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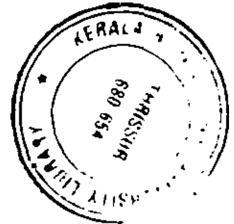
SEROPREVALENCE OF BLUETONGUE IN SHEEP AND GOATS IN KERALA

By

CHINTU RAVISHANKAR



THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

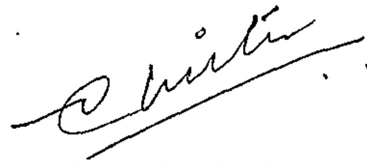
Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

Department of Microbiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR - 680651
KERALA, INDIA

2003

DECLARATION

I hereby declare that the thesis entitled **"SEROPREVALENCE OF BLUETONGUE IN SHEEP AND GOATS IN KERALA"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.



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CHINTU RAVISHANKAR

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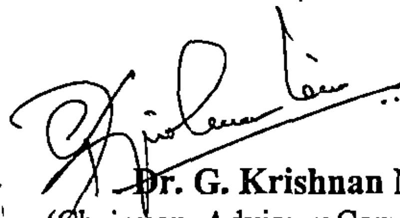
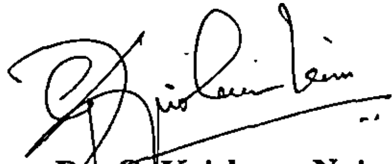


Mannuthy
20-1-03

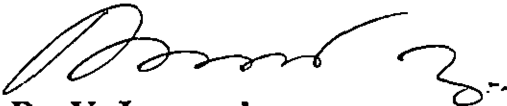
Dr. G. Krishnan Nair,
(Chairman, Advisory Committee)
Associate Professor,
Department of Microbiology,
College of Veterinary and Animal Sciences,
Mannuthy.

CERTIFICATE

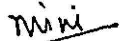
We, the undersigned, members of the Advisory Committee of **Mr. Chintu Ravishankar**, a candidate for the degree of **Master of Veterinary Science in Microbiology** agree that the thesis entitled **"SEROPREVALENCE OF BLUETONGUE IN SHEEP AND GOATS IN KERALA"** may be submitted by **Mr. Chintu Ravishankar** in partial fulfilment of the requirement for the degree.



Dr. G. Krishnan Nair,
(Chairman, Advisory Committee)
Associate Professor,
Department of Microbiology,
College of Veterinary and Animal Sciences,
Mannuthy.



Dr. V. Jayaprakasan,
Associate Professor and Head,
Department of Microbiology.
(Member)



Dr. M. Mini,
Assistant Professor,
Department of Microbiology.
(Member)

Dr. M. R. Saseendranath,
Associate Professor and Head,
Department of Veterinary Epidemiology and Preventive Medicine.
(Member)

EXTERNAL EXAMINER

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Chintu Ravishankar

" The most beautiful thing we can experience is the mysterious. It is the source of all true art and science."

- Albert Einstein.

Dedicated to

Smt. E.K. Santha

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Introduction

1. INTRODUCTION

From ancient times, agriculture had played a vital role in sustaining the Indian economy. The livestock sector is one of the flourishing fields under the agricultural sector today. Of the income generated by the agricultural sector in the country, a sizeable portion comes from the livestock sector.

In Kerala, among the livestock, cattle population is very high when compared to that of goat and sheep. Moreover, the sheep population is mainly restricted to Palakkad and Thrissur districts in Kerala. Hence, not surprisingly, diseases of cattle had been studied extensively than the ones which affect sheep and goats.

Many bacterial and viral diseases affect the ruminant population. One such viral disease is bluetongue (BT), which is considered as one of the most important diseases affecting the pastoral industry world-wide. The classical clinical form of BT is often expressed in sheep, while in other ruminant livestock the disease has variant clinical forms ranging from mild febrile illness to local inflammation and ulceration of mucous membrane. Bluetongue viral infection in large ruminants, especially cattle, can produce clinical manifestations identical to foot and mouth disease (FMD) and epizootic haemorrhagic disease. In Kerala there are reports of

outbreaks of an FMD-like disease in the ruminant population immunised against FMD.

Kerala is flanked by states in which bluetongue is endemic and also there is unrestricted inflow of animals from these areas. Eventhough there are no confirmed outbreaks of the disease in sheep and goats in Kerala, the probability that the disease might be prevalent in our state cannot be ruled out. Moreover there had been unconfirmed reports of the occurrence of a bluetongue-like disease in goats in the northern parts of the state.

Hence this study was conducted with the following objectives:

i) to assess the seroprevalence of BT in sheep and goats in Kerala, employing the agar gel immunodiffusion (AGID) test, indirect enzyme linked immunosorbant assay (I-ELISA) and dot enzyme linked immunosorbant assay (dot-ELISA).

ii) to compare the efficacy of AGID test, I-ELISA and dot-ELISA in detecting antibodies against bluetongue virus (BTV).

Review of Literature

2. REVIEW OF LITERATURE

2.1 History

Bluetongue was first recorded as a disease entity in South Africa in 1876 and the first confirmed BT outbreak outside Africa was in Cyprus in 1943 (Leudke *et al.*, 1964).

The condition was called as "Fever" or "Epizootic Catarrah" in order to distinguish it from other clinical conditions of similar nature and later it was referred to as " Malarial Catarrhal Fever", a designation which was influenced by the mistaken belief that an intracorpuscular parasite caused the disease (Howell and Verwoerd, 1971). Various synonyms have been given to the disease such as Sore mouth, Sore muzzle and Ovine catarrhal fever (Andrewes *et al.,* 1978).

Bluetongue was first reported in India among sheep and goat in Maharashtra state in 1961 (Sapre, 1964). Later, the virus had been isolated from different parts of the country (Bambani and Singh,1968; Uppal and Vasudevan,1980; Kulkarni and Kulkarni,1984; Jain *et al.,* 1986 and Aruni *et al.,* 1997a).

2.2 Classification

Verwoerd (1970) described BTV as a member of the group Diplornavirus. Later, BTV with a large number of similar viruses were

brought under the genus *Orbivirus* (Borden *et al.*, 1971). Baltimore (1971) classified BTV along with the Class III viruses by virtue of its double stranded RNA genome. The virus is now classified in the bluetongue virus subgroup of the genus *Orbivirus* under the family *Reoviridae* and 25 serotypes of the virus have been identified so far (Murphy *et al.*,1999).

2.3 Structural proteins of BTV

The double shelled icosahedral capsid of BTV is composed of an inner core of five structural proteins (VP1, VP3, VP4, VP6 and VP7), surrounded by an outer diffuse layer formed by two structural proteins VP2 and VP5 (DuPleiss, 1992). The proteins responsible for group specificity are VP7 and VP3 and that for the serotype specificity is VP2 (Arita *et al.*, 1993).

2.4 Serological cross reactions

Antibodies against bluetongue virus could cross react either directly or indirectly with a variety of other orbiviruses such as Epizootic haemorrhagic disease virus (EHDV), Ibaraki virus, Eubenangee virus, Tilligery virus and some of the Palyam viruses. (Campbell, 1985). He also opined that the cross reaction of BTV antibodies with EHDV was the most significant one.

2.5 Distribution

Taylor (1986) indicated the global distribution of bluetongue in an equatorial belt approximately between parallels 40°N and 35°S. Bhatnagar *et al.* (1997) in their review of the association between the occurrence of competent vector and BT stated that the disease had been reported from America, Africa, Australia, the South East Asian and European countries, the Indian subcontinent and from the Middle East.

In India, Sreenivasulu and Rao (1999) described the disease as one of the common diseases of sheep in South India, being reported from the states of Andhra Pradesh, Tamil Nadu, Karnataka and Maharashtra. Aruni *et al.* (2000) in their review of BT in India concluded that the disease had been reported in 11 states either on the basis of virus isolation or serology. Of the 21 serotypes reported in India, 11 (serotypes 1, 2, 3, 4, 8, 9, 12, 16, 17, 18 and 23) were determined by virus isolation and 10 (serotypes 5, 6, 7, 10, 11, 13, 14, 15, 19 and 20) by detection of neutralising antibodies (Prasad, 2000).

2.6 Epizootiology

Erasmus (1975) in his discussion about the clinical manifestation of BT in sheep and goat described it primarily as a disease of sheep. He also pointed out that viraemia lasted longer in goats which might be of epizootiological significance.

Nadagouda *et.al.* (1998) studied the epidemiology of BT in migratory sheep in Karnataka and reported that lambs less than six months of age were not susceptible to the disease. Sheep in the age group of six months to one year were found to be most susceptible to BT (Sreenivasulu and Rao, 1999).

Among the domestic ruminants, Metcalf *et al.* (1981) considered cattle as the most important reservoir of the virus.

Mortality, morbidity and case fatality rate ranging from 9.66 to 31.17 per cent, 1.11 to 25.14 per cent and 11.52 to 20.66 per cent respectively have been reported in sheep (Harbola *et. al.,* 1982; Govindarajan *et al.,* 1993 and Joshi *et al.,* 1996).

Taylor (1986) considered *Culicoides imicola,* to be the most important vector of BTV in the Mediterranean region. Of the more than 1400 species of midges belonging to the genus *Culicoides* identified the world over, only eight were proved to transmit BTV to the vertebrate host (Bhatnagar *et al.*, 1995). In India, Prasad (2000) studied the role of *Culicoides oxystoma* as a potential vector of BTV.

In Punjab, Saini *et al.* (1992) compared the month wise prevalence of precipitating antibodies to BTV in sera of various animals and found a higher prevalence rate in August. He attributed this to the increased vector population in the area, because of rainfall during the period. Increased prevalence of BT during the rainy season was also reported in Maharashtra

(Joshi *et al.*, 1996), Tamil Nadu (Aruni *et al.*, 1999) and Andhra Pradesh (Sreenivasulu and Rao, 1999).

Baylis and Mellor (2001) assessed the status of BT in areas around the Mediterranean and stated that wind played a critical role in carrying infected vectors across short stretches of water.

2.7 Disease in domestic ruminants

2.7.1 Disease in sheep

Increased respiratory rate, pyrexia, hyperaemia of buccal and nasal mucosa, frothy salivation, cyanosis of the tongue, mucopurulent nasal discharge and diarrhoea were reported in sheep suffering from BT (Erasmus, 1975).

Jain *et al.* (1986) observed swelling of face, muzzle and nostrils and erosion of the oral mucous membrane in BT affected sheep.

In an outbreak of BT in Tamil Nadu, Aruni *et al.* (1999) noticed ulcers in the oral cavity, laminitis, coronitis and limping in diseased sheep. Extensive muscular wasting was also present.

2.7.2 Disease in goats

Sapre (1964) reported the occurrence of clinical BT in goats in Maharashtra state in India. Anorexia, pyrexia, salivation, oedema of muzzle and face, ulceration of tongue, lips and gums, eruptions on the skin,

coronitis, lameness and diarrhoea were noticed in the affected goats. But mostly the virus produced an inapparant infection in goats (Sellers, 1981 and Dubey *et al.,* 1988).

2.7.3 Disease in cattle

In cattle, the symptoms of clinical BT were found to be very similar to that of FMD (Bekker *et al.,* 1934). He observed a transient febrile response, inflammation and necrosis of the buccal mucosa, dermatitis, skin lesion of the udder, excoriation of the epidermis of the interdigital space and coronitis in the affected animals.

Excessive salivation, nasal discharge, laminitis and stiff gait were reported as clinical signs by Hourrigan and Klingsporn (1975).

Erosions or ulcers on the tongue, muzzle and oral cavity were reported in field cases of BT in cattle (Sellers, 1981).

2.8 Seroprevalence in India

Harbola *et al.* (1982) conducted a seroprevalence study in sheep in Maharashtra and obtained 37.5 per cent prevalence based on AGID test.

Bandyopadhyay and Mallick (1983) in a survey of 7 states in India screened a total of 783 samples from sheep, goat and cattle by AGID test and reported a seroprevalence of 61.11, 3.0 , 26.2 and 2.2 per cent in sheep from Haryana, Uttar Pradesh, Rajasthan and Andhra Pradesh respectively. Sera from cattle (3.77 per cent) and goats (3 per cent) from Uttar Pradesh

were also found to be positive. No positive samples were detected in Himachal Pradesh, Tamil Nadu and West Bengal.

Another study conducted by Mehrotra and Shukla (1984) revealed a seroprevalence of 28.8, 45.8, 61.1, 30.0, 16.4 and 44.4 per cent in sheep and goats in Andhra Pradesh, Karnataka, Maharashtra, Haryana, Rajasthan and Jammu Kashmir respectively, with an overall prevalence of 28.3 per cent.

By AGID test, Sharma *et al.*(1985) reported a seroprevalence of 35.0 per cent in sheep reared in an organised farm in Rajasthan.

Tongoankar *et al.* (1986) could detect precipitating antibodies against BTV in 11.90 per cent of the cattle screened in Gujarat by AGID test.

In Haryana, Prasad *et al.* (1987) screened 306 sheep sera and detected antibodies against BTV in 29.2 per cent of the samples.

Another study conducted by Dubey *et al.* (1988) in Rajasthan revealed an overall prevalence of 8.65 per cent in sheep and goats.

Katoch and Sambyal (1991) could detect antibodies to BTV in 1.94 per cent Gaddi sheep and 0.83 per cent Gaddi goats in Himachal Pradesh by AGID test.

In Tamil Nadu, prevalence levels ranging from 2.71 to 54 per cent had been reported in sheep (Janakiraman *et al.*, 1991; Mehrotra *et al.*, 1991 and Aruni *et al.*, 1997b).

In Punjab, Saini *et al.* (1992) reported a seroprevalence of BT in 2.32 and 8.95 per cent of sheep and goats respectively.

Employing I-ELISA, Mishra *et al.* (1998) could detect BT antibodies in 57.6 per cent of sheep screened in Rajasthan.

A serological survey conducted in Andhra Pradesh revealed the presence of antibodies to BTV in 45.71 per cent sheep, 43.56 per cent goats, 33.11 per cent cattle and 20 per cent buffaloe screened by competitive ELISA (Sreenivasulu and Rao, 1999).

Chandel *et al.* (2001) reported that 8.12 per cent of sheep and 28 per cent of goats in Gujarat were found to harbour BT antibodies.

2.9 Diagnosis

2.9.1 Isolation of the virus

Mason *et al.* (1940) described the isolation of BTV by inoculating clinical materials into embryonated chicken eggs (ECE) by the yolk sac route. Goldsmit and Barzilai (1968) used the intravenous route of inoculation and opined that the method was 100-1000 times more sensitive than the yolk sac method. Yolk sac route and the intravenous route of inoculation of ECE were used by Aruni *et al.* (1997b) for isolation of BTV from clinical specimens.

Clavijo *et al.* (2000) reported that BTV caused cherry red discolouration of inoculated embryos due to haemorrhage and associated oedema.

Many scientists have reported the successful adaptation of egg passaged BTV to cell lines (Osburn *et al.*, 1981; Aruni *et al.*, 1997b and Deshmukh and Gujar, 1999). However, isolation of the virus by direct inoculation of the clinical material into cell lines have also been reported (Jain *et al.*, 1986 and Mehrotra *et al.*, 1996).

Rounding and increased refractility of the cells, granulation and detachment of the cells from the glass surface were the major cytopathic effects produced by BTV in baby hamster kidney-21 (BHK 21) cell lines (Jain *et al.*, 1986).

2.9.2 Detection of Antigen

Pini *et al.* (1966) and Davies *et al.* (1992) used the direct fluorescent antibody technique to detect bluetongue virus in tissue culture.

Jochim *et al.*(1974) described an indirect immunofluorescence test (IIFT) using hyperimmune rabbit antibody to identify and differentiate between BTV and EHDV.

Indirect fluorescent antibody technique was used by Concha-Bermejillo *et al.* (1992) and Roy and Mehrotra (1999) to detect BTV protein in mononuclear cells and cell culture respectively.

Cherrington *et al.* (1985) opined that the peroxidase-antiperoxidase technique could be used to visualise BTV antigen in paraffin embedded sections of infected chorio-allantoic membranes.

Cloned DNA probes were also employed for the detection of BT viral antigen in cell cultures and *in vivo* conditions (Squire *et al.*,1986 and Ventor *et al.*, 1993).

Bluetongue virus in clinical samples were also detected by polymerase chain reaction (Wade-Evans *et al.*, 1990 and Tiwari *et al.*, 2000).

Dot immunoperoxidase assay (DIPA) was described by Afshar *et al.* (1991) for the detection of BTV in tissue culture fluid. He concluded that the DIPA was an effective and less time consuming technique compared to conventional methods used for virus detection.

Stainslawek *et al.* (1996) described an antigen capture ELISA technique to detect BT antigen in sheep blood. Hosseini *et al.* (1998) modified this technique and used it to reveal the presence of BT antigens in inoculated embryonated chicken eggs. He opined that the method had identical sensitivity and specificity to virus isolation in cell cultures.

Prasad and Minakshi (1999) evaluated a RNA polyacrylamide gel electrophoresis for the detection of BTV in tissue culture and found that the detection limits of the test were similar to dot immuno assay.

2.9.3 Detection of Antibody

Fixation of complement by BTV antigen - antibody complex was first described by South African scientists (Van den Ende, 1954). Boulanger *et al.* (1967) developed the modified direct complement fixation test (MDCF) and found that it was more sensitive than the direct complement fixation test in detection of group specific antibodies to BTV. Pearson *et al.* (1985) used the MDCF to detect BTV antibodies in sera along with the AGID test.

Pini *et al.* (1968) described the IIFT and opined that it was a simple alternative to the complement fixation test (CFT). Bowen (1987) used the IIFT to detect group specific antibodies to EHDV and BTV in calf sera.

The precipitation of BT antigen by BTV specific antibodies was first described in 1962 (Klontz *et al.*, 1962). Jochim and Chow (1969) described the microgel diffusion test for the detection of group specific antibody against BTV, using non infective antigen, and opined that this method was a rapid and reproducible one for studying the BT antigen-antibody precipitin reaction.. An improvement of the above method was done by Jochim (1976) in which reference sera were placed in wells adjacent to the test sera. Such a placement of the samples enhanced the weak precipitation reaction and aided in detecting weak positive samples.

AGID test had been used by many scientists for the detection of group specific antibodies against BTV in both domestic and wild animals (Harbola *et al.*, 1982; Gibbs *et al.*, 1983; Sharma *et al.*,1985; Tongaonkar *et al.*, 1986; Prasad *et al.*, 1987 and 1998; Babu *et al.*, 1988; Dubey *et al.*, 1988; Katoch and Sambyal,1991 and Chandel *et al.*, 2001).

Lavakumar *et al.* (1997) evaluated a simple method for extraction of soluble tissue culture antigen for use in AGID and reported that it gave 94 to 96 per cent sensitivity and 100 per cent specificity, when compared to the standard antigen.

Blue *et al.* (1974) described the passive haemagglutination test for the detection of BTV antibodies. Van der Walt (1980) developed the haemagglutination inhibition (HI) test employing a purified preparation of the virus and found it to be serotype specific.

Jochim and Jones (1980) described the hemolysis in gel test for the detection of group specific antibodies to BTV. They opined that the test was sensitive, accurate and reproducible and had the additional advantage over CFT in that it could discriminate between group specific antibodies to EHDV and BTV.

Adkinson *et al.* (1987) used the immunoblotting technique for the detection of BTV protein specific antibodies in sheep sera. Zhou and Chan

(1996) used this test as a confirmatory test along with competitive ELISA (C-ELISA).

The enzyme immunoassays are now widely used in diagnostic medicine (Voller *et al.,* 1975). Manning and Chen (1980) first described an I- ELISA for detecting BTV antibodies using a partially purified antigen, while a highly purified virus preparation was used as antigen in I-ELISA for detection of group specific antibodies by Hubschle *et al.* (1981) and Poli *et al.* (1982). Bhalodiya and Jhala (2002) used the avidin-biotin ELISA (A-B ELISA) for the detection of BT antibodies in cattle and sheep sera.

Anderson (1984) described the blocking ELISA (B-ELISA) technique using monoclonal antibodies against BTV for detecting BTV antibodies. He opined that this technique eliminated cross reaction by antibodies to EHDV and non specific cellular components which gave false positive reaction in AGID test and I-ELISA. Later, Afshar *et al.* (1987b) modified this technique to develop the C-ELISA for bluetongue in which the test sera and monoclonal antibodies were added simultaneously as against the B-ELISA where the monoclonal antibodies were added after the removal of the test sera. Dayakar *et al.* (2001 and 2002) developed a C-ELISA using polyclonal antibodies for screening sera for group specific antibodies to BTV.

The finding that protein antigens could directly be applied to nitrocellulose membranes (NCM) led to the development of the dot immunobinding assay. Dot-ELISA for the detection of group specific

antibodies against BTV had been described (Afshar *et al.*, 1987a and Chander *et al.*, 1991). Naresh *et al.* (1996) compared the group specific VP7 protein expressed in yeast and baculovirus with a commercially available conventional antigen in a dot-ELISA and opined that the recombinant VP7 protein could also be used as antigen in the test.

Neutralisation tests were also employed for the detection of serotype specific antibodies to BTV (Ward *et al.*, 1995).

2.9.4 Comparison of serological tests

Thomas *et al.* (1976) compared the plaque neutralization (PN), MDCF and AGID tests and opined that the PN test was the most sensitive and detected antibodies earlier than the other two. The PN test, in addition to detecting MDCF and AGID test reactors, also picked up the non-reactors.

The plaque neutralisation test and the HI test were equally efficient in detecting serotype specific antibodies to BTV. However, the titres obtained for HI test were 2-4 times lower than those for PN test, making it less sensitive (Van der Walt, 1980).

Afshar *et al.* (1987a) compared a blocking dot-ELISA with the standard AGID test to detect BTV antibodies in bovine and ovine sera. Blocking dot-ELISA could detect BTV antibodies in eight sera samples which were negative by AGID, indicating the higher sensitivity of the former test. They opined that blocking dot-ELISA was simple and that the

antigen coated nitrocellulose membrane strips blocked with gelatin buffer could be stored at ambient temperature without loss of activity.

Lunt *et al.* (1988) evaluated a blocking ELISA, using monoclonal antibodies which specifically detected the major core protein VP7, to detect group specific antibodies to BTV. He could observe that the specificity of the B-ELISA was absolute for antibodies to BTV and that no cross reaction was observed with antibodies to EHDV. The test was also more sensitive than I-ELISA.

From the data obtained by screening sera from BT free areas and experimentally inoculated animals, Afshar *et al.* (1989) could conclude that the specificity of C-ELISA (99.92 per cent) was superior to that of the I-ELISA (99.85 per cent) and AGID test (99.0 per cent). Competitive ELISA detected BTV antibodies earlier than I-ELISA and AGID test. In another study, AGID test was found to be less sensitive than I-ELISA in detecting BTV antibodies, but comparison of I-ELISA and C-ELISA yielded similar sensitivities (Das *et al.*, 1997).

Gupta *et al.* (1990) screened 384 sheep sera using I-ELISA and dot immuno assay (DIA) and found that DIA was more sensitive than the former test in detecting antibodies to BTV. To check the specificity of I-ELISA, C-ELISA was performed and the results showed that DIA was more specific than I-ELISA. However in another study, Mishra *et al.* (1998)

reported that I-ELISA and dot-ELISA were equally sensitive in detecting group specific antibodies to BTV.

Afshar *et al.* (1991) compared B-ELISA and C-ELISA and opined that either of the two tests could be adopted as an international test for serological diagnosis of BT.

A comparison among AGID test, SNT, I-ELISA and dot-ELISA indicated a similar sensitivity between dot-ELISA and I-ELISA and that these tests were superior to the first two in detection of immune response to BTV (Chander *et al.*, 1991).

Koumbati *et al.* (1999) compared AGID test and C-ELISA and opined that C-ELISA was more sensitive in detecting BTV antibodies in sera. Similar results were obtained by Hinsu *et al.* (2000) who could detect BTV antibodies in 87 (53.70 per cent) and 126 (77.78 per cent) of the samples by AGID test and C-ELISA respectively. Relative sensitivity and specificity of AGID test and C-ELISA were 68.25 and 97.22 per cent respectively and the overall agreement between the tests was 74.69 per cent.

Materials and Methods

3. MATERIALS AND METHODS

Borosil brand of glassware and Tarson brand of plasticware were used in the study. Only Analar grade chemicals were used.

3.1 Materials

3.1.1 Reagents and buffers

3.1.1.1 Phosphate Buffered Saline (PBS) [10x Stock Solution]

Sodium chloride	80 g
Potassium chloride	2 g
Disodium hydrogen phosphate ($Na_2HPO_4 . 12 H_2O$)	11.32 g
Potassium dihydrogen phosphate (KH_2PO_4)	2 g
Distilled water	1000 ml

pH was adjusted to 7.4 by 1N NaOH and sterilised by autoclaving at 121°C for 15 min at 15 lbs pressure.

3.1.1.2 PBS (1x Working Solution)

PBS (10x) (3.1.1.1)	100ml
Distilled water to make	1000 ml

pH adjusted to 7.4 using 1 N HCl.

3.1.1.3 Agar Gel Immunodiffusion (AGID) test

3.1.1.3a Gel for AGID test

Agarose	1.0 g
Sodium chloride	0.85 g

Sodium azide	0.01 g
Distilled water	100 ml

To dissolve the agarose in saline, the solution was boiled for five minutes.

3.1.1.3b Stain for AGID test

Amidoblack 10B	0.1 g
Sodium chloride	0.85 g
Distilled water	100 ml

3.1.1.3c Decolouriser I

Methanol	120 ml
Acetic acid	30 ml
Distilled water	30 ml

3.1.1.3d Decolouriser II

Absolute alcohol	140 ml
Acetic acid	20 ml
Distilled water	40 ml.

3.1.1.3e Agar coated slides

Clean glass slides were coated by smearing 0.5 per cent melted agar in distilled water and drying in air by keeping the slides horizontally over glass rods.

3.1.1.4 Indirect ELISA

3.1.1.4a ELISA plates

Flat bottomed ELISA plates with 96 wells (Tarsons Pvt. Ltd.) were used.

3.1.1.4b Bovine serum albumin (BSA)-1 per cent

BSA	1 g
PBS (3.1.1.2)	100 ml

3.1.1.4c PBS-Tween-20 (PBST) - 0.05 per cent

Tween 20	500 µl
PBS (3.1.1.2)	1000 ml

pH was adjusted to 7.4 using 1 N HCl.

3.1.1.4d BSA-PBST - 1 per cent

BSA	1 g
PBST (3.1.1.4c)	100 ml

A fresh solution was prepared just before use.

3.1.1.4e Carbonate-bicarbonate buffer (Coating buffer), pH 9.6

Sodium carbonate (Na_2CO_3)	1.59 g
Sodium bicarbonate ($NaHCO_3$)	2.93 g
Triple distilled water	1000 ml

pH was adjusted to 9.6 using 1 N HCl and the solution was kept at 4°C.

3.1.1.4f Citrate buffer, 0.01 M, pH 5.0

0.2 M Sodium phosphate dibasic ($Na_2HPO_4 . 2 H_2O$)	25.7 ml
0.1 M Citric acid	24.3 ml
Distilled water	50 ml

Adjusted the pH to 5.0 when found necessary and stored at 4°C.

3.1.1.4g Chromogen

O-phenylenediamine dihydrochloride (OPD) obtained from Sigma-Aldrich.

3.1.1.4h Chromogen/substrate solution

OPD	4 mg
Citrate buffer (3.1.1.4f)	10 ml

Just before use, 5 µl of 30 per cent hydrogen peroxide was added to the freshly prepared substrate solution.

3.1.1.4i Stopping solution (1.25 M Sulphuric acid)

Conc. sulphuric acid	34.7 ml
Distilled water to make	500 ml

3.1.1.5 Dot-ELISA

3.1.1.5a Nitrocellulose membrane (NCM)

Probind 45 (Amersham Pharmacea) brand of nitrocellulose membrane having an average pore diameter of 0.45µm was used.

3.1.1.5b PBST - 1 per cent

Tween-20	1 ml
PBS (3.1.1.2)	1000 ml

pH was adjusted to 7.4 using 1 N HCl.

3.1.1.5c Skim milk powder - 5 per cent

Skim milk powder (Amul India Ltd.)	5 g
PBST (3.1.1.5b)	100 ml

The solution was made fresh just before use

3.1.1.5d BSA- PBST- 1 per cent

BSA	1 g
PBST (3.1.1.5b)	100ml

3.1.1.5e Chromogen

Diaminobenzidine (DAB) obtained from Sigma-Aldrich.

3.1.1.5f Chromogen/substrate solution

DAB	5 mg
PBS (3.1.1.2)	10 ml

The solution was made fresh and 10 µl of 30 per cent hydrogen peroxide was added just before use.

3.1.1.6 Competitive ELISA

Stock solutions of diluting buffer and washing buffer and substrate-buffer capsules were supplied along with the C-ELISA kit obtained from Veterinary Diagnostic Technology Inc., Wheat Ridge, Colorado, United States of America.

3.1.1.6a Washing buffer (1X)

Washing buffer (20X)	50ml
Distilled water	950 ml

3.1.1.6b Diluting buffer (1X)

Diluting buffer (10X)	7.5 ml
Distilled water	67.5 ml

3.1.1.6c Buffer- Substrate solution

One buffer-substrate capsule containing sodiumperborate as substrate was dissolved in 100 ml of distilled water and mixed well.

3.1.1.6d Chromogen /substrate solution

OPD (3.1.1.4g)	4 mg
Buffer-substrate solution (3.1.1.6c)	10 ml

3.1.1.6e Stopping solution (3N sulphuric acid)

3.1.2 Biologicals

3.1.2.1 Antigen

Baby hamster kidney-21 cell line based bluetongue AGID test antigen marketed by Vaccine Research Centre, Centre for Animal Health Studies, Madhavaram Milk Colony, Tamil Nadu University of Veterinary and Animal Sciences, Chennai as component of the BT-AGID test kit was used in the study.

For competitive ELISA, BTV antigen coated ELISA plates (each plate consisting of six strips of 16 wells each) which were obtained along with the bluetongue C-ELISA kit.

3.1.2.2 Positive sera

Reference positive sheep serum was supplied along with the BT-AGID test kit.

Goat serum which gave a strong positive reaction in AGID test was used as positive serum in the ELISA test for goat. The strong positive and weak positive sera for C-ELISA were provided along with the kit.

3.1.2.3 Negative sera

Agar gel immunodiffusion test negative sheep/goat serum was used as negative control in the serological tests as the case may be. For calculating the cut off optical density (OD) in I-ELISA ten AGID test negative sera samples from each species were taken.

3.1.2.4 Monoclonal antibody

Mouse monoclonal antibody specific to BTV antigen was supplied along with the C-ELISA kit.

3.1.2.5 Conjugates

Anti-immunoglobulin G horse radish peroxidase (Anti-IgG HRP) conjugates against sheep and goat were obtained from Sigma-Aldrich and Bangalore Genei respectively.

Peroxidase labelled anti-mouse immunoglobulin was supplied along with the C-ELISA kit.

3.2 Methods

3.2.1 Collection of sera samples

A total of 1010 sera samples, 109 (10.79 per cent) from sheep and 901 (89.2 per cent) from goats were collected from the field, farms and slaughter houses throughout the State (Table 1). Samples were collected on

Table 1. District-wise details of sheep and goat sera collected in Kerala

Sl. No.	Name of District	Samples collected						District Total
		Sheep			Goats			
		Farm	Field*	Total	Farm	Field**	Total	
1	Thiruvananthapuram				5	99	104	104
2	Kollam				20	75	95	95
3	Pathanamthitta					46	46	46
4	Alappuzha					40	40	40
5	Kottayam					79	79	79
6	Idukki				5	76	81	81
7	Ernakulam					80	80	80
8	Thrissur	12	18	30	18	61	79	109
9	Palakkad		79	79	5	52	57	136
10	Malappuram					49	49	49
11	Kozhikode					68	68	68
12	Wayanad					20	20	20
13	Kannur				6	51	57	57
14	Kasaragode				5	41	46	46
	Total	12	97	109	64	837	901	1010

* Includes samples from slaughter houses
** Includes samples from veterinary hospitals and slaughter houses

the basis of the sheep and goat population in each district. In case of farms, at least five per cent of the animals housed were screened.

3.2.2 Agar Gel Immunodiffusion (AGID) test

Agar gel immunodiffusion test was done as per the method of Jochim and Chow (1969) with minor modifications. Three to four millilitres of melted agarose was poured onto precoated glass slides and allowed to set. One central well and five peripheral wells, each with three millimetre diameter were punched in such a way that the distance between the central well and any peripheral well was three millimetres. Distance between the adjacent peripheral wells were kept equal. The central well was filled with 20 μl of antigen. Twenty microlitres each of positive and negative serum were loaded separately in two of the peripheral wells. The remaining wells were filled with 20μl each of test sera. The slides were incubated at room temperature in a humid chamber for 48 h and were examined in diffuse light for the presence of precipitin lines.

The slides were rinsed first in two changes of normal saline for 24 h each and then in distilled water for another 24 h to remove the unreacted proteins. Then they were dried slowly, stained with amidoblack 10B (3.1.1.3b) for 15 minutes and decolourised for 20 minutes each in decolouriser I and II. The decolourised slides were dried and mounted in DPX.

3.2.3 Indirect ELISA (I-ELISA)

Indirect ELISA was performed as described by Das *et al.* (1997) with minor modifications. The group specific antigen having a total protein concentration of 3.39 mg per ml was used to coat the plate. For screening sheep sera, optimum concentration of the coating antigen (1:50), positive serum (1:800), negative serum (1:100) and anti-sheep IgG HRP conjugate (1:5000) were arrived at by preliminary checker board titrations.

Test proper

All the wells of the ELISA plate except two were coated with 50µl each of 1:50 dilution of antigen in coating buffer (3.1.1.4e), and incubated overnight at 4°C. The plate was washed thrice with PBST (3.1.1.4c) and tapped dry onto a lint free absorbent towel. The uncoated sites were blocked with 100µl of one percent BSA (3.1.1.4b) and incubated at 37°C for one hour. After incubation the BSA solution was thrown off and the plate tap dried. Fifty microlitres each of sheep sera samples diluted 1:100 in BSA-PBST (3.1.1.4d) were added to the wells in duplicate. Positive and negative serum controls and a conjugate control without any serum were also kept and the plate was incubated at 37°C for 2 hours.

It was then washed and dried as before and each well was charged with 50 µl of 1:5000 anti-sheep IgG HRP conjugate diluted in BSA-PBST

(3.1.1.4d) and was incubated at 37°C for one hour. Washing and drying of the plate was done as mentioned earlier.

Hundred microlitres each of freshly prepared chromogen / substrate solution (3.1.1.4h) was charged into all the wells including the controls. The plates were incubated in the dark at 37°C for 30 minutes.

The reaction was stopped by adding 100 µl of 1.25 M sulphuric acid to each well.

The OD values were read at 492 nm against blank in a software based Multiskan Ascent ELISA reader and the data were interpreted as described by Hubschle *et al.*(1981). This consisted of taking the OD values of few negative sera (ten in the present study) from which the arithmetic mean and the standard deviation were calculated. The standard deviation was multiplied by a factor (which varied with the number of negative sera taken) and the product was added to the arithmetic mean to get the cut off value.

Goat sera were also screened in the same manner using 1:50 dilution of antigen,1:800 dilution of positive serum and 1:100 dilution of negative/ test serum. The dilution of anti-goat IgG HRP conjugate used was 1:10,000.

The cut off OD values calculated as per the method given above were 1.204 and 1.812 for sheep and goat respectively.

3.2.4 Dot-ELISA

The test was done as described by Gupta *et al.* (1990) with slight modifications.

A plastic template with eight strip-like projections, each with five millimetres width and separated from its adjacent one by a distance of four millimetres , were made. Nitrocellulose membrane pieces (0.5 cm x 0.5 cm) were attached to the tip of each projection with adhesive (Fevibond, Pidilite Industries, Ltd.). Two microlitres of neat/undiluted antigen was deposited on to the NCM and allowed to dry at room temperature. The unbound sites were blocked with five per cent solution of skim milk powder (3.1.1.5c) for one hour. The membrane was rinsed 3 times in PBST (3.1.1.5b) and incubated at 37°C for 1hour in 1:20 positive/negative /test sera diluted in BSA-PBST (3.1.1.5d). The membrane was again rinsed as before and incubated in 1:1000 anti-sheep/goat IgG-HRP conjugate, diluted in BSA-PBST (3.1.1.5d), at 37°C for 30 minutes. It was rinsed and immersed in chromogen/substrate solution (3.1.1.5f) for 2-3 minutes and the reaction was stopped by rinsing the membrane in PBS.

3.2.5 Competitive ELISA

Competive ELISA was performed as per the protocol supplied along with the kit.

Washed the wells of the antigen coated ELISA plate two times with washing buffer (3.1.1.6a). Added 100µl of diluting buffer (3.1.1.6b) to duplicate wells and covered with tape. This formed the diluent control wells. Then added 50 µl each of 1:5 dilution of negative, weak positive, strong positive or test serum in diluting buffer to duplicate wells. Immediately added 50 µl of 1:100 diluted mouse monoclonal antibody to all wells except the diluent control wells. Mixed and held for two hours at room temperature. Removed the tape from the diluent control wells and washed all the wells three times with washing buffer. Added 100µl of 1:300 diluted peroxidase labelled anti-mouse immunoglobulin conjugate to all wells and held for one hour in the dark. Washed wells five times with washing buffer and added 100µl of chromogen / substrate (3.1.1.6d) solution to all wells and incubated the plate in the dark for ten minutes. Added 50µl of stopping solution (3.1.1.6e) to all wells and read the absorbance at 492 nm.

A modification of formula described by Afshar *et al.* (1987b) was used to determine the percentage inhibition (PI) of the monoclonal antibody for each serum control and test serum sample.

PI = 100 - [(adjusted average OD of test sample) / (adjusted average OD of the negative control serum) x 100]

The average OD values of the diluent control wells represent the background OD. This is subtracted from the average OD values of the

control and test sera to yield their adjusted OD values. For the test to be valid, the adjusted OD value of the negative serum must be with in a range of 0.4 to 1.4. Percentage inhibition of the weak positive control serum must be between 50 per cent and 80 per cent and the PI of the strong positive serum must be 85 per cent or above. Under these circumstances, a test sample is considered to be positive if the PI is equal to or more than 50 per cent. Samples with PI less than 50 per cent are negative.

3.2.6 Statistical analysis

To determine any significant difference between the tests used in the detection of antibodies to BTV, Z-test was done as described by Rangaswamy (1995).

Results

4. RESULTS

4.1 *Collection of sera samples*

Sheep sera samples were collected from Thrissur and Palakkad districts only. Of the 109 sheep samples collected, 12 (11.0 per cent) were from an organised farm and the rest were from sheep brought to the slaughter house and from animals brought to Kerala from Tamil Nadu to browse in the uncultivated fields in Palakkad district.

Of the goat sera collected, 64 (7.10 per cent) were from organised farms throughout the state. The rest of the samples were collected from the field, slaughter houses and veterinary hospitals (Table 1).

In either species, none of the animals showed clinical signs suggestive of bluetongue during the period of study.

4.2 Serological tests

4.2.1 AGID test

On viewing the gel against diffuse light, a precipitin line was observed between the reference positive serum and antigen wells. The reference negative serum did not give any precipitin line against the antigen.

None of the sheep sera were detected as positive in the AGID test. But two goat sera were tested positive, as evidenced by the formation of a single precipitin line showing complete identity with the

line obtained for the reference positive serum. The precipitin lines were located slightly towards the side of the test serum well.

Clear visualisation of the precipitin line was possible by staining the gel with amido black 10B (Fig. 1).

4.2.2 ELISA

The total protein concentration of the AGID test antigen used for I-ELISA and dot-ELISA was 3.39 mg per ml.

4.2.2.1 I-ELISA

Fifteen (13.76 per cent) of the sheep sera screened had OD values above 1.204 and were taken as positive for the presence of BT antibodies. In case of goat samples, 68 (7.54 per cent) samples were tested positive. Accordingly a total of 83 (8.21 per cent) samples of both the species were detected as positive for the presence of antibodies to BTV by I-ELISA (fig.2).

4.2.2.2 Dot-ELISA

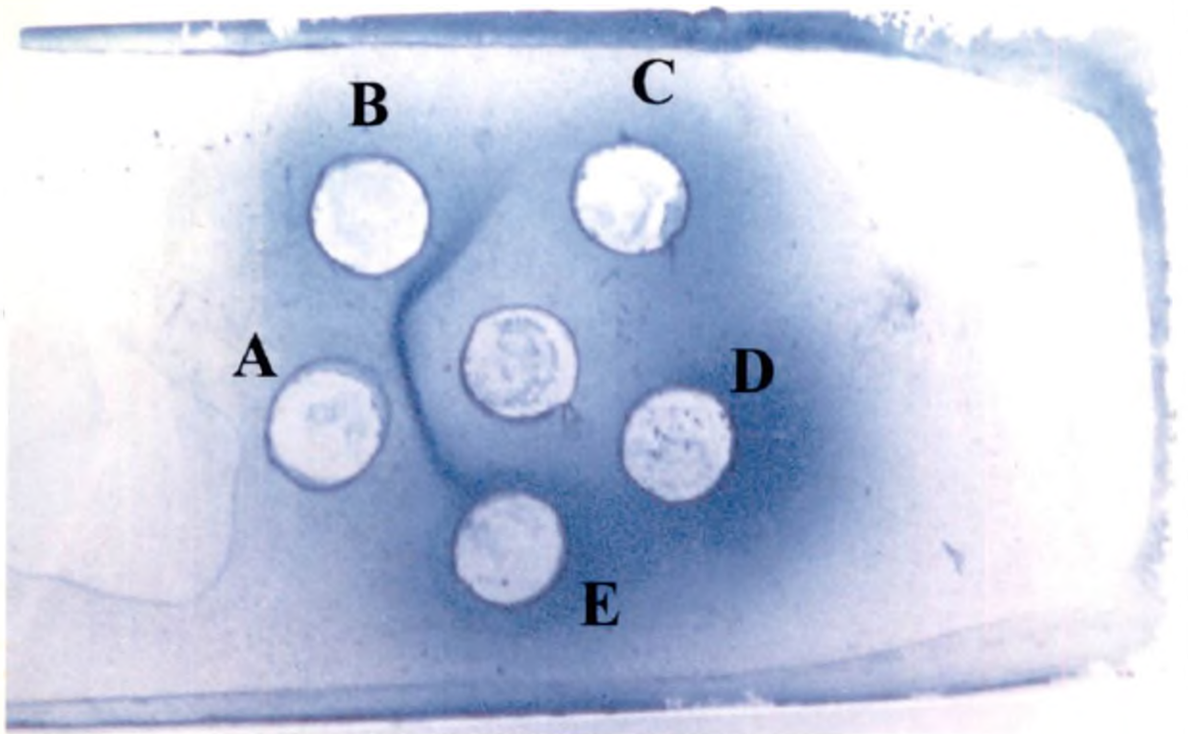
Of the samples screened, 11 (10.09 per cent) sheep and 46 (5.10 per cent) goat samples were found to be positive, as evidenced by the

Fig.1 Agar gel immunodiffusion test

A – Positive control
B to D - Test sera
E – Negative control
Central well – Antigen

Fig.2 Indirect-ELISA

Wells A1 and A2 - Blank
Wells B1 and B2 - Positive control
Wells C1 and C2 - Negative control
Wells D1 and D2 - Conjugate control
All other wells – Test sera in duplicate


B
C
A
D
E

appearance of a brown dot at the site of antigen deposition (fig.3). All the samples obtained as positive in dot-ELISA were positive in I-ELISA also.

The antigen bound to the NCM was found to be stable for a period of one month at room temperature and at 4°C. Further evaluation of the stability of the antigen on storage could not be carried out.

The positive dots remained without fading for 3 months, *i.e.*, till the termination of the study.

4.2.2.3 C-ELISA

All the sera samples positive by I-ELISA and two negative sera from each species, were cross-checked by C-ELISA. Of the 83 positive samples screened, four were found to be false positive by C-ELISA. At the same time, all the negative samples remained negative in C-ELISA also (fig.4).

The adjusted OD of the negative control was 0.642 and the PI values for the weak positive and strong positive controls were 72.5 per cent and 92.68 per cent respectively. Percentage inhibition values of the positive samples ranged from 50.26 per cent to 99.48 per cent.

4.3 Seroprevalence

Based on the results of the C-ELISA test, the results of the I-ELISA test was modified to arrive at the district-wise seroprevalence of bluetongue in Kerala state. Prevalence of the disease could be detected in 12 districts of the state. No samples were tested positive in Alappuzha and Wayanad

Fig.3 Dot-ELISA

A - Positive control
B - Negative control
C to H - Test sera

Fig.4 Competitive-ELISA

Wells A1 and B1 - Diluent control
Wells C1 and D1 - Negative control
Wells E1 and F1 - Weak positive control
Wells G1 and H1 - Strong positive control
All other wells – Test sera in duplicate

A B C D E F G H

districts. The highest prevalence of the disease was seen in Thrissur disrict in which 23 (21.10 per cent) of the 109 animals screened were found to be positive and the lowest was in Ernakulam (1.25 per cent). The overall prevalence of the disease in the state was 7.82 per cent (Table 2). The district-wise seroprevalence of BT is represented diagramatically in figure 5.

The prevalence of the disease in sheep in field and farms were 12.37 and 25.0 per cent respectively. In the state, the prevalence of bluetongue among sheep was 13.76 per cent (Table 3).

Out of the 837 goat sera samples collected from the field, 37 (4.42 per cent) were positive for the presence of antibodies to BTV. Twenty seven out of the 64 samples collected from the farms were found to be positive, giving a prevalence level of 42.18 per cent. When goat sera samples from the field and farms were considered together, the prevalence of BT was found to be 7.10 per cent (Table 4).

4.4 Comparison of Serological tests

Among the 109 sheep sera samples screened none were detected as positive by AGID. In I-ELISA and dot -ELISA, 15 (13.76 percent) and 11 (10.09 per cent) samples respectively were tested positive. Comparison of the proportion of positive samples obtained by I-ELISA and dot-ELISA using the Z-test revealed that there was no significant difference between

Table 2. District-wise seroprevalence of bluetongue in Kerala

Sl. No.	Name of District	Samples collected	Samples positive	Seroprevalence (%)
1	Thiruvananthapuram	104	8	7.69
2	Kollam	95	8	8.42
3	Pathanamthitta	46	2	4.34
4	Alappuzha	40	-	---
5	Kottayam	79	4	5.06
6	Idukki	81	5	6.17
7	Ernakulam	80	1	1.25
8	Thrissur	109	23	21.10
9	Palakkad	136	11	8.08
10	Malappuram	49	4	8.16
11	Kozhikode	68	5	7.35
12	Wayanad	20	-	---
13	Kannur	57	3	5.26
14	Kasaragode	46	5	10.86
Total		1010	79	7.82

Fig. 5 District-wise seroprevalence of bluetongue

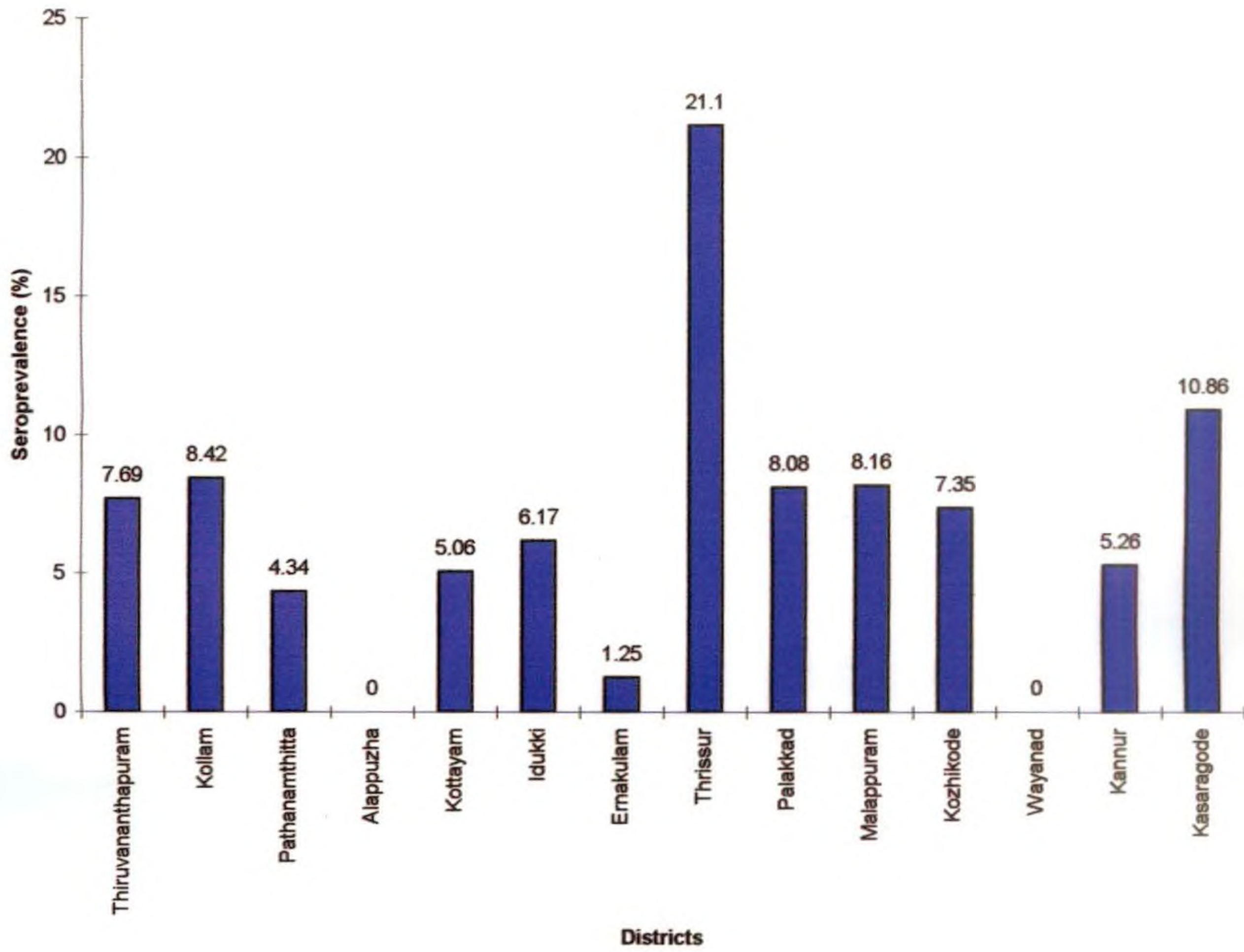


Table 3. Seroprevalence of bluetongue in sheep in Kerala

Sl. No.	Name of district	Samples collected	Samples positive	Seroprevalence (%)		
				Farm	Field	Total
1	Thrissur	(12+18)**	(3+4)**	25.00	22.22	23.33
2	Palakkad	(0+79)	(0+8)	---	10.12	10.12
Total		(12+97) =109	(3+12) =15	25.00	12.37	13.76

Table 4.Seroprevalence of bluetongue in goats in Kerala

Sl. No.	Name of district	Samples collected	Samples positive	Seroprevalence (%)		
				Farm	Field	Total
1	Thiruvananthapuram	(5+99)**	(2+6)**	40.0	6.06	7.69
2	Kollam	(20+75)	(7+1)	35.0	1.33	8.42
3	Pathanamthitta	(0+46)	(0+2)	---	4.34	4.34
4	Alappuzha	(0+40)	(0+0)	---	---	---
5	Kottayam	(0+79)	(0+4)	---	5.06	5.06
6	Idukki	(5+76)	(2+3)	40.0	3.94	6.17
7	Ernakulam	(0+80)	(0+1)	---	1.25	1.25
8	Thrissur	(18+61)	(9+7)	50.00	11.47	20.25
9	Palakkad	(5+52)	(1+2)	20.0	3.84	5.26
10	Malappuram	(0+49)	(0+4)	---	8.16	8.16
11	Kozhikode	(0+68)	(0+5)	---	7.35	7.35
12	Wayanad	(0+20)	(0+0)	---	---	---
13	Kannur	(6+51)	(3+0)	50.0	---	5.26
14	Kasaragode	(5+41)	(3+2)	60.0	4.87	10.86
Total		(64+837) =901	(27+37) =64	42.18	4.42	7.10

** First figure in parenthesis pertains to samples from farms and second to samples from the field.

these two tests in the detection of antibodies to BTV (Z-value 0.837 at five per cent significance).

In the case of goats, 2 (0.22 per cent), 64 (7.10 per cent) and 46 (5.10 per cent) samples out of 901 were tested positive by AGID, I-ELISA and dot-ELISA respectively. On comparison of the tests it was found that there was significant difference between AGID and I-ELISA (Z value = 8.0) and between AGID and dot-ELISA (Z value = 6.51) in detecting BT antibodies in serum. However, as in the case of sheep, there was no significant difference between I-ELISA and dot-ELISA (Z value = 1.77) in the detection of positive samples. A comparison of the three tests on the basis of number of positive samples detected is given in table 5.

Table 5. Comparison of the serological tests

Species	Number tested	Number found positive		
		AGID test	I-ELISA	Dot-ELISA
Sheep	109	0	15 (13.76%)	11 (10.09%)
Goat	901	2 (0.22%)	64 (7.10%)	46 (5.10%)

Discussion

5. DISCUSSION

5.1 Seroprevalence

In the present study, AGID test, I-ELISA and dot-ELISA were used to detect the prevalence of BT antibodies in sheep and goats in Kerala state. Of all the sera screened, only two samples from goats were detected as positive for the presence of antibodies to BTV by the AGID test. Indirect-ELISA detected 15 (13.76 per cent) sheep and 68 (7.54 per cent) goat sera as positive. In case of dot-ELISA, the corresponding figures were 11(10.09 per cent) and 46 (5.10 per cent) respectively. Of the 68 goat sera samples tested positive by I-ELISA four were confirmed as false positive by C-ELISA.

The results of I -ELISA modified based on the result of the C-ELISA were used to calculate the seroprevalence of BT in the state (Tables 2, 3 and 4). Prevalence of BT could be detected in 12 out of the 14 districts in the state. In the case of sheep, antibodies against BTV could be detected in sera samples collected from Thrissur and Palakkad districts where in the sheep population of the state is concentrated. The prevalence in these two districts were 23.33 per cent and 10.12 per cent respectively, with an overall prevalence of 13.76 per cent.

Prevalence levels of 37.5 per cent, 3.0 per cent, 29.2 per cent, 1.94 per cent, 2.32 per cent, 57.6 per cent, 45.71 per cent and 8.12 per cent had been reported in sheep in Maharashtra, Uttar Pradesh, Haryana, Himachal

Pradesh, Punjab, Rajasthan, Andhra Pradesh and Gujarat respectively (Harbola *et al.*, 1982; Bandyopadhyay and Mallick, 1983; Prasad *et al.*, 1987; Katoch and Sambyal,1991; Saini *et al.*, 1992; Mishra *et al.*, 1998; Sreenivasulu and Rao, 1999 and Chandel *et al.*, 2001).

Reports from Tamil Nadu, indicated that the prevalence levels ranged from 2.71 to 54 per cent in sheep (Janakiraman *et al.*, 1991. Mehrotra *et al.*, 1991 and Aruni *et al.*, 1997b).

In case of goats, the highest number of BT seropositive animals were detected in Thrissur district (20.25 per cent) and the lowest in Ernakulam district (1.25 per cent).

The prevalence of BT reported in goats in Uttar Pradesh, Himachal Pradesh, Punjab and Gujarat were 3.0 per cent, 0.83 per cent, 8.95 per cent and 28 per cent respectively (Bandyopadhyay and Mallick, 1983; Katoch and Sambyal, 1991; Saini *et al.*, 1992 and Chandel *et al.*, 2001).

Higher prevalence levels of BT were observed in organised sheep and goat farms. The prevalence in the sheep farm surveyed was 25 per cent. In case of goat farms the levels ranged from 20 per cent to 60 per cent, with an average of 45.31 per cent. High prevalence rates in flocks maintained in organised sheep farms had been reported previously also (Sharma *et al.*, 1985 and Mishra *et al.*, 1998).

The overall prevalence of the BT in sheep and goats in the state was found to be 7.82 per cent. This indicates that the disease has already established its hold in the small ruminant population of the state.

The maximum number of seropositive samples in sheep and goats were from Thrissur district. This might be because of the nearness of this district to Tamil Nadu, where the disease is endemic. Also there is unrestricted movement of large number of animals from Tamil Nadu to Thrissur district. This is in agreement with the findings of Sreenivasulu and Rao (1999) who attributed the higher number of BT cases in Telengana region in Andhra Pradesh to the proximity of the region to BT affected areas of the neighbouring states.

Of the serological tests employed, AGID test could detect only two BT positive goat samples and hence was the least sensitive of the three tests. The poor sensitivity of the AGID test in detecting group specific antibodies to BTV had been reported by many scientists (Anderson, 1984; Afshar *et al.*, 1987b and Naresh and Prasad, 1995).

Indirect ELISA was more sensitive than dot-ELISA in that it detected more number of positive samples than the latter. But, there was no significant difference in the level of detection of positive samples by both the tests. Similar observation was made by Chander *et al.* (1991) and Mishra *et al.* (1998) while comparing I-ELISA and dot-ELISA in the detection of group specific antibodies to BTV.

However, this observation is in contradiction with that of Gupta *et al.* (1990) who reported the superior sensitivity of dot -ELISA over I-ELISA in detecting BT seropositive samples.

So based on the number of positive samples detected, I-ELISA could be considered as the most sensitive test among the three. Though dot-ELISA detected slightly lower number of positive cases, it had the advantage of being a quick, simple and specific test which could be carried out in the field conditions, with detection levels comparable to I-ELISA. It does not require sensitive and costly equipment and the results can be visualised by the naked eye. Similar observations were also made by Afshar *et al.* (1992) who also recommended the use of dot-ELISA in surveillance studies.

5.2 Serological tests

5.2.1 AGID test

Of all the samples screened by AGID test, only two goat sera samples were positive. The precipitin line observed was confluent with that obtained for the reference positive serum indicating that it was due to BT specific antibodies. Jochim (1976) suggested that incorporation of reference positive serum in wells adjacent to the test sera wells greatly enhanced the sensitivity of the test. In the present study, this was not done, due to shortage of reference positive serum, which might be the reason for detection of only less number of positive samples.

5.2.2 Indirect-ELISA

Many scientists have used purified BTV derived from BHK 21 cell lines as antigen in the I-ELISA. Afshar *et al.* (1987b) and Afshar *et al.* (1991) used stock tissue culture antigens having total protein content of 13.71 mg per ml and 4.1 mg per ml respectively in appropriate dilutions in I-ELISA.

Chander *et al.* (1991) used standard AGID test antigen for coating the plate in I-ELISA. In this study also, the AGID test antigen having total protein concentration of 3.39 mg per ml was used in 1:50 dilution to coat the ELISA plate, with good results. This indicated that the tissue culture based AGID test antigen could be used in I-ELISA, in the absence of highly purified BT antigen. But I-ELISA detected four true negative samples as positive. This may be due to the use of semi-purified AGID test antigen for conducting I-ELISA. Similar reason was also given by Afshar *et al.* (1989) to explain the lower specificity for I-ELISA when compared to C-ELISA.

The detection of positive samples based on cut-off value as described by Hubschle *et al.* (1981) was found to be satisfactory in this study also. Gupta *et al.* (1990) and Chander *et al.* (1991) also used this method to evaluate their samples.

5.2.3 Dot-ELISA

The AGID test antigen was used to coat the NCM. Chander *et al.* (1991) also had used AGID test antigen to coat the NCM in dot-ELISA.

Afshar *et al.* (1987a) observed that the antigen bound to NCM stripes blocked with gelatin was stable for long periods under ambient temperature. In this study also the antigen coated NCM remained stable at 4°C and at room temperature for one month. Further studies on the stability of the antigen could not be carried out.

The use of skimmed milk powder (five per cent solution) as blocking agent was found to work satisfactorily in this study. Chander *et al.* (1991) and Afshar *et al.* (1992) also had used five per cent and three per cent skimmed milk powder respectively as blocking agent. Skimmed milk powder had an advantage over BSA in that it was cheaper and readily available.

The positive dots were found to be stable without fading for a period of three months, which enabled them to be stored and used in retrospective studies. Similar observations on the stability of the dots were made by Lin and Halbert (1986).

The use of the comb shaped plastic template instead of the conventional strips to fix the NCM pieces was found to be very useful in the sense that dilutions of the reagents and sera could be made in microtitre

plates in which the NCM combs could be incubated. Such an arrangement avoided the direct handling of the NCM and also large numbers of samples could be tested at a time.

5.2.4 Competitive ELISA

Competitive ELISA proved helpful to find out the true status of the samples found positive by I-ELISA. There was good correlation between the results of these tests as only four samples were detected as false positive in the I-ELISA.

5.3 Epizootiology

During the period of study, no clinical cases of bluetongue were reported in either sheep or goats. However, antibodies against BT virus could be demonstrated in both the species. In the case of goats, this might be because of the inapparent nature of the disease in them. Kulkarni *et al.* (1984) could detect the presence of antibodies to BTV in sheep even two years after the outbreak of the disease. In the present study, the demonstration of antibodies in clinically normal sheep might be due to the persistance of the antibodies in them subsequent to an earlier infection.

The present study has proved conclusively the prevalence of BT in sheep and goat population in Kerala. But the cattle population , the largest reservoir of BTV, was not screened for the presence of antibodies to BTV. Considering the prevalence of antibodies to BTV in sheep and goats, there is

every possibility that cattle may also harbour antibodies against the virus. So to get a better picture of the disease in the state, cattle should also be screened.

The outbreaks of FMD or FMD like diseases should be studied more critically. In addition to screening for FMD in the affected animals, tests to detect BT antibodies should also be carried out to ascertain the true aetiology of these outbreaks.

Summary

6. SUMMARY

Bluetongue is one of the most important diseases affecting the pastoral industry. The disease has been reported from many countries world wide and also from India. The prevalence of the disease has been reported in all South Indian states except Kerala. In Kerala, there are reports of foot and mouth disease, which causes clinical manifestations similar to bluetongue in ruminants. Also there are unconfirmed reports of a bluetongue-like disease in goats in the northern parts of the state. Moreover, there is unrestricted animal movement from the neighbouring states to Kerala. Hence this study was undertaken to detect the seroprevalence of the disease in sheep and goats in Kerala, using AGID test, I-ELISA and dot-ELISA.

A total of 1010 sera samples were collected from all the 14 districts of the state.

Sheep and goats were found to harbour group specific antibodies to BTV. The results of I-ELISA modified on the basis of the results of C-ELISA was used to arrive at the seroprevalence of bluetongue in the State. Of the 12 districts from which seropositive samples were obtained the highest prevalence of the disease was in Thrissur district (21.10 per cent) and the lowest in Ernakulam (1.25 per cent). The higher number of seropositive samples in Thrissur district might be due to the proximity of the district to Tamil Nadu and due to the inflow of animals from that state. The

overall prevalence of the disease among sheep and goats in the state were found to be 13.76 and 7.10 per cent respectively, while the overall prevalence of the disease in Kerala was found to be 7.82 per cent. Higher prevalence of BT was recorded in organised sheep and goat farms.

Tissue culture derived AGID test antigen having a total protein content of 3.39 mg per ml was used for these tests.

In the AGID test, the positive samples gave a single precipitin line confluent with the one obtained for positive control. Of the total samples screened, AGID test could detect only two goat sera samples as positive.

In the I-ELISA, 1:50 diluted AGID test antigen was used satisfactorily to coat the plates. The cut off OD values for sheep and goats were 1.204 and 1.812 respectively. A total of 83 sera samples were detected as positive by this test.

In the dot-ELISA, the use of a comb shaped template greatly improved the ease of performing the test. Skimmed milk powder was used as blocking agent with good results. The positive samples were evidenced by the presence of a brown dot at the site of antigen deposition. The antigen coated NCM stripes remained stable at 4°C for one month. The positive dots were found to remain without fading for a period of three months. Dot-ELISA detected 11 sheep and 46 goat sera as positive.

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On cross verifying the I-ELISA positive samples by C-ELISA, only four samples were tested negative in the C-ELISA, indicating a good correlation between the results of the two tests. The I-ELISA negative samples screened were found to be negative in C-ELISA also. In the C-ELISA, the percentage inhibition values of the positive samples ranged from 50.26 to 99.48 per cent.

On comparison of the three tests, AGID was found to be least sensitive. I-ELISA detected more positive samples than dot-ELISA. However there was no significant difference between I-ELISA and dot-ELISA in the detection of group specific antibodies to BTV. Though dot-ELISA detected lesser number of positive samples than I-ELISA, it could be recommended as a simple, quick and specific test for detection of group specific antibodies to BTV.

The prevalence of the disease in sheep and goats in the Kerala warrants the screening of cattle also, which is the largest and the most important reservoir of the virus, to get a comprehensive picture of the disease in the state. Moreover, the FMD or FMD-like outbreaks should be more critically analysed to ascertain the true cause of these outbreaks.

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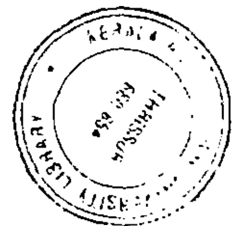

SEROPREVALENCE OF BLUETONGUE IN SHEEP AND GOATS IN KERALA

By

CHINTU RAVISHANKAR

ABSTRACT OF THE THESIS

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Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

Department of Microbiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR - 680651
KERALA, INDIA

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ABSTRACT

A study was conducted to assess the seroprevalence of BT in sheep and goats in Kerala, employing AGID, I-ELISA and dot-ELISA tests. A total of 1010 samples collected from all the 14 districts of the State were screened for the presence of group specific antibodies to BTV. Seropositive samples were obtained from 12 out of the 14 districts. Both sheep and goats were found to harbour antibodies to BTV. Based on the results of the I-ELISA test, the highest prevalence was observed in Thrissur district (21.10 per cent) and the lowest in Ernakulam district (1.25 per cent). Higher prevalence rates were observed in organized farms. The overall prevalence of the disease in the state was found to be 7.82 per cent.

Of the tests employed, AGID test was found to be the least sensitive. The maximum number of positive samples were detected by I-ELISA. To check the validity of the I-ELISA results C-ELISA was done and there was good correlation between the results of the two tests as only four samples were detected as false positive in the C-ELISA. Dot-ELISA was found to be a simple, quick and specific test with detection levels comparable to I-ELISA and could be used in the field conditions.

Screening of the cattle population also should be done to get a better picture of the disease in the State as they comprise the largest reservoir of BTV in nature.