

# Optimization and Characterization of Bluetongue Virus in Embryonated Chicken Egg

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## Abstract

Bluetongue (BT) is an infectious, non contagious arthropod-borne disease of ruminants caused by Bluetongue virus (BTV), prototype species of the genus *Orbivirus*, within the family *Reoviridae*. Bluetongue outbreak was studied in detail in six western districts of Tamilnadu namely, Namakkal, Salem, Erode, Coimbatore, Karur and Dindigul during 2003-2006. The classical signs of cyanosis of the tongue and reddening of the coronary band are a common feature of the disease in native sheep, Macheri breed were observed. 165 blood samples were collected from the field outbreak (95 numbers) have been used to demonstrate BTV and further inoculated in embryonated chicken eggs (11 - 13 day old). In ECE inoculation technique, 13 positive results were obtained in a total of 165 samples (7.87 % positive). The 13 BTV positive samples further inoculated in ECE in two different routes namely, yolk sac route and intravenous route for studying infectivity titres of Bluetongue virus upto 12 passages and compared. High infectivity titre value, i.e., 6.49 and 7.13 was observed in 12<sup>th</sup> passage in yolk sac and intravenous route of inoculation which was confirmed by higher mean difference (0.642  $p=0.019$ ). Intravenous route of inoculation exhibited better infectivity titre value than yolk sac. The thirteen isolated BTV isolates were acid labile, stable at 37°C and resistant to ether, chloroform and RNase.

**Key Words:** Bluetongue Virus, Embryonated Chicken Egg, EID<sub>50</sub>,

Tamil Nadu rear sheep and goats for meat and skin, which fetch Rs.200 for an individual animal, and the current total number of sheep and goats for meat and skin records 5.2 and 6.4 million respectively. The outbreak of the bluetongue disease in Tamil Nadu and its occurrence in many parts of India over the last few decades have affected millions of sheep, goats and other livestock (Ilango, 2006). Sustained growth of the sheep and goat industry depends on eradication of such emerging disease. In Tamil Nadu, 22 out of 24 districts were reported to be affected by the bluetongue virus.

The clinical form of the disease is usually observed among sheep. Infection among cattle and goats are generally unnoticeable, although clinical bluetongue has been reported. The infection causes inflammation, swelling, and haemorrhage of the mucous membranes of the mouth, nose, and tongue. Inflammation and soreness of the feet are also associated with bluetongue during the terminal stages. In sheep, the tongue and mucous membranes of the mouth become swollen, hemorrhagic, and may look red or dirty blue in color, thus giving the disease its name 'Bluetongue' (APHIS, 2003).

The development of laboratory based systems for the investigation of animal disease such as bluetongue is crucial to understand the infectious agent and the disease process globally. As a result, of adaptation of bluetongue virus to grow in laboratory system, such as cell culture and embryonated chicken eggs it has been possible to develop a range of diagnostic tests and vaccines (Bowne

## Introduction

Bluetongue (BT) is an infectious, non-contagious arthropod-borne disease of ruminants caused by Bluetongue virus (BTV), prototype species of the genus *Orbivirus*, within the family *Reoviridae*. Twenty four serotypes of bluetongue virus have been identified till date (Davies *et al.*, 1992). India has significant population of domestic and wild ruminants, which are known to be susceptible to bluetongue virus infection and total sheep population is 51 million, accounting to 5 % of world's sheep population and 123 million goats accounting for 20 % of the total global livestock (FAO,

2003). Hence bluetongue has become one of the important sheep diseases of the Indian subcontinent. The disease was first reported in India in 1964 (Sapre, 1964). Bluetongue in India is endemic in Tamil Nadu, Andhra Pradesh, Karnataka, Maharashtra, Gujarat, Rajasthan, Haryana, Himachal Pradesh, Jammu and Kashmir. However the recent epidemics in Tamil Nadu were devastating (Wilson *et al.*, 1997). India earns Rs.8500 crores annually through the production of meat, wool and skin from 485 million livestock, of which Tamil Nadu accounts for 24 million livestock (Seventeenth Livestock Census, 2003). Small and medium farmers in

*et al.*, 1970). It is essential that diagnostic laboratories use the most appropriate test methods available to achieve the desired result and therefore they must have a clear understanding of the test uses and limitations. So, the above backdrop, in this current investigation focus has been given to isolation, characterization and adaptation of BTV and cultivation of different native isolates in different routes in embryonated chicken eggs.

## Materials and Methods

### Isolation, Cultivation of Bluetongue Virus in Embryonated Chicken Egg

#### Study Area

Blue Tongue outbreak was studied in detail in six western districts of Tamilnadu namely, Namakkal, Salem, Erode, Coimbatore, Karur and Dindigul during 2003-2006.

#### Preparation of Inoculum

Inoculum was prepared from fresh blood. Fresh blood was collected from the infected sheep, washed in PBS and lysed in distilled water. After lysing the samples were centrifuged at 10,000 rpm for 20 min. The supernatant was used for further inoculation.

#### Yolk Sac Route

The experiment was carried out as per the procedure of Wilson *et al* (1997). Washed blood cells were lysed in distilled water and centrifuged. 0.1 ml of supernatant was inoculated via yolk sac route of 9 - 12 day-old-ECEs. The eggs were incubated at 38°C and candled daily. Embryo deaths within 24 hours of post-inoculation (PI) were regarded as nonspecific, hence discarded. Embryo mortalities between 2nd and 7th days were cooled at 4°C and live embryos after seven days were examined for specific lesions. The embryos were homogenized in a mortar and pestle with sterile sand and Eagles MEM supplemented with antibiotics viz., streptomycin sulphate 200 mg/ml and benzyl penicillin 200 mg/ml, and antifungal agent, nystatin 200 IU / ml. The tissue homogenates were clarified by centrifugation at 3000 rpm for 30 minutes at 4°C.

**Intravenous Route** (Goldsmith and Berzilai, 1965)

One in ten dilution of virus inoculum was prepared by reconstituting one ml of the virus suspension in 10ml of PBS. For intravenous route, embryo of 11-12 days old was used. Candling was performed prior to inoculation and an area 1-1.5 cm around a large straight vein embedded in chorioallantoic membrane was marked. The shell piece in marked area was removed without disturbing the shell membrane. The egg was illuminated in a dark room using aperture at bases of an opaque perpendicular cylinder containing a 60 Watt bulb. The needle was introduced at an acute angle, while the egg was held in the other hand beneath the light source. The cut area was sealed with sterile paper to the shell with molten paraffin. The egg was incubated at 33.5°C and observations were made for 7 days. Death within 48 hours was usually taken as non-specific.

#### Titration of BTV isolates in Embryonated Eggs

Serial ten fold dilution of the viral materials were made in PBS and inoculated into 7 days old ECE. For each virus dilution 5 ECE were used and three eggs were used as control. The ECE were inoculated by Yolk sac route and incubated at 38°C. The ECEs were candled next day and dead ones were discarded, since death before 24 h was considered as non specific, the candling was done at every 24 h. The dead embryos were chilled and examined for the presence of characteristic lesions. The Embryo infective dose 50 (EID<sub>50</sub>) was calculated according to the method of Reed and Muench (1938) on the basis of the presence of pathological lesions in embryos, which died following the inoculations. Titration values were expressed as EID<sub>50</sub> per milliliter (log 10).

#### Physico Chemical Characterization of BTV Isolates

##### pH Stability

To test the pH stability, the viral material was mixed with 1% HCl to obtain pH 2.0 and 6.0, with PBS to obtain pH 7.0 and with 1M NaOH to obtain pH 8.5 and pH 12.0 (Verwoerd, 1969). The mixtures were kept for 60 min at room temperature and titrated for infectivity in ECE and the EID<sub>50</sub> was determined as per the method of Reed and Muench (1938).

##### Thermostability

All the BTV isolates were incubated at 37°C, 46°C and 56°C for a period of 1 h (Verwoerd, 1969) and titrated for infectivity in ECE and EID<sub>50</sub> was determined as per the method of Reed and Muench (1938).

##### Storage Temperature

All the BTV isolates were stored at 4°C and -20°C with suitable control and titrated for the infectivity in ECE and the EID<sub>50</sub> was determined as per the method of Reed and Muench (1938).

##### Ether sensitivity

Two ml BTV preparation and 0.5 ml diethyl ether (20%) (Svehag *et al.*, 1966) were placed in airtight tubes, shaken and incubated at 5°C for 24 h during which time the tubes were intermittently shaken. The mixtures and virus controls were then centrifuged at 400 g for 5 min and the clear top phase was removed and titrated for infectivity in ECE and the EID<sub>50</sub> was determined as per the method of Reed and Muench (1938).

##### Chloroform sensitivity

Four volumes of the viral isolates material were mixed with one volume of chloroform and shaken gently for 10 min. Chloroform was removed by centrifugation at 500 g for 15 min (Svehag *et al.*, 1966). After chloroform treatment, the material was titrated for infectivity in ECE in comparison with untreated material and EID<sub>50</sub> was determined as per the method of Reed and Muench (1938).

##### RNase sensitivity

To test the sensitivity of the viral RNA components to degradation by RNase, the preparations were treated with the enzymes at a concentration of 2 g / ml for 30 min at 37°C (Verwoerd, 1969). After RNase treatment, the viral materials were titrated for their infectivity in ECE and the EID<sub>50</sub> was determined as per the methods of Reed and Muench (1938).

##### Statistical Method

All the experiments were subjected to appropriate statistical analysis (Zar, 1974) as

ANOVA and t-test and the means were compared by using Bonferroni multiple comparison test using SPSS statistical software.

### Results

Clinical disease was slightly different in native sheep; the major difference being that swelling of the lips and face was less conspicuous. The classical signs of cyanosis of the tongue and reddening of the coronary band are a common feature of the disease in native sheep. The blood samples were collected from the field outbreaks for the isolation of BTV. In the four year study period there were 95 field outbreaks from which 165 samples were collected for the isolation purpose. In that 13 isolates of BTV were made and further confirmed by characteristics lesion on infected chicken embryos. The lesions found were haemorrhages, oedema and cherry red discolouration of the embryos (Plate I). The thirteen isolates were adapted in embryonated chicken eggs via two routes namely, yolk sac route and intravenous route for comparison of which route of inoculation gave more titre value.



Infectivity titres of Bluetongue virus isolates in embryonated chicken egg yolk sac and intravenous route were done up to 12 passages and compared. The data are given in Table 1

and Figures 1 and 2. In both the routes of inoculation the first two passages did not give marked level of titre. In yolk sac route inoculation, the infectivity titre value between 2.48 and 3.76 up to 7<sup>th</sup> passage. But the titre value was increased to 4.53 and 6.49 in 9<sup>th</sup> and 12<sup>th</sup> passage (Table 1). As a whole, in this route a minimum titre value of 2.1 and 6.3 in 3<sup>rd</sup> and 12<sup>th</sup> passage to a maximum of 2.8 and 6.8 in 3<sup>rd</sup> and 12<sup>th</sup> passage (Figure 1) was observed in all the thirteen isolates. Lower titre values were observed (2.50 - 3.93) (Figure 1) in intravenous route inoculation up to 7<sup>th</sup> passage but higher titre value was

registered in 9<sup>th</sup> (4.90) and 12<sup>th</sup> passage (7.13) (Table 1 and Figure 1). In both routes of inoculation, infectivity titres increased with the increasing passages (Figure 2). Until the 7<sup>th</sup> passage the mean difference observed was not significantly higher. In the 9<sup>th</sup> and 12<sup>th</sup> passage mean infectivity titre difference between yolk sac route and intravenous route were higher, i.e., 0.367 and 0.642 respectively. The p value was significant at 0.019 level. Between the two routes, intravenous route of administration exhibits maximum infectivity titre in all the passages than yolk sac route (Figure 2).

**Table 1. Comparison of infectivity titre value in embryonated egg yolk sac and intravenous route**

| S.No. | Passage          | Infectivity titre value of BTV# |                                 | Mean difference     | P-Value |
|-------|------------------|---------------------------------|---------------------------------|---------------------|---------|
|       |                  | Inoculated in yolk sac route    | Inoculated in intravenous route |                     |         |
| 1     | 3 <sup>rd</sup>  | 2.48±0.24<br>(2.1-2.8)          | 2.50±0.26<br>(2.1-2.8)          | 0.017 <sup>ns</sup> | P>0.05  |
| 2     | 5 <sup>th</sup>  | 3.33±0.15<br>(3.2-3.6)          | 3.41±0.27<br>(3.1-3.8)          | 0.083 <sup>ns</sup> | P>0.05  |
| 3     | 7 <sup>th</sup>  | 3.76±0.17<br>(3.5-4.0)          | 3.93±0.29<br>(3.6-4.4)          | 0.167 <sup>ns</sup> | P>0.05  |
| 4     | 9 <sup>th</sup>  | 4.53±0.24<br>(3.5-4.0)          | 4.90±0.43<br>(5.6-4.90)         | 0.367*              | P=0.019 |
| 5     | 12 <sup>th</sup> | 6.49±0.18<br>(6.3-6.8)          | 7.13±0.29<br>(6.8-7.5)          | 0.642*              | P=0.019 |

# Titres are shown as median embryo lethal dose per ml (Log10)

<sup>ns</sup> - Not significant difference

\* - Significant difference

Values in parenthesis are minimum and maximum in infectivity titre value

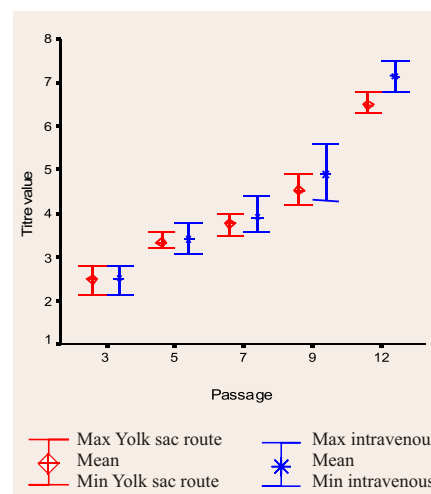


Figure 1- Comparison of Bluetongue virus titre values inoculated into yolk sac and intravenous route

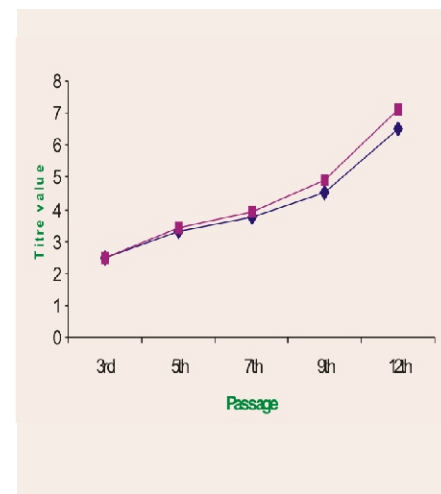


Figure 2 -Mean titre score value of Bluetongue virus inoculated in yolk sac and intravenous route

## Characterization of BTV Isolates

### pH Stability

Thirteen isolates of BTV were exposed to different pH environment to assess their

stability and infectivity. The results are summarized in Table 2 and the titre values expressed as median embryo lethal dose per ml (Log10). The infectivity titre value was nil in all the thirteen isolates from pH 2 to pH 6 and pH 11. All the isolates were found to be unstable at acidic (pH 2.0 and 6.0) and

alkaline pH (pH 11). Titre values were observed only in pH 7 (5.2 - 5.8), pH 8 (4.0 - 4.6) and pH 10 (4.0 - 4.4) (Table 2). Comparatively better titre values were observed only in pH 7 and confirmed through two-tailed t-test ( $p < 0.0001$ ). These results indicated that the BTV isolates are sensitive to acidic pH.

| Isolate | INFECTIVITY TITRE VALUE * |     |     |     |        |         |       |        |          | Inference   |
|---------|---------------------------|-----|-----|-----|--------|---------|-------|--------|----------|-------------|
|         | pH2                       | pH3 | pH4 | pH5 | pH6    | pH7.0   | pH8.0 | pH10.0 | pH11.0   |             |
| BTV1    | -                         | -   | -   | -   | -      | 5.2     | 4.0   | 4.4    | -        | Acid labile |
| BTV 2   | -                         | -   | -   | -   | -      | 5.5     | 4.6   | -      | -        | Acid labile |
| BTV 3   | -                         | -   | -   | -   | -      | 5.8     | 4.0   | 4.0    | -        | Acid labile |
| BTV 4   | -                         | -   | -   | -   | -      | 5.4     | 4.5   | 4.3    | -        | Acid labile |
| BTV 5   | -                         | -   | -   | -   | -      | 5.8     | 4.3   | -      | -        | Acid labile |
| BTV 6   | -                         | -   | -   | -   | -      | 5.2     | 4.0   | 4.4    | -        | Acid labile |
| BTV 7   | -                         | -   | -   | -   | -      | 5.5     | 4.6   | -      | -        | Acid labile |
| BTV 8   | -                         | -   | -   | -   | -      | 5.8     | 4.0   | 4.0    | -        | Acid labile |
| BTV 9   | -                         | -   | -   | -   | -      | 5.4     | 4.5   | 4.3    | -        | Acid labile |
| BTV 10  | -                         | -   | -   | -   | -      | 5.8     | 4.3   | -      | -        | Acid labile |
| BTV 11  | -                         | -   | -   | -   | -      | 5.2     | 4.0   | 4.4    | -        | Acid labile |
| BTV 12  | -                         | -   | -   | -   | -      | 5.5     | 4.6   | -      | -        | Acid labile |
| BTV 13  | -                         | -   | -   | -   | -      | 5.5     | 4.6   | -      | -        | Acid labile |
| SEM (±) |                           |     |     |     |        | 0.064   | 0.075 | 0.613  |          |             |
| T-value | pH 7 & pH 8               |     |     |     | 12.084 | DF - 24 |       |        | P<0.0001 |             |
|         | pH 7 & pH 10              |     |     |     | 5.211  |         |       |        |          |             |

# - Two tailed t-test.

\* Titres are shown as median embryo lethal dose per ml (Log10)

SEM Standard Error Mean; DF- Degrees of freedom

| Isolate | INFECTIVITY TITRE VALUE *       |       |       |        | Stability |          |
|---------|---------------------------------|-------|-------|--------|-----------|----------|
|         | Temperature °c                  |       |       |        |           |          |
|         | 37°C                            | 40°C  | 46°C  | 50°C   |           |          |
| BTV1    | 5.5                             | 4.1   | 3.5   | -      | Stable    |          |
| BTV 2   | 5.8                             | 3.9   | 3.1   | -      | Stable    |          |
| BTV 3   | 5.3                             | 4.2   | 3.4   | -      | Stable    |          |
| BTV 4   | 5.1                             | 4.0   | 3.0   | -      | Stable    |          |
| BTV 5   | 5.8                             | 4.4   | 3.3   | -      | Stable    |          |
| BTV 6   | 5.5                             | 4.1   | 3.5   | -      | Stable    |          |
| BTV 7   | 5.8                             | 3.9   | 3.1   | -      | Stable    |          |
| BTV 8   | 5.3                             | 4.2   | 3.4   | -      | Stable    |          |
| BTV 9   | 5.1                             | 4.0   | 3.0   | -      | Stable    |          |
| BTV 10  | 5.8                             | 4.4   | 3.3   | -      | Stable    |          |
| BTV 11  | 5.5                             | 4.1   | 3.5   | -      | Stable    |          |
| BTV 12  | 5.8                             | 3.9   | 3.1   | -      | Stable    |          |
| BTV 13  | 5.6                             | 3.2   | 2.8   | -      | Stable    |          |
| SEM±    | 0.073                           | 0.083 | 0.063 |        |           |          |
| T-value | Temperature between 37 and 40°C |       |       | 13.467 | DF - 24   | P<0.0001 |
|         | Temperature between 37 and 46°C |       |       | 23.050 |           |          |

# - Two tailed t-test.

\* Titres are shown as median embryo lethal dose per milli litre (Log10)

SEM Standard Error Mean; DF- Degrees of freedom

### Thermostability

All the BTV isolates were subjected to different temperature regimen to determine the thermostability of the BTV. Four temperature regimen was imposed and the findings are shown in Table 3. When the thirteen isolates were exposed at 37°C, 40°C, 46°C and 50°C the obtained infectivity titre value was 5.1 - 5.8, 3.2 - 4.4, 2.8- 3.5 and nil respectively. Higher titre value was registered at 37°C. The titre value was inversely proportional to the temperature ( $p < 0.0001$ ) (Table 3). With regard to storage conditions of all the BTV isolates, kept at 5°C was found to be ideal rather than storing at -20°C which is considered to be the optimal temperature for storage of the viral agents. The infectivity titre (log10) value at 5°C and -20°C ranged between 5.3 and 5.8 and 4.0 and 4.6.

### Ether, Chloroform and RNase Sensitivity to BTV isolates

The results of ether, chloroform and RNase sensitivity of BTV isolates are presented in Table 4. It was evident from the results obtained that before treatment all the isolates were found to have titre value between 5.2 and 5.3, 5.8 and 5.2 and 5.6 and 5.1 in case of ether, chloroform and RNase but after treatment the titer value varied between 5.0 and 5.2, 5.0 and 5.6 and 5.0 and 5.3 respectively. It showed all the isolates were found to be resistant to ether chloroform and RNase treatment. However a slight reduction in infectivity titre was observed after treatment with ether for some of the isolates (Table 4).

### Discussion

Bluetongue has been recognized as an economically significant vector borne, non-contagious, viral disease of domestic and wild ruminants in many parts of the world (Bowne, 1971; Erasmus, 1975; Jones *et al.*, 1981).

**Table 4. Sensitivity of bluetongue against ether, chloroform and RNase**

| INFECTIVITY TITRE OF BTV * |                  |                  |                  |                  |                  |                  |           |
|----------------------------|------------------|------------------|------------------|------------------|------------------|------------------|-----------|
| Isolate                    | Ether            |                  | Chloroform       |                  | RNase            |                  | Stability |
|                            | Before treatment | Before treatment | Before treatment | Before treatment | Before treatment | Before treatment |           |
| BTV1                       | 5.2              | 5.0              | 5.5              | 5.0              | 5.6              | 5.3              | Resistant |
| BTV 2                      | 5.3              | 5.2              | 5.8              | 5.6              | 5.3              | 5.2              | Resistant |
| BTV 3                      | 5.2              | 5.2              | 5.2              | 5.0              | 5.2              | 5.0              | Resistant |
| BTV 4                      | 5.3              | 5.1              | 5.2              | 5.2              | 5.5              | 5.3              | Resistant |
| BTV 5                      | 5.2              | 5.2              | 5.6              | 5.4              | 5.1              | 5.0              | Resistant |
| BTV 6                      | 5.2              | 5.0              | 5.5              | 5.0              | 5.6              | 5.3              | Resistant |
| BTV 7                      | 5.3              | 5.2              | 5.8              | 5.6              | 5.3              | 5.2              | Resistant |
| BTV 8                      | 5.2              | 5.2              | 5.2              | 5.0              | 5.2              | 5.0              | Resistant |
| BTV 9                      | 5.3              | 5.1              | 5.2              | 5.2              | 5.5              | 5.3              | Resistant |
| BTV 10                     | 5.2              | 5.2              | 5.6              | 5.4              | 5.1              | 5.0              | Resistant |
| BTV 11                     | 5.2              | 5.0              | 5.2              | 5.2              | 5.6              | 5.3              | Resistant |
| BTV 12                     | 5.3              | 5.2              | 5.6              | 5.4              | 5.3              | 5.2              | Resistant |
| BTV 13                     | 5.2              | 5.0              | 5.2              | 5.2              | 5.1              | 5.0              | Resistant |

Titres are shown as median embryo lethal dose per milliliter (Log 10)

Bluetongue continues to be a major disease-affecting sheep in all countries in the tropics and subtropics including India. The economic loss due to bluetongue outbreaks in sheep is very high. Control of the disease is very difficult because of the plurality of the serotypes that occur and no single vaccine may be effective (Nachimuthu *et al.*, 1992). In the absence of vaccines available for the control of Bluetongue virus, the other measures are needed to be adopted such as control of the vector population, treatment of animals for secondary bacterial infection and nursing of the animals. These control measures would be worthwhile if early diagnosis of the disease is accomplished (Prasad *et al.*, 1994).

In the present study, a total of 13 isolates of BTV were made from 165 samples collected 95 different field bluetongue outbreaks in sheep with characteristic clinical signs of Bluetongue. The identities of these 13 isolates were confirmed by characteristics lesion on infected chicken embryos. The lesions found were haemorrhages, oedema and cherry red discolouration of the embryos. The above symptoms were observed as noticed by earlier research workers (Pearson *et al.*, 1991; Nachimuthu *et al.*, 1992; Clavijo *et al.*, 2000). In the present study all the BTV isolates when inoculated into 11 day old ECE by Yolk sac

route and intravenous route produced death of the embryos and a characteristic cherry red coloration as a result of extensive haemorrhage all over the body of the embryos due to degenerative changes of the blood vessels.

For primary isolation of BTV from natural disease outbreaks, the yolk sac, and intravenous inoculation of ECE were found to be the most sensitive and practical methods. However, intravenous route of ECE inoculation detected even small quantities of viral antigen and gave better viral titres than yolk sac route of inoculation. The successful inoculation of BTV in ECE by the intravenous route requires a degree of technical expertise. Mortality of the chick embryo due to inappropriate handling of the eggs or poor inoculation techniques should be reduced as much as possible. It has been found that cooling the eggs to room temperature (20-22°C) by removing them from the incubator 1-2 h before inoculation considerably reduces non specific embryo mortality which results from bleeding at the site of inoculation as reported by Clavijo *et al.* (2000).

Goldsmith and Barzilai (1965, 1967) obtained the maximum titre of 10<sup>5</sup> C.E. LD<sub>50</sub> in field samples of all three types of BTV when titrated intravenously and 30% of the samples

was positive by the intravenous route, whereas, nil in yolk sac inoculation. Further, he proved intravenous route inoculation gave 100 % mortality within 10 days whereas, 7 to 8 weeks required in yolk sac route inoculation (Goldsmith and Barzilai, 1968). It is shown in the present study that by using the intravenous route the time required for the isolation and typing of the BTV is shortened considerably when compared with other methods.

Physical and chemical properties in combination with antigenic relationship serve as more precise criteria for virus identity and classification than pathogenicity and tissue affinity (Svehag *et al.*, 1966). In the present study all the BTV isolates were subjected to various physico-chemical properties such as pH stability, thermostability, ether, chloroform and RNase sensitivity.

The results of pH stability of BTV isolates revealed that all the isolates were found to be unstable at acidic from pH 2.0 to pH 6.0 and alkaline pH 10. Better infectivity titre was obtained at pH 7.0 as compared to pH 8 and pH 10.0. The infectivity titre (Log 10) ranged between 5.2 and 5.8 and 4.0 and 4.4 at pH 7 and pH 8 indicated that the BTV isolates were sensitive to acidic pH. However, since the higher infectivity titres were observed at pH 7.0, the optimum pH has been taken into account as pH 7.0. The same findings were reported by Howell (1963), Owen (1964), Svehag *et al.* (1966), Verwoerd (1969), Inaba *et al.* (1970) and Howell and Verwoerd (1971). All the BTV isolates were found to get inactivated at 56°C. However at 46°C the isolates retained partial infectivity and the EID<sub>50</sub> (Log 10) ranged between 5.1 and 5.8. At 37°C the titres were found to be between 5.0 and 5.8. Hence temperature of 37°C was ideal to maximize the infectivity titres of BTV. With regard to storage conditions, keeping at 5°C was found to be ideal rather than storing at -20°C which was considered as the normal storage temperature for most of the other viral agents. These observations are in agreement with the findings of earlier workers (Alexander, 1947; Neitz, 1948; Kipps, 1958; Svehag, 1962; Howell *et al.*, 1967).

Ether, RNase and chloroform sensitivity of BTV isolates revealed that all the isolates were found to be resistant to ether and chloroform treatment and these observations

are in accordance with the earlier findings of Howell (1963), Andrews (1964), Studdert (1965), and Svehag *et al* (1966), Verwoerd (1969) and Bowne (1971). However a slight reduction in infectivity titers was observed after treatment with ether for some of the isolates BTV1, BTV6, BTV11 and BTV13. After RNase treatment a slight reduction in infectivity titre has been observed with some of the isolates BTV3, BTV5, BTV8, BTV10 and BTV13.

The occurrence of BTV disease in India became endemic in native breeds. In Tamil Nadu there are 58 lakhs of sheep in which 12 lakhs are Macheri breed. In 2005 around 2 lakh sheep were suffering from BT disease. BTV is often difficult to isolate in the laboratory. The success of virus isolation is enhanced if blood is collected from sheep showing clinical signs and at the early stage of the disease. Viraemia is primarily associated with red blood cells and leucocytes and the virus coexist in infected sheep with high concentrations of neutralizing antibody. The routine method of BTV isolation is through embryonated chicken eggs, due to the lack of infrastructural facilities, embryonated chicken eggs method is widely used in India. In the present investigation was helpful for which route of inoculation was produced more amount of viral titre. Further it was used for attenuation of virus for vaccination purpose.

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