10 -30	21	274	57
BTV-seg-5/R-NS1	AAGCCAGACTGTTTCCCGAT	264 -283	20		
BTV-nested seg-5/F-NS1	GCAGCATTTTGAGAGAGCGA	169-188	20	101	55
BTV-nested seg-5/R-NS1	CCCGATCATACATTGCTTCCT	249-269	21		

volume of 25 µl and the optimized PCR protocol consisted of an initial template denaturation at 95°C for 30 minutes, followed by 35 'constant' cycles of amplification at 95°C for 30 seconds for melting, 57°C for 30 seconds for annealing of primers, 72°C for 1 minute for cycle extension, and a final extension at 72°C for 10 minutes to complete the reaction. The details of group specific NS1 primer sequences were given in Table I.

Nested-PCR

The nested-PCR was done for the second round amplification of the 101 bp PCR product using nested BTV NS1 gene specific primers. Five µl of first round amplified product (274 bp) were used as a template for the second round of PCR amplification. The thermal cycling conditions for the second round of PCR amplification were an initial template denaturation at 94°C for 5 minutes, followed by 40 'constant' cycles of amplification at 95°C for 30 seconds for melting, 55°C for 30 seconds for primers annealing, and 72°C for 1 minute for extension with a final extension at 72°C for 10 minutes to complete the reaction.

Table II. *Prevalence of Bluetongue virus antibodies in ruminants in India.*

S. No.	Species*	No. of samples	Positive	Prevalence (%)
1.	Cattle	416	304	73.08
2.	Sheep	160	87	53.30
	Total	576	391	67.88

* Cattle serum samples were collected from various districts in Rajasthan and Uttar Pradesh State, and sheep serum samples were collected from various districts in Rajasthan and Karnataka State, India during 2013.

Results

Whole blood in EDTA and the serum samples from cattle and sheep were collected from different agro-climatic zones of India. In the present study, 576 serum samples from cattle (416) and sheep (160) were screened by c-ELISA for the presence of BTV specific antibodies. The prevalence of BTV infection in ruminants was calculated on a percentage basis. Out of 416 cattle sera tested, 304 (73.08%) were found to be positive for BTV-specific antibodies. Similarly in sheep, out of 160 sera tested, 87 (53.30%) were sero-positive for BTV-specific antibodies. The rate of sero-positivity was therefore higher in the cattle population than in sheep (Table II).

The prevalence of BTV-specific antibodies in cattle in different agro-climatic zones ranged between 60 to 80% in Rajasthan, and between 66 to 70% in Uttar Pradesh. Seropositivity was highest (83.09%) in the irrigated Northwestern plain of Suratgarh, 72.30% in the semi arid Eastern plain of Jaipur, and 63.76%

Table III. *Agro-climatic zone / regional sero-prevalence of Bluetongue virus antibodies in Indian cattle during 2013.*

Agro-climatic zones	Region (State)	Total number of samples	Positive	Negative	Seropositivity (%)
Irrigated north western plain	Suratgarh (Rajasthan)	71	59	12	83.09
Semi arid eastern plain	Jaipur (Rajasthan)	65	47	18	72.30
Arid western plain	Bikaner (Rajasthan)	69	44	25	63.76
Semi arid bundelkhand plain	Jhansi (Uttar Pradesh)	84	61	23	72.61
Mid western gangetic plain	Bareilly (Uttar Pradesh)	127	84	43	66.14

Table IV. *Agro-climatic zone / regional sero-prevalence of Bluetongue virus antibodies in Indian sheep during 2013.*

Agro-climatic zones	Region (State)	Total number of samples	Positive	Negative	Seropositivity (%)
Semi arid and sub-tropical zone	Avikanagar (Rajasthan)	76	34	42	44.73
Central dry zone	Chitradurga (Karnataka)	84	53	31	63.09

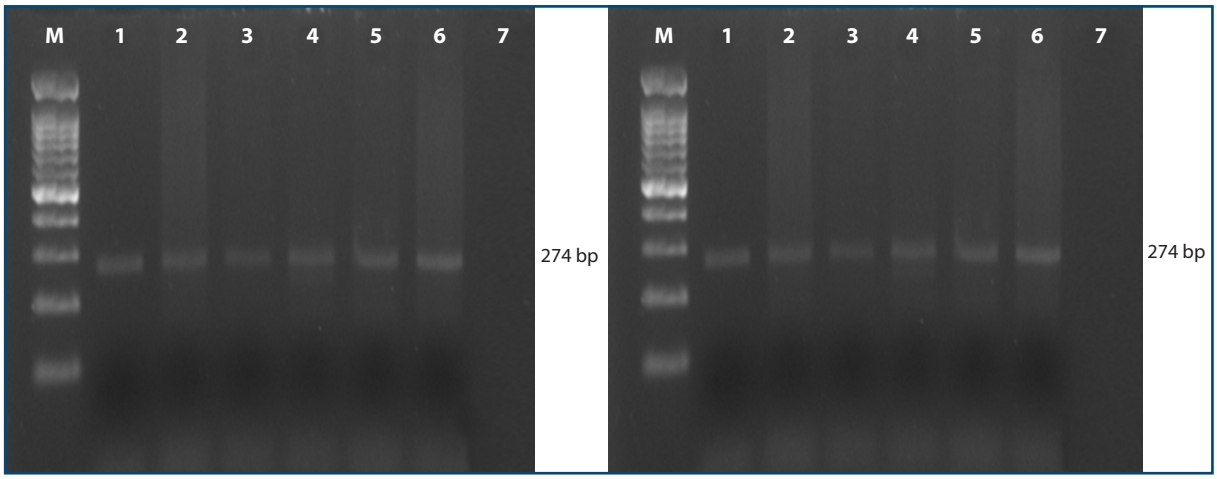

Figure 1. *Bluetongue virus NS1 based diagnostic polymerase chain reaction assay.* **A**. Agarose gel elctrophoresis showing 274 bp amplicon specific for the NS1 gene of BTV; Lane M = 100 bp DNA ladder; Lane 1 = positive control; Lane 2-4 = cattle blood samples; Lane 5 and 6 = sheep blood samples; Lane 7 = non template control. **B**. Agarose gel showing 101 bp amplicon specific for the NS1 gene of BTV. Lane M = 100 bp DNA ladder; Lane 1-3 = cattle blood samples; Lane 4-6 = sheep blood samples; Lane 7= positive control.

in the arid western plain of the Bikaner districts of Rajasthan. In the semi arid Bundelkhand plain of Jhansi, 72.61% of cattle were found seropositive, while 66.14% of the samples collected in the Western Gangetic plain of the Bareilly districts of Uttar Pradesh were positive for BTV-specific antibodies.

The prevalence of BTV antibodies in sheep was 44.73% in the semi arid and sub-tropical zone of the Avikanagar area of Rajasthan. In the central, dry zone of the Chitradurga district of Karnataka State, 63.09% of sheep were positive for BTV-specific antibodies. The results of seroprevalence of BTV-specific antibodies in cattle and sheep from different agro-climatic zones are summarised in Tables III and IV.

During the present study, the nested PCR-based assay, using the NS-1 gene specific outer and inner primers, was optimized for detection of the RNA of BTV from a cell culture adapted strain of BTV-23 and blood samples from animals showing high titers of BTV-specific antibodies by c-ELISA. The RT-PCR and nested PCR assays amplified outer and inner primer specific products of 274 bp and 101 bp sizes, respectively, from the cell culture adapted BTV and blood samples (Figure 1, A and B). Nineteen out of the 70 cattle blood samples and 9 out of 30 sheep blood samples tested positive for BTV genomic RNA, whereas all of the BTV negative serum samples were also found to be negative by nested BTV PCR assays run on matched blood samples.

Discussion

Bluetongue is endemic in India (Ranjan *et al.* 2015, Rao *et al.* 2016). The occurrence of BT has been reported from several states from the Northen (Haryana, Himachal Pradesh, Jammu and Kashmir, Punjab, Rajasthan, and Uttar Pradesh), Central (Madhya Pradesh), Western (Gujarat and Maharashtra), and Southern areas of the country (Andhra Pradesh, Karnataka, Kerala and Tamil Nadu) (Ranjan *et al.* 2015, Rao *et al.* 2016). The results of the present study revealed an overall 67.88% seroprevalence of BTV in ruminants, which is consistent with 60.26% seroprevalence of BTV in West Bengal (Panda *et al.* 2011) and 50.59% seroprevalence in Rajasthan (Tripathi *et al.* 2012) in domestic ruminants.

In our study, cattle showed higher sero-positivity (73.08%) in comparison to sheep (53.30%). Similar observations were made by Oberoi and colleagues (Oberoi *et al.* 1988), who reported BTV-specific antibodies in 70% of cattle sera from the Punjab, as well as by Raut and colleagues (Raut *et al.* 2013) who reported a 89.80% seropositivity of BTV in cattle in Maharashtra state. Joardar and colleagues (Joardar *et al.* 2013) reported 58.82% seroprevalence in sheep serum samples for BTV-specific antibodies in the Northeastern Indian state of Assam.

The seroprevalence of BT in cattle and sheep shows a wide variation in different agro-climatic zones covering the irrigated plains to arid/semi-arid and dry-subtropical zones in different regions of India. However, each of these zones clearly provides favourable climatic conditions for vector multiplication and, hence, for the propagation of the virus. The higher seroprevalence in cattle may reflect their potential role as major subclinical carriers of BTV and their involvement in the micro-epidemiology of the disease. An improved sero-surveillance system for BTV in endemic areas would be of great help in understanding the epidemiology of the virus and disease, and for formulating effective control and preventive strategies in India.

Nucleic acid based assays for BTV have revolutionized diagnostic procedures by being quick, sensitive, and serogroup (or serotype) specific. NS1-gene based BTV nested PCR assays have been used to detect the RNA of BTV directly in clinical samples (Aradaib *et al.* 1998, Tiwari *et al.* 2000, Billinis *et al.* 2001). The segment 5 of BTV, encoding NS1, is highly conserved among different BTV serotypes (Huismans and Cloete 1987, Gould *et al.* 1988) and was recommended as RT-PCR assay target by OIE (2008) for the international trade in livestock.

In the present study, the NS1-gene based BTV nested PCR was standardized using viral RNA extracted from a cell culture adapted strain of BTV-23 and field blood samples. The assay amplified outer and inner primer specific products of 274 bp and 101 bp sizes, respectively. Our findings are in line with BTV NS1-gene based nested PCR based primer-specific amplification carried out by Katz and colleagues (Katz *et al.* 1993) and Chauhan and colleagues (Chauhan *et al.* 2009). The use of the BTV NS1-gene nested PCR assay to analyse clinical field blood samples provides a rapid, sensitive, and reliable diagnostic method for detection of infection to study the viral epidemiology BTV during outbreaks.

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