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Serological and molecular evidence of bluetongue in sheep and goats in Uttar Pradesh, India

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Cross-sectional experimental study was conducted with the objective to estimate the seroprevalence on the basis of antibodies to VP7 protein of bluetongue virus by competitive enzyme linked immunosorbent assay (c-ELISA), to test the neutralizing ability of the antibody to reference strains of 4 BTV serotypes (BTV-1, 2, 10 and 23) by micro serum neutralization assay (m-SNT), to check the presence of BTV dsRNA and to isolate and characterize bluetongue virus (BTV). A total of 91 serum and 26 whole blood samples were obtained from sheep and goat. The study was conducted between September and November, 2012 when the culicoides midges' activity is maximal. The animals were observed for clinical signs of BTV infection and serum samples were obtained from all animals for c-ELISA and m-SNT. Further, blood samples were collected from the c-ELISA positive animals and subjected to virus isolation and nested RT-PCR. Out of 91 animals tested, 26 (28.6%) were found to be seropositive by c-ELISA and one sheep showed neutralizing antibody against BTV-1 serotype at a titer of 1.2 $\log_{10}$. Multivalent logistic regression analysis of risk factors like age, sex, body condition and species of animals were considered during the serological study and found that species of animal had significant influence (χ^2 = 17.111, P<0.05) in seropositivity of BTV. Goats showed more seropositivity to bluetongue as compared to sheep (OR = 0.233). Other risk factors had no significant influence (P>0.05) on seropositivity. It was worth enough to conclude that higher seroprevalence among goats indicated that goats would be the most important animals in the epidemiology of BTV with less clinical manifestation due to development of acquired immunity as the result of continuous exposure.

Key words: Bluetongue virus (BTV), competitive enzyme linked immunosorbent assay (c-ELISA), goat, seroprevalence, sheep, micro serum neutralization assay (m-SNT), nested reverse transcription polymerase chain reaction (RT-PCR).

INTRODUCTION

Bluetongue (BT) is an arthropod-transmitted hemorrhagic disease of wild and domestic ruminants. It is enzootic in many tropical, subtropical and some temperate regions, including much of the Americas, Africa, southern Asia and northern Australia, coincident with the geographic distribution and seasonal activity of competent *Culicoides* vector insects (Purse et al., 2005, 2009).

Bluetongue virus (BTV) is the prototype of the genus

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Abbbreviation: BTV, Bluetongue virus; **c-ELISA,** competitive enzyme linked immunosorbent assay; **m-SNT,** micro serum neutralization assay; **BT,** Bluetongue.

Orbivirus in the family *Reoviridae* (Van Regenmortel, 2003; Mertens et al., 2004). Currently, there are 26 recognized serotypes (BTV-1 to -26) with recent additions of the 25th serotype ("Toggenburg orbivirus") from Switzerland in goat and 26th serotype from Kuwait in sheep and goat (Hofmann et al., 2008; Maan et al., 2011, 2012; Batten et al., 2013). There is only low level of cross-protection among the BT virus serotypes and making vaccination strategies and control programmes a daunting task (Hofmann et al., 2008; Eschbaumer et al., 2009).

BTV is almost exclusively spread by *Culicoides* spp. biting midges (*Diptera*) and occurs worldwide and its spread increased in association with the climate change (Purse et al., 2005). All 26 serotypes can cause BT, a non-contagious hemorrhagic disease of domestic and wild ruminants and camelids with no known zoonotic potential (Verwoerd and Erasmus, 2004; Batten et al., 2013). BT is a multiple species disease to the OIE, World Organisation for Animal Health (OIE, 2013) and notifiable to veterinary authorities in many countries (Eschbaumer et al., 2009). BT primarily affects sheep and deer, but clinical disease in cattle does occur. The worldwide economic losses due to BT have not been expressed in exact numbers, but the estimate is 3 billion US$ a year (Tabachnick, 1996; Sperlovaand Zendulkova, 2011). It can have considerable economic impact, both directly by deaths and decreased productivity and indirectly by trade losses through animal movement restrictions (Verwoerd and Erasmus, 2004).

The OIE recommended complement fixation test (CFT), Agar gel immunodiffusion (AGID) and competitive enzyme linked immunosorbent assay (c-ELISA) as tests for international trade for BT disease screening (OIE, 2008). Many research results has declared that the competitive ELISA technique that detect antibodies directed against the VP7 core protein can be applied to all 26 BTV serotypes, and this technique is highly sensitive and specific (Khalid et al., 2012).The specificity is gained by the use of the monoclonal anti-VP7, which is the protein that distinguishes the BT serogroup from other Orbivirus serogroups, representing the preferred method to determine and monitor BTV circulation (Afshar et al., 1992; Afshar et al.,1993; De et al., 2009; Khalid et al., 2012). The diagnostic sensitivity and specificity of c-ELISA is 87.8% (85.1 to 91.1) and 98.2% (96.3 to 99.6) respecttively, and it is considered as the first choice for the serological surveillance of BT in susceptible animals/herd (Vandenbussche et al., 2008).

Based on virus isolation and serological studies, 21 serotypes have been recorded in different parts of India to date (Ramakrishnan et al., 2005; Dahiya et al., 2004). Realizing the importance of BT in India, Indian Council of Agricultural Research (ICAR) has launched an All India Network Program on BT in an attempt to isolate the virus in embryonated chicken eggs and cell culture system, molecular characterization, maintenance of repository, development of serological and molecular diagnostics besides epidemiology, prevention and control.

This study was carried out with the objectives to determine the seroprevalence of BT by c-ELISA and the neutralizing ability of the antibody to 4 reference BTV serotypes (BTV-1, 2, 10, and 23) by micro serum neutralization assay (m-SNT), to check the presence of the virus by nested RT-PCR and finally to isolate and identify the virus from the blood samples.

MATERIALS AND METHODS

Study animals and experimental design

Cross-sectional experimental study was conducted between September and November 2012 in sheep and goats in Uttar Pradesh, India. A total of 91 (58 sheep and 33 goats) were included in this study. Both males and females of different age groups were part of the study.

Sample collection and testing

10 ml of blood was collected aseptically from the jugular vein of each animal of which 5 ml was mixed with anticoagulant (EDTA1.5 mg/ml) for antigen detection and 5 ml for serum separation for BTV antibody detection. Serum and whole blood were collected from animals and serum was stored at 4°C until analyzed by c-ELISA, m-SNT and whereas blood samples were immediately sent for nested RT-PCR and virus isolation.

Competitive enzyme linked immunosorbent assay (c-ELISA)

The c-ELISA was carried out in this study because it is less subjecttive than serum neutralization test (SNT) techniques and is able to discriminate BTV from another closely associated *Orbivirus*, Epizootic Hemorrhagic Disease virus (EHDV). Serum samples were centrifuged at 3,000 x *g* for 5 min and serum was removed using a sterile transfer pipette. The detection of BTV specific antibodies in serum was carried out using competition ELISA assays (Pourquier c-ELISA kit (IDEXX, UK) according to the manufacturer's instructtions. Results were reported as positive or negative and percent competition based on optical density readings at 450 nm by ELISA reader machine when compared with positive and negative controls.

Micro-serum neutralization test (m-SNT)

The micro serum neutralization test (m-SNT) was used to further confirm the positive c-ELISA results. The m-SNT was performed according to the method of Haig et al. (1956), Oura et al. (2009) and Batten et al. (2013). The reference viruses (BTV 1, 2, 10 and 23) maintained in the virus laboratory of CADRAD, IVRI, Izatnagar, Uttar Pradesh were used in the study. 50 µl of serial 2 fold sera dilutions starting from 1:2 to 1:256 were added to each well of flat-bottomed microtitre plates and mixed with an equal volume of standard reference BTV serotypes 1, 2, 10, and 23 which are prevalent in India containing 100 TCID50 followed by incubation at 37°C in 5% CO_2 for 2 h. 100 µl of BHK-21 cell suspensions having 2×10^5 ml^{-1} concentration were added per well and incubated for 72 h. The plates were examined under the inverted microscope for the presence of cytopathic effect (CPE). A sample was considered positive when it showed a CPE of more than 50% neutralization at the lowest dilution (1:2). The neutralization titer was determined as

Table 1. Nested PCR primers and cycling conditions.

Genes and primer position	Primer sequence (5'-3')	Length (bp)		Annealing temperature (°C)	Nucleotide position
		Primer	Product		
VP5(NS1)	GTTCTCTAGTTGGCAACCACC	21	274	58	10 -30
	AAGCCAGACTGTTTCCCGAT	20			264 -283
Nested VP5(NS1)	GCAGCATTTTGAGAGAGCGA	20	101	57	169-188
	CCCGATCATACATTGCTTCCT	21			249-269

the dilution of serum giving a 50% neutralization end point. m-SNT antibody titers were expressed as log10 reciprocal of the highest positive serum dilution.

Viral dsRNA extraction

Blood samples were subjected to the following pre-treatment before RNA extraction: 250 µl of red blood cells were washed with PBS and lysed with 1 ml distilled water. It was centrifuged at 10,000 × *g* for 10 min, the supernatant was discarded and the pellet was resuspended in 250 µl DEPC water. Total RNA was extracted either from 250 µl lysed red blood cells or 250 µl supernatant from infected BHK-21 cells with 750 µl Trizol-LS reagent (Life Technology, USA), according to the method recommended by the manufacturer. The precipitated RNA was suspended in 30 µl DEPC water.

Nested reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was done with two steps as described by Wade Evans et al. (1990). Reverse transcription was done in a final volume of 20 µl from BTV dsRNA using segment 5 (NS1) gene specific primers (OIE, 2008) and MMLV-RT (Promega, USA) according to manufacturer's protocols. The primers were custom synthesized by M/s Eurofins, Bangalore. The details of the primer set are given in Table 1.

The cDNA mix containing 4 ul of BTV ds RNA (500 ng), 1 ul of forward primer (20 pmol), 1 ul of reverse primer (20 pmol) and 3 ul of DPEC treated water was prepared in 0.5 ml centrifuge tube. This reaction mixture was incubated at 94°C for 4 min for denaturation of dsRNA and to remove secondary structure and then chilled on ice for annealing of primer. 10 µl of the master mix contains 4 µl of 5×Reaction buffer (50 mM Tris/HCl, pH 7.5, 3mM $MgCl_2$ and 75 mM KCl) (Thermoscientific, USA), 1 µl of dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP), 2 µl DTT (0.1 M), 1.5 µl of DEPC water, 0.5 µl of Ribolock™ (Ribonuclease-inhibitor) (Fermentas, USA) (40U/ul) and 1 µl of Reverse transcriptase (Revert Aid H minus) (200 units/µl) (Thermoscientific, USA). 10 µl from the master mix was aliquated to each reaction mixture tube and then mixed properly. Reverse transcription was carried out by incubating the reaction mixture at 42°C for 60 min and MMLV-RT was inactivated by incubating the tube at 70°C for 10 min.The cDNA was stored at -20°C till further use in PCR.

The PCR master mix consisted of 2.5 µl of 10X magnesium free PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X), 1.5 µl of 25 mM $MgCl_2$, 1.0 µl of 10 mM dNTP, 1.0 µl each of (20 pmol) forward and reverse primers, 0.5 µl of 1.0 U/µl Taq DNA polymerase (promega, USA), 11.5 µl of NFW to which 1 µl of cDNA was added. The PCR was carried out by GeneAmp® PCR system 9700 thermal cycler machine (Applied biosystems®, USA). The cycling conditions were initial denaturation at 95°C for 5 min, 35 cycles of 94°C/30 s denaturation, 58°C/30 s primer annealing and 72°C/30 s extension followed by final extension of 72°C for 10 min. The RT-PCR product was resolved on 1.5% agarose gel in 1X TAE buffer. 100 bp DNA ladder (Fermentas, USA) was used as marker to determine the size of RT-PCR products. Gel was stained with ethidium bromide (Life Technologies, USA) and visualized by UV illumination using Gel Documentation System (Gel Doc ™ XR+, imaging system, BIORAD, USA).

Virus isolation and identification from blood sample

Virus isolation was carried out according to the procedure described by Oura et al. (2009). Briefly, blood samples were washed three times with PBS and sonicated as described in the OIE manual (OIE, 2008). BHK-21 cells were inoculated with 500 µl of washed blood and incubated overnight at 26°C. The following day, the inoculum was removed and replaced with fresh DMEM (GIBCO ™, UK) (1% pen/strep, 1% Amphotericin B, 10% FCS). Cells were incubated 26°C for 7 days and then harvested by centrifugation at 2400 ×g for 5 to 10 min, supernatant was tested by nested RT-PCR for the presence of BTV RNA.

Data management and analysis

The data was collected and stored in Microsoft excel spreadsheet format and analyzed using SPSS version 18 software program (SPSS Inc. Chicago, IL, USA). The prevalence was calculated as the number of sample positive, divided by the total number of sample examined. The association between the risk factors and the outcome variables was assessed using chi-square (x^2) test. Uni-and multivariate logistic regressions were used to investigate possible associations between the seroprevalence and the explanatory variables. For all analysis, a P-value less than 0.05 were taken as significant.

RESULTS AND DISCUSSION

Of the 91 small ruminants examined, 26 (28.6%) were found to harbor BTV antibody by c-ELISA. Similarly, species level prevalence of BTV antibody was 8 (13.8%) and 18 (54.5%) in sheep and goats respectively. Multiva-lent analysis of risk factors showed that risk factors like sex, age and body condition had no significant (P>0.05) impact on the c-ELISA result. However, significant (P<0.05) difference was observed in the prevalence of BTV antibody between sheep and goat (Table 2).

Of the 91 sheep and goat serum tested for neutralizing antibody for the four locally prevalent serotypes of blue tongue virus (BTV 1, 2,10, and 23) serotypes, only one sheep (animal code number 3) had detectable neutralizing antibody against BTV 1 serotype measured by SNT

Table 2. Multivariate analysis of risk factor with seroprevalence of the BT virus antibody in sheep and goats.

Risk factor	Prevalence (%)	Total	OR	χ^2(P-value)
Sex				
Female	12 (34.3)	35	2.1	0. 91(0.36)[a]
Male	14 (25.0)	56	1.5	
Total	26 (28.6)	91		
Body condition				
Good	14 (23)	61	7.5	2.864(0.091)[a]
Poor	12 (40)	30	6.03	
Total	26 (28.6)	91		
Age				
Adult	26 (31.3)	83	1.002	3.508(0.061)[a]
Young	0 (0)	8	6.52	
Total	26 (28.6)	91		
Species of animal				
Sheep	8 (13.8)	58	0.521	17.111(0.0000)[b]
Goat	18 (54.5)	33	0.233	
Total	26 (28.6)	91		

[a] No significant association (P>0.05), [b] significant association (P<0.05).

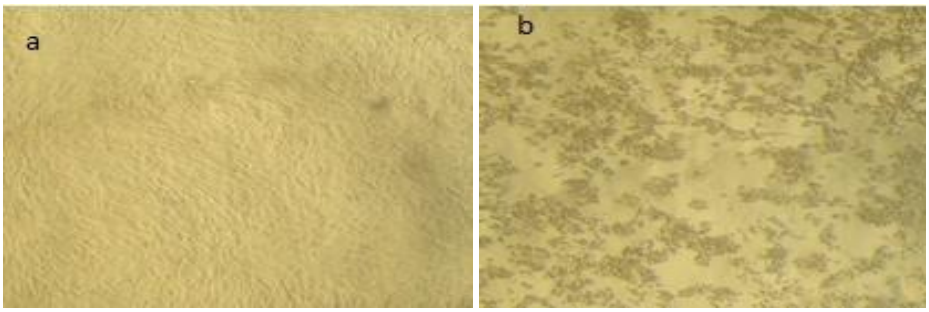

Figure 1. (a) Cell growth and no CPE in the serum neutralization test showing confluent growth of BHK-21 cells after 4 days of incubation at 1:16 serum dilution taken from animal code number 3. **(b)** CPE observed at 1:2 dilutions in other sheep and goat sampleS against all four serotypes.

with titers 1.2 log10 (Figure 1).

The blood sample was collected from 26 c-ELISA positive animals for the detection of dsRNA of BT virus and found negative in nested RT-PCR (Figure 2).

The study also attempted to isolate BT virus from the blood sample; however, there was no growth of virus in the BHK-21 cells after 7 days of incubation and three blind passages.

BT has been reported in several states of India, including northern (Haryana, Himachal Pradesh, Jammu and Kashmir, Punjab, Rajasthan and Uttar Pradesh), central (Madhya Pradesh), western (Gujarat and Maharashtra) and southern areas (Andhra Pradesh, Karnataka, Kerala and Tamil Nadu). However, there are no reports on BT in the north-eastern states of India (Prasad, 2000; Mertens et al., 2008)

In the present study, an overall seroprevalence of 28.6% (26) BTV antibody in sheep and goats was found. It was also found that 13.8% (8) sheep and 54.5 % (18) goats were seropositive in the study. Significant (P<0.05) difference was observed in the prevalence of BTV antibody between sheep and goat in which goats showed more seropositivity than sheep (Table 2). This finding is in agreement with different authors in India and abroad. Studies conducted in Andhra Pradesh during 1991 revealed a higher prevalence of antibodies to BTV in sheep

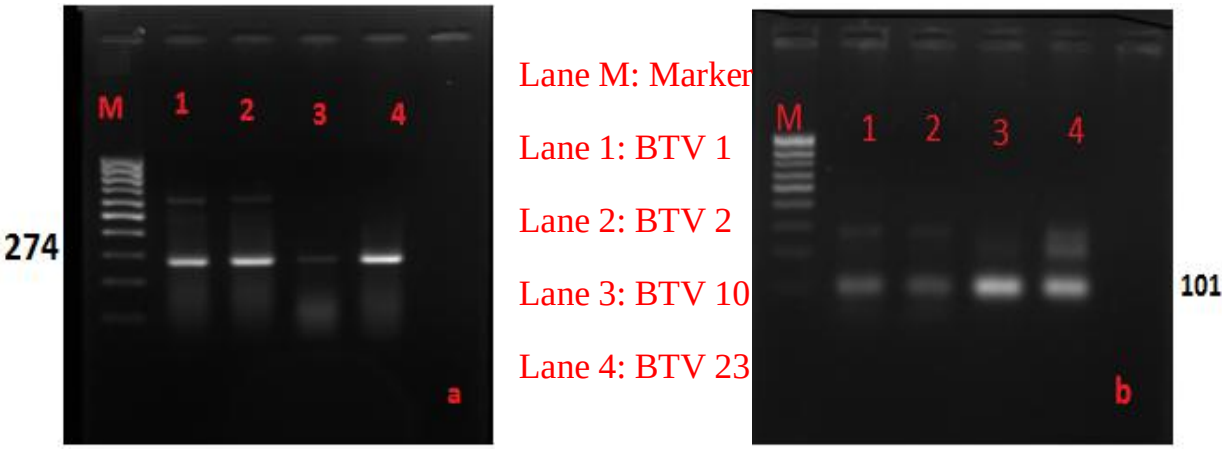

Figure 2. (a) Agarose gel showing 274 bp amplicon specific for NS1 gene of BTV 1, 2, 10 and 23 serotypes in RT-PCR. **(b)** Agarose gel showing 101 bp amplicon specific for NS1 gene of BTV-1, 2, 10 and 23 serotypes in nested RT-PCR.

and goats (45.71 and 43.56%, respectively) than in cattle (33.4%) and buffalo (20%) (Sreenivasulu et al., 2004). This higher prevalence in small ruminants may reflect their involvement in the basic ecology of the virus (Sreenivasulu et al., 2004). Khalid et al. (2012) reported a total seroprevalence of 43.82% (1436) in sheep and goat in Iraq by c-ELISA. According to Khalid et al. (2012), 43.97% (1391) sheep were seropositive by c-ELISA which is very high as compared to the present study (13.8%). This could be due to the geographic nature of Iraq and the presence of rivers, wet lands such as the marshes, lakes, oasis and pools, and the humid hot climate especially in the southern parts of Iraq which provided a very suitable condition for the propagation of the *Culicoides imicola,* the main biting midges of the virus in Iraq (Khalid et al., 2012).

In this study, 54.5% (18) goats were seropositive to BT virus antibodies which were in agreement with the result of Sreenivasulu et al. (2004) who reported 43.6% seropositivity in goats in Andhra Pradesh, India and Khalid et al. (2012) who reported 39.47% positivity in goats in Iraq. On the contrary, low levels of antibody (2.63 and 5.3%), were also detected by Ravishankar et al. (2005) and Doddamani and HariBabu (2006) in Kerala and Karnataka, respectively. Vengadabady et al. (2006) reported the occurrence of BT in goats in the Coimbatore District of Tamil Nadu, with 13% morbidity and 2.3% mortality; however, these data are based mainly on historical information and clinical diagnosis.

This study identified higher seroprevalence among goats indicating that goats would be the most important animals in the epidemiology of BTV with less clinical manifestation due to development of acquired immunity as a result of continuous exposure. Sheep which are highly susceptible animals to BT show clinical signs and die due to the infection. However, in the goats, clinical manifestation is less and they can be tested positive.

On the basis of multivalent logistic regression analysis, it was also detected that risk factors like sex, age and body condition had no significant (P>0.05) impact on the seroprevalence (Table 2). This is in contrast with the results of Christie (2010) who found that flock level seroprevalance was higher among the adult population (25.93%) than young animals (3.7%). This disagreement is due to the fact that the numbers of young animals included in this study are small and all are seronegative which in turn has impact on the data analysis.

Of the 91 sheep and goat serum tested for neutralizing antibody for the four locally prevalent serotypes of bluetongue virus (BTV 1, 2, 10 and 23) serotypes, only one sheep (animal code number 3) had detectable neutralizing antibody against BTV 1 serotype measured by m-SNT with titers 1.2 (log_{10}). This is because antibodies detected by the c-ELISA are directed against the group-specific inner capsid protein (VP-7) and may not be neutralizing antibodies which are directed against serotype-specific VP-2 (Huismans and Erasmus, 1981; Oura et al., 2009). Oura et al. (2009) reported that there is a good correlation between the presence of neutralizing antibodies at the time of challenge and both clinical and virological protection suggests that, if an animal has neutralizing antibodies to a particular serotype present at the time of challenge, it is likely to be protected against that serotype.

The blood sample was collected from c-ELISA positive animals for the detection of BT genomes and found negative in RT-PCR. This result is in accordance with Christie (2010) who detected viral RNA and clinical signs higher in the lamb population than adults. All our lambs were seronegative so that lamb blood samples were not tested by nested RT-PCR. The c-ELISA positive animals were adult and it could be due to the development of group specific antibody after long time and frequent exposure of small amount of antigen. However, the study did not identify any active clinical disease and infection so, there was no active virus in the body of study animals.

The study was also attempted to isolate BT virus from the blood sample; however, there was no growth of virus in the BHK-21 cells after 7 days of incubation. The reason for this result is also the same with the absence of dsRNA detection stated in the above paragraph.

Conclusions

BT is a reportable disease of considerable socioeconomic concern and of major importance for the international trade of animals and animal products. BT has been reported in several states of India including in this study area. BT affects a substantial number of sheep within India and the prevalence is distributed heterogeneously throughout most states of the country. This study reveals high seroprevalence of BT in goats by c-ELISA and goats are very important in the epidemiology of the disease because they do not show any clinical sign; however, they could be source for transmission of the virus through appropriate *Culicoides* biting midges. m-SNT result in this study also shows that there are antibodies against BT which are not neutralizing against locally prevalent viral strains. Higher seroprevalence among adults suggests continued exposure with the development of an acquired immune response, whereas no detection of nucleic acid, virus isolation and no concurrent observation of clinical signs is due to the absence of active viral infection.

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REFERENCES

Afshar A, Eaton BT, Wright PF, Pearson JE, Anderson J,Jeggo M, Trotter HC (1992). Competitive ELISA for serodiagnosis of bluetongue:evaluation of group-specific monoclonal antibodies and expressed VP7 antigen. J. Vet. Diagn. Invest. 4:231-237.

Afshar A, Trotter HC, Dulac GC, Reddington JJ (1993). Evaluation of a commercial competitive ELISA test kit for the detection of group-specific antibodies to bluetongue virus. J. Vet. Diagn. Invest. 5:336-340.

Batten CA, Henstock MR, Steedman HM, Waddington S, Edwards L, Oura AL (2013). Bluetongue virus serotype 26: Infection kinetics, pathogenesis and possible contact transmission in goats. Vet. Microbiol. 162: 62–67.

Christie M (2010). Prevalence and risk factors associated with bluetongue virus among colorado sheep flocks, MSc theis, Department of Clinical Sciences, Colorado State University. pp. 45-60.

Dahiya S, Prasad G, Kovi RC (2004).VP2 gene based phylogenetic relationship of Indian isolates of Bluetongue virus serotype 1 and other serotypes from different parts of the world. DNA Seq. 15(5–6):351–61.

De A, Batabyal S, Biswas SK, Chand K, Singh RK, Mondal B (2009).Surveillance of Bluetongue virus antibody in goats using a recombinant VP7- based indirect ELISA in the coastal saline area of west Bengal, India. Vet. Ital. 45 (2):339-346.

Doddamani RS, HariBabu Y (2006). Study of prevalence of bluetongue in sheep and goats in north Karnataka. Tamil Nadu J. Vet. Anim. Sci. 2: 229–33.

Eschbaumer M, Hoffmann B, Konig P, Teifke JP, Gethmann JM, Conraths FJ, Probst C, Mettenleiter TC and Beer M(2009). Efficacy of three inactivated vaccines against bluetongue virus serotype 8 in sheep. Vaccine 27(31):4169–75.

Haig DA, McKercher DG, Alexander RA (1956). The cytopathic action of bluetongue virus in tissue culture and its application to the detection of antibodies in the serum of sheep. Onderstepoort J. Vet. Res. 27:171–7.

Hofmann MA, Renzullo S, Mader M, Chaignat V, Worwa G (2008). Genetic characterization of toggenberg orbivirus, a new bluetongue virus, from goats, Switzerland. Emerg. Infect. Dis. 14:1855-1861.

Huismans H, Erasmus BJ (1981). Identification of serotype-specific and group-specific antigens of Bluetongue virus. Onderstepoort J.Vet. Res. 48(2):51–8.

Khalid HS, Laith MS, Abdul-RasoulMazin MN, Mahir HH(2012). A serologicalsurveillance of bluetongue disease in sheep and goats in Iraq by using competitive ELISA Technique, Proceeding of the Eleventh Veterinary Scientific Conference. pp. 89-94.

Maan NS, Maan S,Belaganahalli MN, Ostlund EN, Johnson DJ, Nomikou K, Mertens PC (2012). Identification and differentiation of the twenty six bluetongue virus serotypes by RT–PCR amplification of the serotype-specific genome segment 2. PLoS ONE 7(2): e32601. doi:10.1371/journal.pone.0032601.

Maan S, Maan NS, Nomikou K, Batten C, Antony F, Belaganahalli M N. Samy AM, Reda A A, Al-Rashid SA, Batel M El, Oura CAL, Mertens P PC(2011). Novel bluetongue virus serotype from Kuwait. Emerg. Infect. Dis. 17: 886–889.

Mertens PP, Diprose J, Mann S, Singh KP, Attoui H (2004). Bluetongue virus replication, molecular and structural biology. Vet. Ital. 40: 426-437.

Mertens P, Baylis M, Mellor P (2008). Bluetongue, Academic Press. pp.167-195.

Office International des Epizooties (OIE) (2008). Bluetongue. In: Manual of diagnostic tests and vaccines for terrestrial animals. Paris, OIE, Available from: http://www.oie.int/eng/normes/mmanual/2008/pdf/2.01.03 BLUETONGUE.pdf. accessed in Feb. 2013.

Office International des Epizooties (OIE) (2013). OIE-Listed diseases, infections and infestations in force in 2013, http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2013/, accessed in Feb 23, 2013.

Oura CAL, Wood JLN, Sanders AJ, Bin-Tarif A, Henstock M, Edwards L, Floyd T, Simmons H, Batten CA (2009). Seroconversion, neutralising antibodies and protection in bluetongue serotype 8 vaccinated sheep. Vaccine 27:7326–7330.

Prasad G (2000). Whither bluetongue research in India. Indian J. Microbiol. 40:163–75.

Purse BV, Brown HE, Harrup L, Mertens PP, Rogers DJ(2008). Invasion of bluetongue and other orbivirus infections into Europe: the role of biological and climatic processes. Rev. Sci. Tech. 27: 427–442.

Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PP, Baylis M(2005). Climate change and the recent emergence of bluetongue in Europe. Nat. Rev. Microbiol. 3: 171–181.

Ramakrishnan MA, Singh KP, Pandey AB, Singh R, Nandi S, Mehrotra ML(2005). Genomic diversity of Indian isolates of bluetongue virus. Acta Virol. 49:285–6.

Ravishankar C, Nair GK, Mini M and Jayaprakasan V (2005). Seroprevalence of bluetongue virus antibodies in sheep and goats in Kerala State, India. Rev. Sci. Tech. Off. Int. Epiz. 24**:** 953–8.

Sperlova A, Zendulkova D (2011). Bluetongue: a Review. Vet. Med. 56: 430–452.

Sreenivasulu D, SubbaRao MV, Reddy YN, Gard GP (2004). Overview of bluetongue disease, viruses, vectors, surveillance and unique features: the Indian sub-continent and adjacent regions. Vet. Ital. 40 (3):73-77.

Tabachnick WJ (1996). The genetics of Culicoides variipennis and the epidemiology of bluetongue disease in North America. Annu. Rev. Entomol. 45: 20–40.

Van Regenmortel MH (2003). Viruses are real, virus species are man-made, taxonomic constructions. Arch. Virol. 148(12):2481-2488.
Vandenbussche F,Vanbinst T,Verheyden B, van Dessel W,Demeestere L, Houdart P, Bertels G, Praet N, Berkvens D, MintiensK, Goris N, De Clercq K (2008). Evaluation of antibody-ELISA and real-time RT-PCR for the diagnosis and profiling of bluetongue virus serotype 8 during the epidemic in Belgium in 2006. Vet. Microbiol. 129: 15-27.
Vengadabady N, Selvaraj R, Ramaswami AM (2006). A report on the outbreak of bluetongue in sheep and goats in Coimbatore district. Tamil Nadu. J. Vet. Anim. Sci. 2: 246–7.
Verwoerd DW, Erasmus BJ (2004). Bluetongue.In: Coetzer JA, Tustin RC (Eds.), Infectious Diseases of Livestock. , 2nd ed. Oxford Press, Cape Town. pp. 1201–1220.
Wade-Evans AM, Mertens PPC, Bostock CJ(1990). Development of the polymerase chain reaction for the detection of bluetongue virus in tissue samples. J. Virol. Methods 30:15–24.