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Sero Diagnosis of *Bluetongue virus* Infection and Isolation of Virus in Embryonated Chicken Egg and BHK-21 Cell Line

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Abstract: Isolation of *Bluetongue virus* from blood samples of sheep and goat was carried out in the present study. Out of one fifty blood samples screened for seroprevalance of BTV antibodies by Agarose Gel Precipitation Test (AGPT) 42 gave positive results. The overall percentage of virus isolation was 28% from Embryonated Chicken Eggs (ECE). The identities of the isolates were confirmed by cytopathogenicity. All the isolates were passaged twenty one times in embryonated chicken eggs and further passaged in BHK-21 cell lines. The viral isolates adapted well to the cell culture system and produced cytopathic change like grouping of cells, polycaryon, syncytica formation, acidophilic and intracytoplasmic inclusion bodies in BHK-21 cells. This study confirms the BTV incidence in the tested blood sample with a possible means showing that the virus can easily adapt to ECE and BHK-21 cell line.

Key words: *Bluetongue virus*, BHK-21 cell line, sero diagnosis

INTRODUCTION

Bluetongue is an arthropod-transmitted disease of wild and domestic ruminants caused by BT Virus (BTV). *Blutongue virus* is a member of Orbivirus genus, one of nine genera in the family Reoviridae characterized by a double stranded RNA genome. The genome is composed of ten double stranded RNA segments (Verwoeds *et al.*, 1970). Twenty four serotypes of this particular virus have been described by Gibbs and Greiner (1994). Of which 21 serotypes existed in India and out of it 4 serotypes have been reported in TN. The virus is transmitted almost entirely by the bites of certain species of *Culicoides* biting midges and as a result is restricted to areas where these vectors are presented (Mellor and Wittmann, 2002). *Culicoides* sp. are small flies with 27 species recorded in India. However, additional taxonomic studies of Indian *Culicoides* species are needed, because even a small country like Laos has recorded 62 species. It is possible that one or both of these species of *Culicoides* could have been involved in the transmission of BTV in Tamil Nadu, but to date no entomological studies are available (Prasad *et al.*, 1994).

The central role of the insect in BT epidemiology ensures that prevalence of the disease is governed by ecological factors, such as high rainfall, temperature, humidity and soil characteristics, which favor insect survival (Clastri *et al.*, 2003). In many parts of the world therefore, the disease has a seasonal occurrence (<http://www.ncbi.nlm.nih.gov/pubmed/169785>). Epitope on VP2 are the target of neutralizing antibody response and are the determinants of serotype specificity, it is also

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responsible for haemagglutination and the binding of BTV to mammalian cells (Huisman *et al.*, 1987). The 10 RNA segments codes for NS3/3A protein which mediates the release of virus particles from infected cells.

The first sign of illness is a rise in temperature reaching 106-107 F (40.6 to 41.7°C) beginning at 7 or the 8 day following infection. Temperature may be elevated for 4 to 12 days following the initial rise. Within 24 h of the initial rise in temperature, excessive salivation and frothing at the mouth develops and is associated with hyperemia of buccal and nasal mucus membrane. Clinical signs of disease in domestic and wild ruminants range from subclinical in the vast majority of cases to an acute febrile response characterized by inflammations and congestion, leading to oedema of the face, eyelids and ears and haemorrhages and ulceration of the mucous membranes. Extensive erosions can develop in the cheeks and on the tongue opposite to the molar teeth. The tongue may show intense hyperemia and become edematous, protrude from the mouth and, in severe cases become cyanotic. Hyperemia may extend to other parts of the body particularly the groin, axilla and perineum. There is often severe muscle degeneration. Dermatitis may cause wool breaks (Ilango, 2006).

Bluetongue (BT) has become one of the important sheep diseases of the Indian sub continent. The disease was first reported in Pakistan in 1959 and in India the disease was first reported during 1963 in Maharastra. In Tamil Nadu the disease was first recorded by Janakiraman *et al.* (1991). It was more pronounced during the monsoon season when the vector population is more (Wilson, 1991). In many parts of India there was a recent outbreak of BT disease including Tamil Nadu. India earns Rs. 8500 crores annually through the production of meat, wool and skin from 485 livestock, of which Tamil Nadu accounts for 24 million livestock. Small and medium farmers in Tamil Nadu rear sheep and goats for meat and skin, which fetches Rs. 200 and the current total number of sheep and goats records 5.2 million and 6.4, respectively.

Annually Tamil Nadu incurs a loss of Rs. 52 lakhs (European Economic Committee Report). This article presents the occurrence of BT in Tamil Nadu over the past 12 years (1988-99) (Ilango, 2006). BT in India is endemic to Tamil Nadu andhra Pradesh, Karnataka, Maharastra, Gujarat, Rajasthan, Himachal Pradesh and Jammu and Kashmir. In Tamil Nadu, 22 out of 23 districts were reported to be affected by the BTV (*Bluetongue virus*). The reported cases of BTV among sheep and goats occur presumably in an epidemic form during the monsoon. Although, the history of reporting was not continuous, the number of outbreaks, attacks and deaths among ruminants posed the great concern, which needs immediate attention for the protection of live stock and economic growth (Ilango, 2006). The isolation was made by intravascular inoculation of 11 day old embryonated chicken egg (Foster and Leudke, 1968). It has been reported that cells are the more susceptible to BTV with BHK-21 producing more titer compared to other cell lines (Weesler and Mc Holland, 1988). The first isolation in tissue culture of wild type non egg adopted virus from the blood of infected sheep was in 1959 (Fernandes, 1959). In this study we isolated the BTV using embryonated chicken egg and cell line BHK-21 during the out break at Dharmapuri, Namakkal and Krishnagiri districts.

The native *Bluetongue* viral isolates were isolated and adopted to cell line for propagation using the embryonated chicken egg and established cell lines such as BHK-21. Seroprevalance of the disease was studied by the AGPT test.

MATERIALS AND METHODS

Collection of Specimens

During an outbreak at the districts of Namakkal, Krishnagiri and Dharmapuri a total of 150 samples were collected from pyrexia animals in the jugular vein (Fig. 1). The outbreak occurred during the monsoon (from November to January 2005). The samples were collected in sterile vacutainers with EDTA and transported to the laboratory and immediately stored at -70°C. For long term storage of blood samples Oxalate-Phenol-Glycerin (OPG) medium was used.



Fig. 1: Blood collection from infected animal

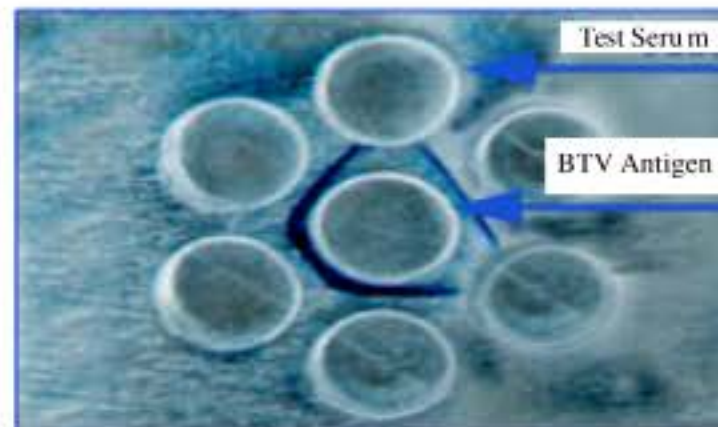


Fig. 2: Agarose gel precipitation test for detection of BTV

Screening for the Presence of BTV Antibody

The collected samples were screened for BTV antibody using Agarose Gel Precipitation Test (AGPT) test (Pearson and Jochim, 1985) (Fig. 2).

Virus Inoculum

Blood samples collected in OPG medium were washed with PBS, sonicated at 20 cycles min^{-1} at 5 w for 2 min and centrifuged at 1000 rpm for 10 min. The resulting supernatant served as viral inoculum.

Virus Isolation Embryonated Chicken Egg (ECE)

Yolksac Route

An aliquot of 0.1 mL viral inoculum was injected into the yolk sac route of embryonated chicken egg (9-12 days) (Fig. 3). The eggs were incubated in a humid chamber at 33.5°C and candled daily. The dead embryos within the first 24 h post inoculation were retained at 4°C and embryos alive after 7 days were sacrificed and processed immediately. Infected embryos often have a hemorrhagic appearance (Fig. 4 a, b). The embryos obtained were homogenized in a mortar and pestle with sterile sand and Eagles MEM supplemented with streptomycin 100 mg mL^{-1} and penicillin 10 mg mL^{-1} , to give a final 10% suspension. The tissue homogenates were clarified by centrifugation at 10,000 rpm for 10 min at 4°C. Virus in the supernatant identified by Agarose Gel Precipitation Test (AGPT) to detect the antibodies (Pearson and Jochim, 1985).

Development and Maintenance of Cell Culture

Minimal Essential Medium (MEM) Preparation

Eagle's minimal essential medium (HI-MEDIA) was prepared in 900 mL of sterile triple distilled water and pH adjusted between 7 to 7.2 with NaHCO_3 and the final volume of the medium

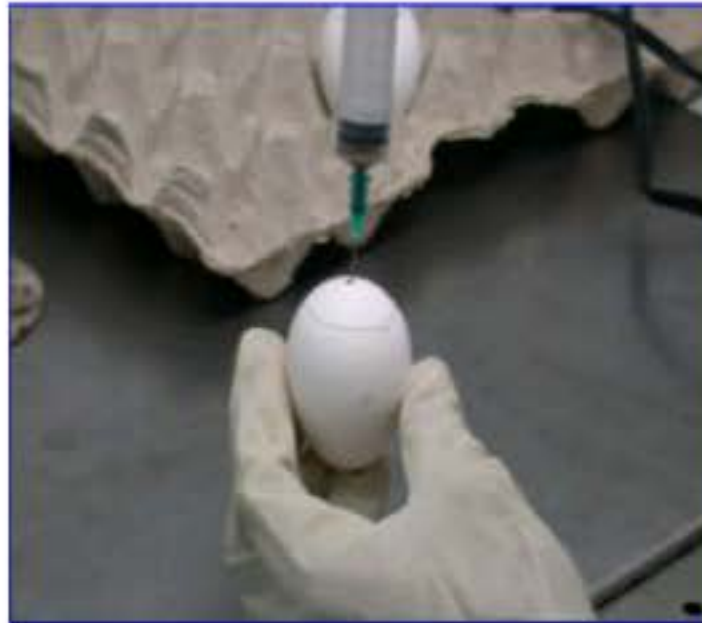


Fig. 3: Inoculation of sample in to yolk sac route of ECE 9-12 days

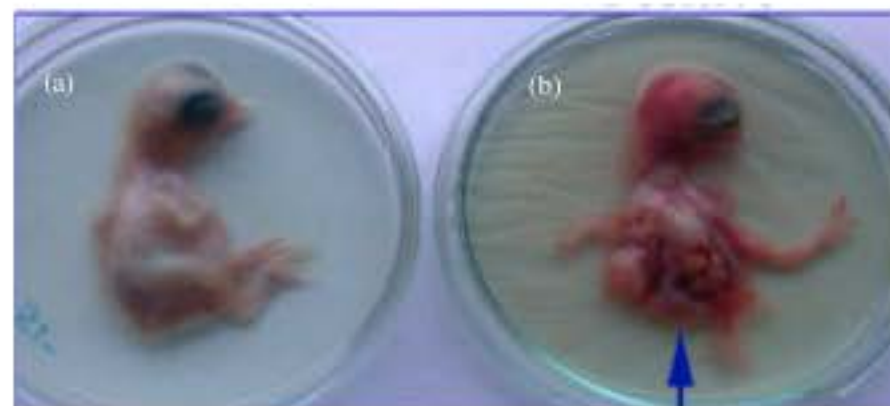


Fig. 4: Chick embryo infected with BTV, (a) control and (b) positive

adjusted to 1 L. Added antibiotic and antimycotic at the given concentration. The prepared medium was filter sterilised through a 0.2 μm pore size membrane filter by creating negative pressure. Filtered medium was stored at 4°C. Sterility check was carried out at regular intervals.

Recovering of Frozen Cells

Preserved vials were taken from cryocan (-196°C) thawed at 37°C. The vial suspension was added to the tissue culture bottles (25 cm²) containing 5 mL of FBS and 3 mL of MEM. The inoculated culture bottles were incubated at 37°C at 5% CO₂ with humidity, for 4 h. Medium was discarded after the monolayer formation. Added the growth medium (10% FBS) and incubated at 37°C at 5% CO₂ with humidity.

Maintenance of BHK-21 Cell Line

Fully formed monolayer of BHK-21 (baby hamster Kidney) was selected for harvesting and further passage. Maintenance medium was discarded and 5 mL of PBS (Phosphate Buffered saline) was added to it. After rinsing (PBS) discarded and 1 mL of Trypsin Versine Glucose (TVG) was added.

The TVG solution was discarded after the detachment of the monolayer. Fifteen milliliter of growth medium (10% FBS) was added and pipetted to get an uniform cell suspension. A sample of suspension was diluted (1:2) with 0.1% trypan blue and checked for viability.

Cell Counting

The trypan blue diluted cell suspension was counted on a hemacytometer. Average number of cells on each squares were obtained and the concentration of the cells mL⁻¹ were determined. For the best result adjust the concentration of the suspension below 50 and 100 cells on each square. The BHK-21 cell suspension had a count of 28×10^{-4} cells mL⁻¹ (Ian Freshny Laboratory Manual).

Propagation of Cells

Cells were diluted in growth medium to contain 1×10^5 cells mL^{-1} and distributed in tissue culture bottles. For each TC bottles, 5 mL of growth medium (10% FBS) was added. The seeded bottles were incubated at 37°C at 5% CO_2 with humidity.

Inoculation of Viruses in to Cell Line

The tissue homogenates were taken clarified by centrifugation at 10,000 rpm for 10 min at 4°C . 0.1 mL of viral sample was inoculated using syringe filter (0.22 μm) to baby hamster kidney (BHK-21) (Wilson, 1991).

Serological Tests (AGPT)

The Immuno Diffusion (ID) test was carried out to detect Bluetongue (BT). 0.9% Agarose was prepared in borate buffer. Six well rosette around the central well was made. Added test serum and bluetongue standard positive serum to identify the weak positive sera. The slide was incubated at room temperature and examined the appearance of precipitation lines. The results were recorded on 3rd day by washing the slide in PBS to give clear lines of precipitation. Stained the slide using coomassie blue and observed the result (Pearson and Jochim, 1985).

Plaque Purification of Viral Isolates

The method of Dulbecco and Copper were followed with slight modification. Monolayer culture were prepared in 45 and 65 mm Petri dishes seeded with 4-6 mL of cell suspension containing $4-5 \times 10^5$ cells mL^{-1} . Plates were incubated at 37°C . Confluent Monolayer was formed in 2-3 days PI. The cells were washed with PBS (pH 7.4) to equilibrate pH and to remove inhibitory substances. Two Petri dishes were used for each dilution of virus inoculums was allowed to adsorb at 37°C for 1 h and the solution was decanted. An overlay containing 1% agarose in Earls saline sterilized by autoclaving at 121°C for 15 min, then cooled to 44°C mixed with equal volume of $1 \times$ basal medium and about 2-3 mL was overlayed and allowed to solidify. A second overlay of 4 mL held at 44°C was poured above the first overlay. The plates were incubated aright on the first day at 37°C (Fig. 5, Table 1) and inverted on the second day. The Petri dishes were stained after 3-4 days using 1:20,000 dilution of neutral red for 18 h. The plaque forming units mL^{-1} was calculated by $\text{pfu mL}^{-1} = \text{Plaque number reciprocal of dilution reciprocal volume in mL}$.

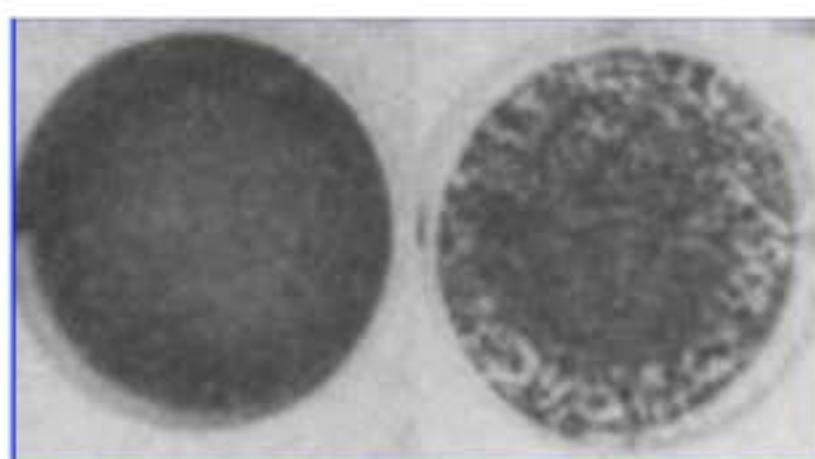


Fig. 5: BTV plaque assay in BHK-21 cell line

Table 1: Plaque assay of BTV with BHK-21cell line

Cell line	Virus conc.	Cell conc /1.5 mL	Plaque description
BHK-21	0.1 mL	4.0×10^5	Not discrete

RESULTS AND DISCUSSION

Bluetongue virus is endemic in many parts of India and an outbreak occurred in Tamil Nadu during the monsoon season (November to January, 2005) (Table 2-4). The outbreak was due to heavy rain fall and the presence of biological vector at the highest (Uppal and Vasudevan, 1980). Thus the severity recorded was attributed to the heavy rain fall which brought about a high vector index of *Culicoides* species that persists through the season. The highest incidence was noted in Dharmapuri District particularly in December month because of heavy rain fall when compared to all other districts, so the climatic condition favored for the growth and multiplication of vectors (*Culicoides* sp). Earlier outbreak of BT was observed in Tamil Nadu during the monsoon season during 1997-98 which resulted in mortality of 300,000 sheep and goats. The reported cases of BTV among sheep and goats occur during the monsoon season (Ilango, 2006).

Previous study demonstrated that BTV could persist in cattle during several months and that these animals would serve as reservoirs for the viruses during the winter (MacLachlan *et al.*, 1994).

A total of 150 samples were collected during the outbreak. Out of these 150 samples collected from Namakkal, Krishnagiri, Dharmapuri districts 42 were found to be positive for *Bluetongue* viral antigen. The isolation of BTV was carried out with the 42 positive samples employing embryonated chicken eggs by yolk sac route. With in 2-3 days of incubation incidence of cherry red color embryo (sign of hemorrhage) followed by death was noticed (Fig. 4).

A total of 21 passages were done individually for all the 42 samples it was shown that the yolk sac route of inoculation of 7-8 days old ECE produced consistently higher virus titers (Alexander, 1947). Egg adapted viral isolates were grown on BHK-21 cell line. All the 42 BTV isolates were adapted to BHK-21(Razi) and they formed monolayers. Though all 42 isolates are adapted to BHK-21, cell line and CPE were noted, the first visible CPE was noted as early as 18 h post infection. Early cytolysis (Fig. 7) was observed during 18-24 h. the results obtained was similar to the observations of Howell and Verwoerd (1971). All the isolates showed characteristic cytopathogenic effect (CPE) within 48 h post infection (Fig. 6, 7) with rounding of cells, extreme vacuolation of cytoplasm with syncytia formation, extreme vacillation of the cytoplasm along with granulation, intranuclear acidophilic inclusion bodies with pyknotic nuclei were seen. Similar changes were observed by earlier Workers also (Bowne and Jochim, 1967; Babu *et al.*, 1993). This study confirmed the presence of BTV in the sample and the virus can easily adapted to ECE and also the BHK-21 cell line, this is helpful for the propagation of virus in high titer using ECE and BHK-21.

Table 2: Month wise seroprevalence of BTV infection in Namakkal District

Month of sample collection	No. of samples	No. of sheep	No. of goat	No. of positive sheep	No. of positive goat	No. of positive samples confirmed by AGPT test	Disease (%)
Nov.	17	10	7	-	2	2	11.70
Dec.	17	11	6	1	3	4	23.00
Jan.	16	12	5	1	-	1	6.20

Table 3: Month wise seroprevalence of BTV infection in Dharmapuri District

Month of collection	No. of samples collected	No. of sheep	No. of goat	No. of positive sheep	No. of positive goat	No. of positive confirmed by AGPT test	Disease (%)
Nov.	17	10	7	2	2	4	23.50
Dec.	17	11	6	6	9	15	88.00
Jan.	16	12	5	-	-	5	31.25

Table 4: Month wise seroprevalence of BTV infection in Krishnagiri District

Month of collection	No. of samples collected	No. of sheep	No. of goat	No. of positive sheep	No. of positive goat	No. of positive confirmed by AGPT test	Disease (%)
Nov.	17	10	7	1	-	1	5.80
Dec.	17	11	6	3	5	8	47.50
Jan.	16	12	5	-	2	2	12.50

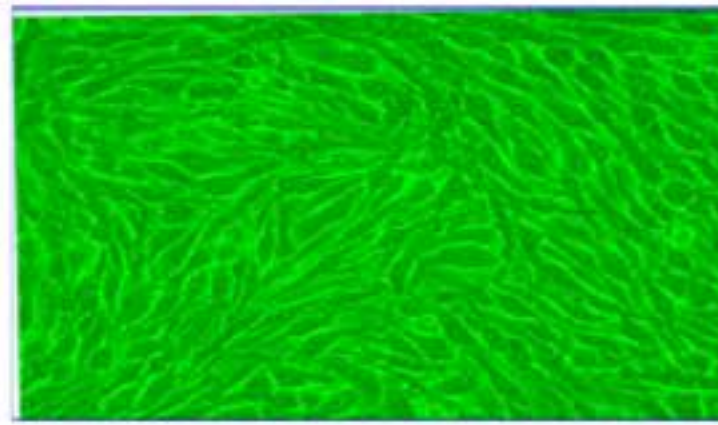


Fig. 6: BHK-21 cell line-control

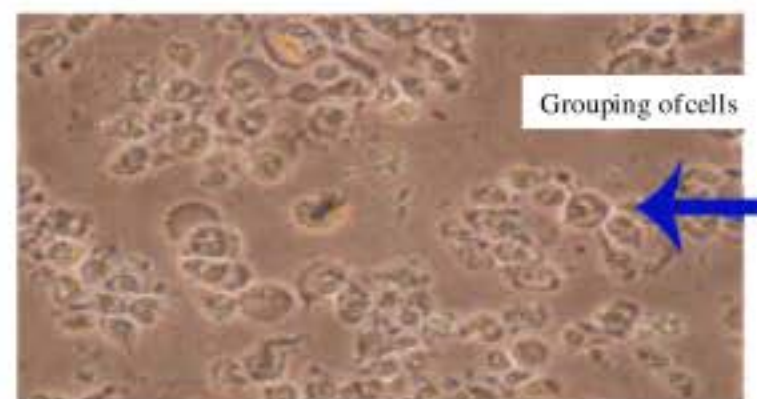


Fig. 7: BHK-21 cell line infected with BTV

Thus, the result of the present study dealing with the adaptation of the virus to cell line was promising in order to develop large scale production of vaccine and virus through fermenters. Thus the major objective of this study was met out by isolating the BTV from the local area which caused a high malady and economic loss to the agricultural industry in Tamil Nadu.

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REFERENCES

- Alexander, R.A., 1947. The propagation of *Bluetongue virus* in the temperature of incubation, Onderstepoort. J. Vet. Sci. Anim. Ind., 22: 7-26.
- Babu, N.G.R., S.M. Byregowda, A.J. Bragitha and T. Gopal, 1993.. Isolations and preliminary characterization of *Bluetongue virus* from sheep. Indian Vet. J., 69: 1071-1074.
- Bowne, J.G. and M.M. Jochim, 1967. Cytopathogenic changes and development of inclusion bodies in cultured cells infected with *Bluetongue virus*. Am. J. Vet. Res., 28: 1091-1105.
- Clastrì, P., M. Goffredo, V. Caporale and R. Meiswinkel, 2003. The distributions of *Culicoides imicola* in Italy: Application and evaluation of current Mediterranean models based on climate. J. Vet. Met. B, 50: 132-138.
- Fernandes, M.V., 1959. Cytopathogenic effects of lamb tissues *in vitro*. Texas Rep. Biol., 17: 94-105.
- Foster, N.M. and A.J. Luedke, 1968. Direct assay of *Bluetongue virus* by intravascular inoculation of embryonated chicken eggs. Am. J. Vet. Res., 29: 749-753.

- Gibbs, E.P. and E.C. Greiner, 1994. The epidemiology of Bluetongue. Comp. Immunol. Microbial. Infect. Dis., 17: 207-220.
- Howell, P.G. and D.W. Verwoerd, 1971. *Bluetongue virus*. Virol Monogr., 9: 35-35.
- Huisman, H., A.A. Van Dijk and J.M. Els, 1987. Uncoating of parental *Bluetongue virus* to core and sub core particles in infected L cells. Virology., 157: 180-188.
- Ilango, K., 2006. *Bluetongue virus* out break in Tamil Nadu, Southern India. Current Sci., 90: 1-5.
- Janakiraman, D., A.T. Venugopalan, V. Ramasamy and R.A. Venkatesan, 1991. Serodiagnostic evidence of prevalence of *Bluetongue virus* serotypes among sheep and Goats in Tamil Nadu. Ind. J. Anim. Sci., 61: 497-498.
- MacLachlan, N.J., R.A. Nunamaker, J.B. Katz, M.M. Sayer, G.Y. Akita, B.I. Osburn and W.J. Tabachnik, 1994. Detection of *Bluetongue virus* in the blood of inoculated calves: comparison of virus isolation, PCR assay and *in vitro* feeding of *Culicoides variipennis*. Arch. Virol., 136: 1-8.
- Mellor, P.S. and E.J. Wittmann, 2002. *Bluetongue virus* in the Mediterranean Basin 1998-2001. Vet. J., 164: 20-37.
- Pearson, J.E. and M.M. Jochim, 1985. An overview of diagnostic for Bluetongue. Bluetongue Related Orbiviruses, : 423-433.
- Prasad, G., A.K. Garg, Minakshi, N.K. Kakkar and R.N. Srivastava, 1994. Isolation of *Bluetongue virus* from sheep in Rajasthan State, India. Rev. Sci. Tech. Off. Int. Epiz., 13: 935-938.
- Uppal, P.K. and B. Vasudevan, 1980. Occurrence of Bluetongue in India. Ind. J. Comp. Microbiol. Immunol. Infec. Dis., 1: 18-20.
- Verwoerd, D.W., H. Louw and R.A. Oellermann, 1970. Characterization of *Bluetongue virus* ribonucleic acid. J. Virol., 5: 1-7.
- Wechsler, S.J. and L.E. Mc Holland, 1988. Susceptibilities of 14 cell lines to *Bluetongue virus* infection. J. Clin. Microbiol., 26: 2324-2327.
- Wilson, W.C., 1991. Molecular comparison of VP3 from bluetongue and epizootic hemorrhagic disease viruses. Virus Res., 21: 225-226.