Chapter - 2  

REVIEW OF LITERATURE

2.1: Bluetongue: A Historical Perspective

Bluetongue, as a disease of cattle and sheep, was first described in the late 18th century as ‘tong-sikte’ by a French biologist Francois de vaillant (Gutsche, 1979). About 40 years later, the clinical features of the disease were noticed in imported merino sheep by the farmers of South Africa and described as “Malarial catarrhal fever of Sheep” (Spreull, 1902). Spreull (1905) came across the disease peculiar to South Africa. On the basis of symptoms and lesions appeared in the mouth and tongue, he suggested common name “Bluetongue” instead of word ‘Malarial’. Spreull demonstrated that the virus was transmitted to goat and cattle but infection remained in apparent and he suspected intra corpuscular parasite or plasmodium as causative agent of the disease.

2.2: Geographical distribution

2.2.1: World

First confirmed outbreak of bluetongue outside the South Africa was in Cyprus (Gambles, 1949). Subsequently the disease was reported in Palestine (1943), Turkey (1944) and Israel (1946 and 1947) (Komarov and Goldsmith, 1951), North America (McKercher et al., 1953), Portugal (1956) and Spain (1957) (Manso –Ribiero et al., 1957), Iberian Peninsula (Lopez and Botija, 1958), Pakistan (Sapre, 1964), Australia (St George et al., 1978). The disease outbreaks

2.2.2: India

The prevalence of the disease was initially noticed in 1963 among indigenous sheep and goats in Maharashtra and the disease was confirmed based on clinical picture, post-mortem lesions and presence of specific antibodies in the sera of recovered animals (Sapre, 1964).

Later the Bluetongue disease was reported from different states including Uttar Pradesh (Bhambani and Singh, 1968), Himachal Pradesh (Uppal and Vasudevan, 1980), Haryana (Vasudevan, 1982), Karnataka (Srinivas et al., 1982), Maharashtra (Kulkarni and Kulkarni, 1984), Tamilnadu (Janakiraman et al., 1991), Madhya Pradesh (Mehrotra et al., 1996), Gujarat (Chauhan et al., 2004), West Bengal (Joardar et al., 2009) and Andhra Pradesh (Rama Rao 1982, Sreenivasulu et al., 1999, Bommineni et al., 2008, Yugendar Reddy et al, 2010).

Sudheer et al., 2008 studied epidemiology of bluetongue in Andhra Pradesh from 1995-2002. Bluetongue outbreaks were most important in 1998 with case fatality rate of 18.65 % and over all bluetongue outbreaks with case fatality rate of 18.85%.

The investigations made by different authors demonstrating BTV antibodies established the fact that BTV infection is present in cattle, buffaloes and goats in India (Sodhi et al., 1981, Harbola et al., 1982, Bandyopadhyay and Mullick, 1983, Sharma et al., 1985, Prasad et al., 1987, Oberoi et al., 1988, Jain et al., 1992, Mehrotra and Shukla, 1990, Hinsu et al., 2000)
In Andhra Pradesh studies were carried out during 1991 revealed the prevalence of BTV antibodies in 45.71% of sheep, 43.56% of goats, 33.11% of cattle and 20% of buffaloes (Sreenivasulu and Subba Rao, 1999).

Jaykar Johnson et al., 2006 studied seroprevalence of bluetongue in various species of domestic animals in Andhra Pradesh from 1995-2005. Results indicated that seropositivity was 9.09% in cattle (n=220), 8.25% in buffaloes (n=291), 35.39% in sheep (n=291), 33.47% (n=239) in goats.

Sairaju (2010) studied seroprevalence of bluetongue in various species of domestic animals during the year 2009. A total of 1113 sera samples from sheep, goat, cattle and buffaloes were collected from 20 districts of Andhra Pradesh. Species wise sero-positivity percentage for sheep (n=427), goat (n=16), cattle (n=3) and buffaloes (n=35) were 72.80, 81.25, 100.00 and 100.00 respectively by cELISA. cELISA was found to be highly sensitive compared to Agarose Gel Immuno Diffusion Assay.

2.3: Bluetongue: The disease

Bluetongue is an infectious, non-contagious, arthropod-borne viral disease of domestic and wild ruminants. It is primarily a disease of sheep but also affects cattle, buffalo, goat, camel, dog and wild ruminants such as Sambar, blue bull, Lamas, white tailed deer, antelope, etc., (Howerth et al., 1988; Akita et al., 1994 and McLachlan, 1994). It occurs through out tropical, semitropical and temperate regions of the world, mostly in a band stretching from latitude 40°N to 35°S. It is prevalent in Africa, Middle East, Asia, America and Australia.

The morbidity and mortality rate depends on severity of the disease in different outbreaks. The severity of the disease depends on number of factors like
susceptibility of the animal, pathogenicity of the virus, competency of the arthropod vector and environmental condition and geographical location. Being highly seasonal, BT mostly occurs during summer and autumn months when there are large numbers of blood sucking midges. Favorable environmental condition includes hot climate and high humidity that are desirable for the development of *Culicoides* larvae.

### 2.4: Clinical signs

The incubation period of BT is 6-9 days. The first sign is the rise in body temperature (104-108°F), which continues for 5-7 days. During the early febrile reaction, there is rise in respiration rate, hyperemia and swelling of buccal and nasal mucosa (Sapre, 1964 and Harbola *et al.*, 1982). There is nasal discharge which may be serous, mucopurulent or bloody, frothy salivation, swollen tongue and lips and licking of the lips and nostrils followed by the cracking of epidermis at the commisures of lips along with edema of face, submaxillary space and occasionally the ears and eyelids. There is also petechial to ecchymotic haemorrhage on the oral mucosa in the vicinity of ventral surface of tongue. Rarely the tongue become swollen, markedly congested and may protrude from the mouth and because of its cyanotic appearance, the name “Bluetongue” has been assigned to this disease (Spreull, 1905), but this sign is transitory and may often be missed. Later there is excoriation of the oral epithelium, which leads to infection and necrosis. There is also arching of the back. During this time the acutely infected sheep develop muscle stiffness, lethargy and anorexia. There is hyperemia and petechial hemorrhage around the coronary band and periople (Skin-Horn junction) and degeneration of skeletal muscle, which leads to extreme
weakness and prostration. Terminally torticollis occurs. Due to smooth muscle lesion on pharyngeal region and esophagus there is vomition leading to aspiration pneumonia ending frequently in death.

2.5: Post-Mortem Lesions

Hemorrhages are seen on the pulmonary artery and aorta near the cardiac end. Hemorrhages on upper gastrointestinal tract (GIT) mostly in oral cavity, esophagus, ruminal pillars and omasum are also found. Necrosis of skeletal and cardiac muscle particularly in myocardium of the papillary muscle of left ventricle has also been noticed.

BTV replicates in endothelial cells and produce coagulation abnormalities such as consumptive coagulopathy, which ultimately predisposes to hemorrhagic diathesis, which is characteristic of fulminant BT (Howerth et al., 1988). So this disease is the result of virus mediated injury to vascular epithelium.

BTV causes necrosis in developing nervous system in 50-80 day old fetal lamb, when the fetus is not yet immunologically competent. After 90 days of gestation period the fetal lamb respond to BTV immunologically by developing neutralizing antibodies. Infection in last trimester of gestation results in development of antibodies but birth of clinically normal offspring is seen.

2.6: Transmission

The epizootiology of virus is very complex in nature involving interaction of types of virus, vector, climate and the host. Bluetongue virus requires appropriate species of vector for its perpetuation in various geographic regions. The vectors are biting midges of genus *Culicoides*. Du Toit (1944) first reported that *C. imicoli* (Pallidepannis) (Fig 2.1) harbored the BTV and was capable to
transmit the virus from infected to susceptible sheep. This finding was confirmed by various workers (Walker and Davis, 1971, Braverman et al., 1981 and Mellor, 1990). In India, Jain et al., (1986) isolated BTV from Culicoides in Rajasthan, India, but were unable to identify the species of Culicoides. However, there is 1000 Culicoides spp. in the world, out of them only 17 are connected to BT (Mellor, 1990, Gibbs and Greiner, 1994). To date, only, C. variipennis, V.imicola, C.fulvus, C. actoni, C. wadai, C.nubeculosus, C. bresvitaris, C. milnei and C. tororensis are known to transmit the disease (Bhatnagar et al., 1995). Jochim and Jones (1966) observed that infected C. variipennis remained infected and adult over wintered, they could constitute reservoir. The virus was transmitted through bite of infected vector and was not transmitted by contact or through infected products (Srivastava et al., 1995).

2.7: Bluetongue Virus

Blue Tongue virus (BTV) is the causative agent of Bluetongue disease in domestic livestock (Sheep, goats, cattle, camels etc.,) and wild ruminants (bison, white-tailed deer, elk, pronghorn, antelope etc.,) (Roy, 1989). It is a member of the genus Orbivirus in the family Reoviridae (Joklik, 1983). The name Orbivirus was derived from the Latin word ‘orbis’ which means ‘ring’ or ‘circle’. The name
was based on electron microscope of the doughnut like appearance of viral particle capsomers (Borden et al., 1971). BTV is a non enveloped virus with a concentric protein shells (Fig 2.2 a and b) (Eaton et al., 1990). It has an icosahedral shape with a diameter of about 68-70 nm (Hyatt and Eaton, 1988). The virus is resistant to lipid solvents and sodium deoxycholate (Borden et al., 1971) and stable at pH 6.0-8.0 but inactivated below pH 6.0 and above pH 8.0 (Owen, 1966). Freezing may cause loss of infectivity of the virus whereas virus is stable at 4°C for more than a year (Neitz, 1948).

There are three morphologically distinct virus particles: Virions, Cores and sub core. The bluetongue virions can be converted into core particles by removing the layer of outer capsid proteins (VP2 and VP5). The cores are made up of sub core particles and the inner capsid proteins (VP3 and VP7). The sub core particles work like a skeleton and consist of BTV proteins VP1, 4 and 6 along with the viral genome segments (Huismans and Cloete, 1987). Based on virus neutralization test (VNT) 24 BTV serotypes were recognized (Mann et al., 2007).
2.8: BTV Serogroups and Serotypes

The *Orbiviruses* have been classified into 21 different serogroups on the basis of cross-reaction in serological tests out of which bluetongue virus is one of the serogroups (Mertens et al., 2007a). The reactivity of different BTV isolates in Complement fixation (CF), Agarose gel immuno diffusion (AGID), and Enzyme linked immuno sorbant assay (ELISA) have been demonstrated using reference antibody preparations of VP7 and VP3 protein which are the group specific antigens in the viral core (Inumaru et al., 1987). Both proteins are recognized by all anti-BTV antisera. The BTV serogroup includes all the known BTV serotypes.

There are 24 confirmed serotypes of BTV, and two more have been recently proposed (Hofmann et al., 2008, Maan et al., 2009 and 2011). In India, BTV serotypes identified based on virus isolation from the sheep were BTV- 1, 2, 3, 4, 8, 9, 10, 16, 17, 18, 22 and 23 (Jain et al., 1992) and based on serological evidence, Prasad et al., (1992) found out the prevalence of serotypes 5, 6, 7, 11, 12, 13, 14, 15, 19, 20, 21 and 24. So, total of 24 serotypes (12 based on isolation and 12 based on serology) have been reported from India (Sreenivasulu et al., 2004). In 2005 outbreaks, BTV-21 was isolated from Andhra Pradesh (Susmitha et al., 2010) and there is evidence for existence of antibodies for serotype 24 in camel in Gujarat (Chauhan et al., 2004). So, currently existing number of serotypes in India is 24. Details of bluetongue virus serotypes prevalent in India are given in table 2.1 and 2.2.
Table 2.1: Prevalence of bluetongue virus serotypes in India

<table>
<thead>
<tr>
<th>Basis</th>
<th>Serotypes</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus isolation</td>
<td>1, 2, 3, 4, 8, 9, 10, 16, 17, 18, 22, 23</td>
<td>12</td>
</tr>
<tr>
<td>Neutralizing antibodies</td>
<td>5, 6, 7, 11, 12, 13, 14, 15, 19, 20, 21, 24</td>
<td>12</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>24</strong></td>
<td></td>
</tr>
</tbody>
</table>


Table 2.2: Various Bluetongue virus serotypes reported in India

<table>
<thead>
<tr>
<th>State</th>
<th>Species</th>
<th>Virus Isolation</th>
<th>Neutralizing antibodies</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andhra Pradesh</td>
<td>Sheep</td>
<td>2, 3, 9, 10, 16, 21</td>
<td>4, 6, 12, 13, 14, 17, 18, 19</td>
<td>Sreenivasulu <em>et al.</em>, 2004 Bommineni <em>et al.</em>, 2008 Susmitha <em>et al.</em>, 2010</td>
</tr>
<tr>
<td>Tamil Nadu</td>
<td>Sheep</td>
<td>1, 3, 16, 23</td>
<td>1, 4, 5, 6, 7, 11, 12, 14, 15, 16, 17, 19, 20</td>
<td>Janakiraman <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Karnataka</td>
<td>Sheep</td>
<td>18, 23</td>
<td>1, 2, 12, 16, 17, 20</td>
<td>Mehrotra and Shukla, 1990</td>
</tr>
<tr>
<td>Maharashtra</td>
<td>Sheep</td>
<td>1, 2, 3, 4, 8, 9, 16, 18, 23</td>
<td>-</td>
<td>Kulkarni and Kulkarni, 1984,</td>
</tr>
<tr>
<td>Madhya Pradesh</td>
<td>Sheep</td>
<td>18</td>
<td>-</td>
<td>Mehrotra <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Gujarat</td>
<td>Cattle, Camel, Sheep</td>
<td>-</td>
<td>1, 2, 3, 4, 10, 12, 14, 15, 16, 17, 18, 20, 21 and 24</td>
<td>Chauhan <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>Uttar Pradesh</td>
<td>Sheep</td>
<td>9, 18, 23</td>
<td>-</td>
<td>Mehrotra <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Haryana</td>
<td>Sheep</td>
<td>1, 4</td>
<td>14</td>
<td>Mahajan <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Himachal Pradesh</td>
<td>Sheep</td>
<td>3, 9, 16, 17</td>
<td>4</td>
<td>Sreenivasulu <em>et al.</em>, 2004</td>
</tr>
</tbody>
</table>
2.9: Bluetongue viral proteins

There are seven structural proteins and four non-structural (NS) proteins encoded by BTV genome. The structural proteins are numbered as VP1 to VP7 in order of decreasing size based on electrophoretic migration in SDS-PAGE. The non-structural proteins are designated as NS1, NS2, NS3 and NS3A. Proteins constitute 88% and 80.5% of the dry weight of the virion and core respectively. The fully infectious virion contains 4 major structural proteins (VP2, VP3, VP5 and VP7) and minor structural proteins (VP1, VP4 and VP6) (Verwoerd et al., 1972). The major capsid proteins constitute 93% of the protein content of the virion. Most of the proteins are synthesized throughout the infectious cycle with a relative frequency, which corresponds to the synthesis of their mRNAs (Huismans et al., 1987c). The first virion specific polypeptides are detected as early as 2-4 hr post infection (hpi). There is a rapid increase in the rate of synthesis of viral proteins until 11-13 hpi, after which the rate remains constant until at least 24 hpi. The different BTV proteins encoded by different gene segments and their functions are given in the table 2.3.

![Molecular structure of Bluetongue virus](Source: bluetonguevirus.net)
2.9.1: Core polypeptides

The BTV core is composed of 2 major polypeptides (VP3 and VP7) and 3 minor polypeptides (VP1, VP4 and VP6). VP7 is the major protein, which comprises almost 1/3 of the total protein of BTV. The surface of core particle consists entirely of 780 copies of VP7 (T=13 symmetry) (arranged in a near perfect example of quasi-equivalence) as a network of 260 trimers containing ‘rings’. VP7 is the main component of the capsomer on the surface of the BTV core particle (Huismans et al., 1987c). Beneath the VP7 layer, the ‘sub core’ is composed of 120 copies of VP3 (T=2 symmetry), displaying “quasi-quasi-equivalence”. VP3 is also the major structural protein (SP) in the BTV sub core and it forms proteins scaffold on which the capsomers (VP7) are arranged (Huismans et al., 1987b). VP3 encloses the 10 linear dsRNA segments and 3 minor proteins on VP1, VP4 and VP6.

VP7 has been identified as the soluble group-specific antigen (Huismans and Erasmus, 1981). The binding of BTV particle to the large number of VP7 monoclonal antibodies indicates that VP7 is accessible on the outer surface of the virus (Hyatt and Eaton, 1988). VP3 is also highly conserved (99% at the amino acid level) among different serotypes and can be used in the diagnosis by indirect ELISA (Inamaru et al., 1987).

The minor polypeptides VP1, VP4 and VP6 are present in very small amounts in the inner core of the virion. The VP1 (150kDa) serves as the viral RNA polymerase involved in elongation of RNA (Roy et al., 1988). VP4 (76 kDa) has guanylyl transferase as well as transmethylase 1 (forming 7mG of the Cap) and transmethylase 2 (forming 2-O mG of the Cap) activity. VP6 (36kDa)
binds to ssRNA and dsRNA, has helicase and ATPase activity. Its binding
capacity to RNA is independent of the tertiary structure.

2.9.2: Non-structural proteins

Three different non-structural proteins have been identified in BTV
infected cells so far. Major NS proteins are NS1 and NS2, first identified by
Huismans (1979). Minor NS proteins are NS3 and NS3A.

NS1 (64kDa) comprises 25% of the virus specified proteins in the infected
cells. Polymerization of NS1 produces high molecular weight tubular structure
with a sedimentation coefficient of about 300S-500S (Huismans, 1979; Urakawa
and Roy, 1988). The presence of these tubules is characteristic feature of BTV
replication in the infected cells. Negatively stained tubules are approximately 68
nm in diameter and 4µm in length. NS1 is highly conserved among different BTV
serotypes. So it can aid in diagnosis of BTV in ELISA and other nucleic acid
based assays. Some NS1 remain associated with the highly purified virus or core
particle and it participate in virus morphogenesis (Eaton et al., 1988).

The other major non-structural protein NS2 (41kDa) is present in a
phosphorylated form. It is an important component of the matrix of viral
inclusion bodies (VIB), which are the site of viral replication and assembly (Eaton
et al., 1988). It has got high affinity for ssRNA (mRNA) suggesting an active role
in replication and encapsidation. A small amount of NS2 remains associated with
highly purified virus on its surface (Mertens et al., 1987a).

The minor non-structural proteins NS3 (25kDa) and NS3A (24kDa) are
encoded by the smallest RNA segment of the BTV (S10). NS3 is the only
glycosylated protein encoded by BTV and is found associated with both VP2 and VP5 (French et al., 1989; Wu et al., 1992, Beaton et al., 2002). These two proteins are related and derived from two different in-frame translation initiation codons in the genome segment S10. Deletion of the first AUG codon abolishes the synthesis of NS3 but not that of NS3A (Wu et al., 1992). Current data suggest that NS3 is involved in both maturation and release of virus (Hyatt et al., 1993, Beaton et al., 2002 and Wirblich et al., 2006).

Table 2.3: BTV Proteins and their functions

<table>
<thead>
<tr>
<th>Genome segment</th>
<th>Size (bp)</th>
<th>Encoded protein(s)</th>
<th>No. of amino acids</th>
<th>Predicted Mr</th>
<th>No. of molecules/virion</th>
<th>Location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>3954</td>
<td>VP1</td>
<td>1302</td>
<td>1,49,588</td>
<td>~ 6</td>
<td>Inner core</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>L2</td>
<td>2926</td>
<td>VP2</td>
<td>956</td>
<td>1,11, 112</td>
<td>180</td>
<td>Outer shell</td>
<td>Type-specific structural protein</td>
</tr>
<tr>
<td>L3</td>
<td>2772</td>
<td>VP3</td>
<td>901</td>
<td>1,03,344</td>
<td>601</td>
<td>Core structural proteins</td>
<td>Forms scaffold for VP7 trimers</td>
</tr>
<tr>
<td>M4</td>
<td>2011</td>
<td>VP4</td>
<td>654</td>
<td>76,433</td>
<td>~ 5</td>
<td>Inner core</td>
<td>Guanyl transferase capping enzyme</td>
</tr>
<tr>
<td>M5</td>
<td>1770</td>
<td>NS1 tubes</td>
<td>552</td>
<td>64,445</td>
<td>NA</td>
<td>Non-structural</td>
<td>Unknown</td>
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<tr>
<td>M6</td>
<td>1639</td>
<td>VP5</td>
<td>526</td>
<td>59,163</td>
<td>120</td>
<td>Outer shell</td>
<td>Structural protein</td>
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<tr>
<td>S7</td>
<td>1156</td>
<td>VP7</td>
<td>349</td>
<td>38,548</td>
<td>780t</td>
<td>Core surface</td>
<td>Group-specific structural protein</td>
</tr>
<tr>
<td>S8</td>
<td>1123</td>
<td>NS2 VIBs</td>
<td>357</td>
<td>40,999</td>
<td>NA</td>
<td>Non-structural</td>
<td>Binds mRNA</td>
</tr>
<tr>
<td>S9</td>
<td>1046</td>
<td>VP6</td>
<td>328</td>
<td>35,750</td>
<td>~ 37</td>
<td>Inner core</td>
<td>Binds ssRNA, dsRNA</td>
</tr>
<tr>
<td>S10</td>
<td>822</td>
<td>NS3</td>
<td>229</td>
<td>25,572</td>
<td>NA</td>
<td>Non-structural</td>
<td>Glyco proteins, aid virus release</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS3A</td>
<td>216</td>
<td>24,020</td>
<td>NA</td>
<td>Non-structural</td>
<td></td>
</tr>
</tbody>
</table>

NA: Not applicable
2.9.3: Outer capsid polypeptides

The outer capsid of BTV consists of two major polypeptides i.e. VP2 and VP5, which together constitute approximately 40% of the total BTV protein. There were 180 copies of VP2 protein (111kDa) arranged as triskelion structures and 360 copies of an inter-dispersed and underlying VP5 protein (59 kDa), which may be arranged as 120 trimers. Both VP2 and VP5 are attached to the core particle (VP7), although it has been reported that VP5 is more closely associated with the core particle than VP2 (Huismans et al., 1987b). VP2 and VP5 show the largest variation in size among the structural proteins of the different BTV serotypes (De Villirs, 1974 and Mecham et al., 1986). Further peptide mapping indicates that VP2 is unique for each of the BTV serotypes whereas VP5 revealed an intermediate level of conservation (Mecham et al., 1986).

The variability of VP2 and VP5 protein reflects the role of these proteins in the induction of serotype-specific neutralizing antibody (Mecham et al., 1986). The first evidence for the role of VP2 in determining serotype specificity was obtained by demonstrating that VP2 immuno-precipitate in serotype specific manner (Huismans and Erasmus, 1981). Huismans et al., (1987b) reported the induction of serotype specific neutralizing antibodies in rabbit and sheep after infection of purified VP2 protein. VP2-specific monoclonal antibodies can neutralize the virus (Appleton and Letchworth, 1983) and provide passive protection against homologous BTV serotype challenge (Letchworth and Appleton, 1983). So VP2 may act as a suitable candidate for a subunit vaccine.

Immunization of recombinant baculovirus expressed VP2 protein induces neutralizing antibodies not only against homologous (BTV-10) serotype but also
against the heterologous serotype (BTV-11 and BTV-17) to a lesser extent (Inumaru et al., 1987). So, more than one serotype specific neutralizing epitopes have been predicted in the VP2 protein (Gould et al., 1988).

The role of VP5 in the neutralization specific immune response has not been fully known. There is no evidence that VP5 by itself can induce neutralizing antibodies (Marshall and Roy, 1990), but possibly has got a contributory role in the induction of neutralizing antibodies by interacting synergistically with VP2, affecting its confirmation and subsequently its immunogenicity (Mertens et al., 1989). A mixture of solubilised VP2 and VP5 elicits a higher titer of neutralizing antibodies than the solubilised VP2 alone (Huismans et al., 1987c).

The outer capsid proteins are associated with the virulence and cell adsorption. VP2 is involved in cellular attachment. Upon removal of VP2, the virus failed to attach to the cellular receptor (Huismans et al., 1983). VP2 is a hemagglutinating protein and cleavage of VP2 results in loss of hemagglutination activity though it does not affect cell attachment. So it has been thought that the sites for hemagglutination activity and cell attachment are not necessarily same (Cowley and Gorman, 1987). The hemagglutination inhibition (HI) antibodies to BTV are also type specific (Tokuhisa et al., 1981b). The presence of large number of conserved cysteine residues suggests that it has a highly ordered structure that involves disulphide bonds (Urakawa et al., 1989).
2.10: BTV Genome

The BTV genome comprises of 10 segments of linear double stranded RNA (dsRNA) that is packaged in exactly equimolar ratios, one of each segment per particle. The viral particle and core contains 1% and 19.5% RNA respectively. The 10 segments of dsRNA are designated as L1, L2, L3, M4, M5, M6, S7, S8, S9 and S10, in order of increasing electrophoretic migration in the PAGE. The length of RNA segments varies from 3954 to 822 bp (total length is 19.2 kbp) (Urakawa et al., 1989). The Mol. Wt. of RNA varies from 0.5 to 2.7x10^6 Da (total size is 13.1x10^6 Da) (Fukususho et al., 1989, Els, 1973 and Verwoerd et al., 1979). The 5' non-coding regions are relatively short varying between 8 (M4) and 34bp (M6) (Roy, 1989) whereas the 3' non-coding region varies from 31bp (M5) to 116bp (S10). A sequence of 6 nucleotides (nt) at both 5’ and 3’ non-coding region is conserved for all the 10 segments of dsRNA of different BTV isolates (5’ GUUAAA…….ACUCA3’) (Rao et al., 1983, Mertens and Sanger, 1985). The different RNA segments are classified as highly variable, moderately conserved or highly conserved. L2 and M5 gene segments are highly variable among all the gene segments (Huismans et al., 1987d). Oligonucleotide fingerprint analysis of individual gene segments among different BTV serotypes also revealed that except L2 and M5, the fingerprint is identical for all genes (Sugiyama et al., 1981, Rao et al., 1983). Cross hybridization studies of RNA segments of different BTV serotypes were conducted to determine the genetic relationship among them. The cross-hybridization occurred between different isolates for all the dsRNA segments except L2 and M5 (Gorman et al., 1983, Squire et al., 1986, Kowalik and Li, 1987). The genes that code for inner core
protein and NS protein (L1, L3, M4, S7, S9, S10) exhibit strongest hybridization among different serotypes.

![Fig 2.4: Genome organization of bluetongue virus](Source: bluetonguevirus.net)

The assignment of the individual RNA segments into the protein that it encodes was determined based on the in vitro translation of the respective genome segment (Mertens et al., 1984 and Grubman et al., 1983). The most highly conserved genome segments are generally considered suitable for serogroup specific genomic probe. The L3 (Roy et al., 1985) and NS1 (Huismans and Cloete, 1987) gene segments have been recommended as the group specific genomic probe for BTV diagnosis. L2 is the most highly variable gene segment among different BTV serotypes but L2 segment is highly conserved in a specific serotype so it is best used as a serotype specific probe.

The mRNA species is an exact copy of the positive strand of one dsRNA segment. The 10 mRNA species are not synthesized at the same rate. The molar ratios in which the different mRNAs are transcribed remains the same through out the infection cycle and ratio of mRNA synthesized in vivo and in vitro is also identical (Huismans and Howell, 1973). The BTV mRNAs are capped at 5΄ end but they lack poly (A) tail at 3΄ end. There is only a single major open reading frame (ORF), which is always on the same strand in all the RNA segments. However, the ORF may have more than one functional initiation site near to the 5΄
end of mRNA resulting in production of two distinct but related proteins. The first AUG codon on the positive strand of each segment initiates a long ORF. But all the three possible termination codons are used. Four gene products terminate with UGA, four with UAG and the rest two with UAA.

2.11: S7 gene

Gene segment S7 encodes a major structural protein VP7, comprising 36% of the core particle protein (Huismans et al., 1987b). This protein contains serogroup specific antigenic determinants (Gunn and Newman, 1982). The BTV S7 genes are 1154-1156 base pairs in length and have a single open reading frame encoding a protein of 349 amino acids. The estimated mol. Wt. of S7 gene product is 38,548 KDa. Substantial conformational changes in VP7 occur at some stage in the viral life cycle. Such changes may be related to the central role of VP7 in cell attachment and membrane penetration.

2.12: NS3 & NS3A

Both NS3 and NS3A proteins are encoded by BTV S10 dsRNA and are synthesized in very low abundance (Mertens et al., 1984). The S10 gene has two conserved in-frame methionine codons, suggesting that NS3 and NS3A are derived from alternative translation initiation sites (Lee and Roy, 1987) and this gene is very highly conserved among BTV strains and serotypes (Roy, 1996 and Bonneau et al, 1999). NS3 functions as a viroporin, facilitating virus release from mammalian cells by inducing membrane permeabilization (Han and Harty, 2004). NS3 also binds to a cellular protein Tsg 101 (Wirblich et al., 2006) allowing BTV
particles to bud from the insect cells (egress) in which BTV do not induce significant cytopathic effect.

2.13: Isolation of Bluetongue virus

For primary isolation of BTV four main systems have been employed - intracerebral inoculation of new born suckling mice (Verwoerd, 1969), intravenous or combined intradermal and subcutaneous inoculation of sheep (Alexander et al., 1947), Yolksac or intravenous inoculation of embryonated chicken eggs (Alexander, 1947, Foster and Luedke, 1968, Goldsmith and Barzilai, 1965) and inoculation directly on primary or continuous cell cultures (Fernandes, 1959, Bando, 1975, Sawyer and Osburn, 1977).

Girard et al., (1967) observed that strains of BTV could be easily propagated in cell cultures after preliminary adaptation to embryonated chicken eggs. Stott et al., (1981) made 305 isolations of BTV from sheep, cattle, and goat and wild animals by intravenous inoculation of heparinised blood samples in 11 day old chicken embryos. Isolates were subsequently adapted to Vero and mouse fibroblast cells.


A total of 113 BT, 50 EHD, 36 Palyam, 12 Simbu, one bovine ephemeral fever virus isolates were recovered from 878 breeds from sentinel cattle in Australia using chicken embryo, baby mice, Aedes albopictus C6/36 cells and
BHK-21 monolayers. In all chicken embryo inoculation was found to be the best for isolating bluetongue virus (Gard et al., 1987).

Nachimuthu et al., (1992) and Ramesh babu et al., (1992) used blood samples to inoculate in chicken embryos to isolate the BTV virus from Tamil Nadu and Karnataka respectively. Sendow et al., (1993) isolated BTV serotype 2 from Culicoides species and serotypes 1, 21 and 23 from healthy sentinel cattle in Indonesia using chicken embryos, Aedes albopictus C6/36 and BHK-21 cell lines.

Yugendar Reddy et al., (2010) isolated serotypes 2, 9 and 10 from blood samples collected during field outbreaks in Andhra Pradesh. Two serotypes 9 and 10 were reported from a single outbreak.

2.14: Propagation of bluetongue virus

Clavijo et al., (2000) described the detection and specific identification of BTV as multi step process. The first step involved isolation of the virus from the animal’s blood or other tissues, followed by inoculation of embryonated chicken eggs (ECE) by intravenous route. After the virus amplification in ECE, it was passaged into BHK-21 cell culture for subsequent replication. The virus was then amplified further and identified in microtitre plates by the immunoperoxidase assay using a group specific monoclonal antibody. Finally, the viral isolate was typed by virus neutralization test.

2.14.1: Animals

Alexander et al., (1947) isolated BTV by intravenous or combined intradermal and subcutaneous inoculation of sheep. Verwoerd (1969) isolated BTV by intracerebral inoculation of new born suckling mice. Roy and Mehrotra
(1999) inoculated BTV isolates into 2-3 days old suckling mice and observed 28.5% mortality at the first passage.

On experimental inoculation of BTV sero-negative lambs with BTV by Deshmukh and Gujar (1999) the animals showed thermal reaction (106° - 108°F) on tenth day post-infection and prominent signs of hyperemia, cyanosis of tongue with loss of most of the epithelium and erosions of mucous membranes of buccal cavity and nasal passages. The heparinised blood collected at height of temperature was used to isolate BTV.

2.14.2: Embryos

Alexander et al., (1947) used 8 day old ECE to study the replication of a strain of BTV. The author noticed that incubation temperature of 33.6°C is essential for optimum results. Haig et al., (1956) described the multiplication of egg adapted BTV in lamb kidney cells. Evidence of virus multiplication associated cell destruction was noticed as early as 24 h PI, though the incubation period of 48 to 72h was more common. Affected cells became enlarged, refractile and detached from glass surface.

The BTV isolates were adapted to ECE through subcutaneous route and intravenous route of inoculation by Aruni et al., (1997) and observed that there is an increase in titre of virus from 0.8 to 1 log₁₀ EID₅₀ on an average for every five passages.

Deshmukh and Gujar (1999) employed ECE for isolation of BTV from processed blood samples of experimentally infected lambs. The embryos dying 24 hours post-inoculation showed haemorrhages, edema and cherry red colour.
Sreenivasulu et al., (1999) inoculated blood samples from BT affected sheep intravenously into ECE. This caused cherry red appearance of dead embryos.

Sudheer (2003) and Yugendar Reddy (2004) also employed ECE for the isolation of BTV from field samples. Similar post-inoculation embryonic lesions were observed by them.

2.14.3: Cell cultures

Fernandes (1959) reported successful propagation of virulent strain of BTV and egg adapted BTV in primary lamb kidney cells. Further, BTV was successfully grown by inoculating into primary explants of kidney cortex and medulla, tongue, nasal mucosa, lip mucosa, conjunctiva, skeletal muscle, liver and spleen.

Five strains of egg adapted BTV were infected into lamb kidney tissue cultures by Haig et al., (1956) and a cytopathic effect (CPE) was observed as early as the third day on the third passage. The effected cells became granular and underwent variable degrees of shrinkage and detachment.

Mc Phee et al., (1982) observed replication of Australian BTV serotypes 20 and 1 in BHK-21, Vero, foetal ovine lung, mouse fibroblast, Aedes albopictus cells and 11-day-old chicken embryos. At high multiplicity of infection (MOI) all vertebrate cell lines showed a clear CPE whereas at low MOI, CPE was almost absent except in BHK-21 cells. The invertebrate cell line (Aedes albopictus) showed no sign of CPE at higher or lower MOI. BHK-21 cells produced much higher yields of virus (8log PFU/0.2ml) than other cell lines (6 log PFU/0.2ml).

Jain et al., (1986) propagated bluetongue virus in BHK-21 cells. The virus titre was found to be $10^{6.5}$TCID$_{50}$/ml. Cytopathic effects observed were
rounding, increased refractivity of cells, granulation and detachment of cells from
glass surface.

Gard (1987) tried a variety of cell cultures like Aedes albopictus C6/36, BHK-21, bovine fetal trachea, bovine turbinate, chicken embryos, Georgia bovine kidney, mouse embryo fibroblast, rabbit kidney, Vero, chick embryo myocardial, lamb testis, sheep choroids plexus, sheep endothelial cells, bovine thyroid, chick embryo fibroblasts for growth of BTV-3 virus. He observed CPE only in lamb testis and chicken embryo rough cells at the first passage level. However, other cell cultures showed CPE on inoculation with chick embryo virus or lamb testis adapted BTV. The CPE was of a degenerative type for all except embryo fibroblasts where proliferate type of CPE was observed with acidic media.

Channel and Stott (1989) reported 90% CPE in 3 to 5 days with virus titres of $2.2 \times 10^5$ PFU/ml in Vero cells grown in RPMI-1640 medium whereas cells maintained in MEM developed CPE in 6-8 days with a virus titre of $7.8 \times 10^4$ PFU/ml.

From BTV infected sheep blood samples were collected and BTV was inoculated BHK-21 cells which upon Giemsa staining revealed granular cytoplasm, vacuoles, distorted pyknotic nuclei, characteristic inclusions and syncytia formation Nachimuthu et al., (1992).

BTV isolates were infected into BHK-21 and vero cells and observed CPE in both cell cultures as rounding of cells, cytoplasmic vacuolations and granulations, karyomegaly and intracytoplasmic inclusion bodies. Intranuclear inclusion bodies were observed only in Vero cells Ramesh Babu et al., (1992). Davies et al., (1992) inoculated blood samples into sero-negative sheep and embryonated eggs by yolk sac and intravenous route. Two blind passages were
routinely carried out in eggs, then spleen and heart suspensions were prepared from embryo tissues and then inoculated into lamb testis cells, BHK-21 and Vero cells. The supernatant from a tube at the limiting dilutions showing only 1-3 foci of viral CPE was passaged once then freeze dried for identification and typing purposes.

BTV serotype -1 was adapted by Bhat et al., (1996) to C6/36, an insect (Aedes albopictus) cell line which grow as round cells in a loosely adherent monolayer. After 24 h. of incubation, thinning of infected monolayer was seen, after 48h of incubation, infected cells aggregated together forming small and large clumps and after 60h detachment of cell sheet and cell lysis were seen. Garg and Prasad (1996) studied the susceptibility of goat peripheral blood mononuclear cells to BTV serotype-1. They noticed that small number adherent and non adherent cells were found positive for BTV antigens by immunoperoxidase and immunofluorescent tests.

BTV isolates were adapted into BHK-21 cells and evidence of cellular destruction from 24 hours post-infection was observed. The H&E staining of infected BHK-21 monolayers by 48h post-infection revealed several intracytoplasmic eosinophilic, perinuclear inclusion bodies, vacuolation of cytoplasm and giant cell formation Roy and Mehrotra (1999). The ECE adapted BTV was adapted to BHK-21 cell lines by Deshmukh and Gujar (1999) and on third passage observed the characteristic CPE within a period of 96 to 120 hours as cell detachment and rounding of cells. Clavijo et al., (2000) isolated BTV virus by inoculation of blood, into embryonating chicken eggs and later passaged in BHK-21 cell culture for subsequent replication and identified by immunoperoxidase assay using a group specific monoclonal antibody.
Gorch et al., (2002) isolated first BTV in Argentina from northeastern bovines without any disease symptoms. Erythrocytes, from these animals which showed seroconversion with cELISA, were processed for virus isolation by inoculation into embryonated chicken eggs and cell cultures. Cells with CPE were positive by direct and indirect immunofluorescence with BTV-specific reagents.

Niranjan babu (2003) propagated BTV-2 and MBN isolate (BTV-9) in BHK-21 cell line. The CPE of the virus isolate was characterized by formation of swollen spindle shaped cells and granulation in cytoplasm and finally detachment of monolayer by 96-120 hours post-infection.

Sudheer (2003) and Yugendar Reddy (2004) reported that inoculation of blood samples collected during bluetongue outbreaks into ECE caused mortality of chicken embryos with cherry red appearance. This embryo fluid when inoculated into BHK-21 cells caused CPE. Later these isolates were confirmed as BTV by Serum Neutralization Test.

Chaitanya (2005) and Prasad (2005) employed BHK-21 cells for propagation of BTV and the viral CPE was observed within 24-72h depending on the virus titres present in the inoculums as cell rounding, aggregation, thinning of monolayer and finally detachment of cells from surface. Infected cell culture fluid was used for further infections and viral RNA extraction.

Meenambagai et al., (2006) propagated an isolate of bluetongue virus in BHK-21 and Vero cells. The cytopathogenic changes were found to be intracytoplasmic eosinopilic inclusion bodies and nuclear fusion with giant cell formation. BHK-21 was found to be a better cell line for bluetongue vaccine production because of better adaptation of BTV to this cell line.
Three serotypes (2, 9, 15(10)) of BTV were propagated successfully in Vero cell lines by Krishna Jyothi (2007) and Sandhya (2007). Distinct cytopathic effect appeared within 4-5 days after inoculation. The earliest sign observed, as early as 72-78 h. post-infection was scattered focal rounding of cells. Within next 24-36 h. cell destruction extended rapidly and was characterized by cell detachment by 100-120h. post-infection.

Ramesh Babu et al., (2009) required 21 passages in Embryonated Chicken Eggs before BTV could be adapted to BHK-21. The adapted virus produced characteristic cytopathic changes like grouping of cells, polykaryon, syncytia formation, acidophilic and intracytoplasmic inclusion bodies.

2.15: Purification of Bluetongue virus

A number of protocols had been described for the purification of BTV. One of the basic problems associated with purification is the marked tendency of the bluetongue virus particles to be tightly bound to cellular material and instability of the virus particles, once purified. (Verwoerd, 1969, Huismans et al., 1979). Verwoerd (1969) exploited the tendency of BTV to remain cell-bound for purification and developed a virus purification method using fluorocarbon for extracting the virus from infected cell pellets. The extracted material yielded two bands on a CsCl –density gradient with respective densities of 1.18 and 1.26 of which, the later contained most of the infectivity. He also observed that sonication, freezing and thawing and treatment with trypsin were found to inactivate bluetongue virus.
Huismans (1979) further modified the Freon extraction method described by Verwoerd (1969) for purification of BTV. More number of Freon extractions was carried out on the combined water phases to dissociate virus from cellular material and the viruses were pelleted by centrifugation for two hours at 24,000rpm through a 5 ml cushion of 40% sucrose in 0.002 M Tris, pH - 8.8. The virus was then banded on sucrose gradients and was considered purified when at least seven BTV polypeptides could be detected on electrophoresis of a purified virus sample.

Patricia Jameson and Grossberg (1981) obtained 10-30 fold concentration of BTV by polyethylene glycol (PEG) precipitation. The concentration of 0.5M NaCl along with PEG 6000 was found to yield maximum virus. The precipitate containing virus is sedimented and the BTV is extracted using 0.01 M Tris HCl containing 0.01 M NaCl and 0.001 M EDTA.

Mertens et al., (1987a) harvested the infected cells by centrifugation at 3000g for 30min at 4°C and resuspended the cellular pellet in TNET buffer and homogenized in a glass homogenizer. The nuclei were then pelleted by centrifugation at 8000g for 10 min and discarded. The supernatants collected were pooled and kept at 0°C. The pooled material was layered onto short discontinuous sucrose gradients and centrifuged at 85,000g for 3hr at 4°C. The virus and cellular components which formed a thick disc at the interface of the sucrose solution were collected and resuspended in 0.2 M Tris HCl, pH 8.0. The resuspended discs were made to 3.0% sodium deoxycholate, layered onto sucrose gradients and centrifuged as described. 0.2 M Tris buffer at 4°C was used for collection and storage of virus.
Hussein et al., (1989) purified BTV-10 virus using polyethylene glycol precipitation, tartarate gradient centrifugation, and pelleting through 30% sucrose cushion. Purified virus was stored in multiple portions at -70°C. Wilson Aruni et al., (1999) used the method of high speed pelleting at 35,000 rpm for 2 hr and 30% and 40% sucrose gradient centrifugation for 1hr at 40,000 rpm for purification of BTV from infected cell culture fluids.

Meenambigai et al., (2003) concentrated BTV to be used as antigen in AGPT. At complete CPE, the cells and medium were harvested by three cycles of freezing- thawing and clarified at 10,000rpm for 20 min at 3°C. The supernatant was transferred to a large beaker in an ice bath on a magnetic stirrer. Sodium chloride and PEG-6000 were added to a final concentration of 2.3% and 7% with constant gentle stirring. The beaker and ice bath were placed at 4°C overnight to allow the virus to get precipitated. The precipitate was collected by centrifuging at 12,000rpm for 20 min at 4°C and reconstituted with minimal volume of TES buffer. PEG was completely removed by centrifuging the reconstituted suspension at 10,000 rpm for 4 min at 4°C.

2.16: Diagnosis

Diagnosis of bluetongue involves the detection and identification of BTV-specific antigens, antibodies or RNA in diagnostic samples taken from animals that are potentially infected, virus isolation and serological or molecular assays to identify the virus serogroup and serotype. BTV serotype is determined by the BTV outer capsid proteins VP2 and VP5, particularly VP2, which primarily controls the specificity of interactions with neutralizing antibodies in serum.
neutralization assays. These existing serotypes can be detected and differentiated by RT-PCR and phylogenetic analyses targeting segment-2 of the virus genome, which encodes for out capsid protein VP2 (Maan et al., 2007a, Mertens et al., 2007a and b). The identification of serotype of BTV can be used to demonstrate the virus belongs to the BTV serogroup/species and can therefore be used to confirm an initial diagnosis. Indeed, the identification of a specific BTV serotype is cited as one of the most reliable methods of BTV diagnosis (Hamblin, 2004).

BTV group-specific assays include serological methods to detect BTV-specific antibodies generated during infection of the mammalian host, serological assays to detect and identify BTV-specific protein antigens and molecular techniques such as RT-PCR and cDNA sequencing/phylogenetic analyses that can be used to detect and identify BTV RNA extracted from diagnostic samples. These assays may also involve or depend on the ‘isolation’ of the virus and its growth in cell culture.

RT-PCR assays (particularly nested or real-time) can be very sensitive and can give positive results at an earlier stage of post-infection (e.g. after 1-3 days) than methods that detect the BTV-specific antibodies in the infected host (e.g. after 7-10 days). BTV-specific antibodies are also detectable for long periods, possibly for the life of the ruminant host, whereas virus particles and viral RNA can be cleared much more quickly from the blood of the infected animals.
2.16.1: Group-specific molecular assays

Many of the earliest BTV group-specific tests are based on conventional gel-based RT-PCR methods (Dangler et al., 1990, Wade-Evans et al., 1990, McColl and Gould, 1991, Akita et al., 1992, Shad et al., 1997, Aradaib et al., 1998, 2003 and 2005, Billinis et al., 2001, Anthony et al., 2007) and were developed before the advent of real-time RT-PCR. Real-time diagnostic assays have rapidly become popular because they are less labour intensive, quicker, can be more sensitive and have a lower risk of cross-contamination in the laboratory. The most conserved regions of the BTV genome segments 1, 3, 4, 5, 8 and 9 (Maan et al., 2008) (coding for the proteins: VP1, VP3, VP4, NS1, NS2 and VP6). These genome segments therefore represent potential targets for the development of additional BTV species/serogroup-specific RT-PCR assays.

2.16.2: Serotype-specific molecular assays

The outer capsid proteins VP2 and VP5 of BTV are encoded by segments 2 and 6 (Mertens et al., 1984), which represent the first and second most variable regions of the virus genome, respectively (Maan et al., 2008). Sequence comparisons of genome segments 2 and 6 from isolates of the 24 serotypes have shown variations in segment 2 that correlate primarily with virus serotype although they also show differences within each serotype that correlate with the geographic origins of the virus isolate (Maan et al., 2007). These studies and sequence comparisons of full length segment 2 from over 300 virus strains belonging to different BTV serotypes have demonstrated that BTV isolates can be reliably ‘typed’ by sequence analyses and phylogenic comparisons of segment 2
The development of a sequence data set for the reference strains of all 24 BTV serotypes (Maan et al., 2007a) confirmed the earlier studies showing that VP2 is the primary determinant of BTV serotype (Mertens et al., 1989 and DeMaula et al., 2000). These sequencing studies also provided a basis for the development of RT-PCR assays to identify the serotype of RNA extracted from virus isolates or directly from diagnostic samples (Zientara et al., 2006, Mertens et al., 2007a and b).

RT-PCR assays are also much faster than conventional antigen-based ‘typing’ methods giving positive serotype identification, compared with conventional VNT typing methods. These molecular assays are also independent of the standardized preparation of neutralizing antisera or reference virus strains that are needed for VNTs and SNTs. The conventional RT-PCRs that have been developed to identify the different serotypes of BTV can also be used to distinguish between field and vaccines strains as well as different topotypes of genome segment 2, in a manner that is impossible using antibody/antigen-based methods alone.

2.17: Genomic relationships

Fukusho et al., (1987) determined the extent and nature of the antigenic variation of four BTV serotypes of USA (BTV-10, 11, 13 and 17) by comparing the nucleotide sequence of the L2 gene. Diagon comparisons, hydropathic plots and analyses of secondary structure of proteins indicated that all four VP2 proteins were structurally similar. However, alignment of the VP2 sequences of the four BTV serotypes showed close relationships between BTV-10, -11 and -17.
serotypes only. Numerous amino acid differences were evident in the BTV-13 sequence when compared with the other three virus serotypes. While 70% of the amino acid sequences of the other three BTV serotypes are conserved, only 39 to 40% of the aligned amino acids were found identical for BTV-13 and BTV-10, BTV-13 and BTV-11 and BTV-13 and BTV-17. These studies suggested that BTV-13 of USA might have evolved independently. Gould and Pritchard (1990) compared VP2 nucleotides and deduced amino acid sequences of BTV from North America, Australia and South Africa. BTV-10 US, BTV-11 US, BTV-17 US and BTV-20 AUS were found closely related to each other. A similar relationship was also observed between BTV-3 SA, BTV-3 AUS, BTV-13 US and BTV-16 AUS. Homologies of VP2 segments of these viruses were approximately 70% identical at the nucleotide level and 80-90% at amino acid level.

Gould et al., (1992) studied the inter–relationships between the BTV serotypes using DNADIST in the KITSH program by the comparison of nucleotides 975-1190 and hyper-variable virus-neutralization site of VP2. Based on these comparisons, certain serotypes which showed close relationships by both nucleotide and aminoacid sequence homologies (i.e. BTV-3, BTV-13 and BTV-16 or BTV-10, BTV-11, BTV-17 and BTV-20) were grouped under a single “nucleotype”. Within a nucleotype, VP2 nucleotide or aminoacid sequence homologies were ~70% while in comparisons done between nucleotypes, homologies dropped to ~55%. It is also observed that within a nucleotype both 5’ and 3’ noncoding regions were highly conserved in both length and sequence which were highly variable between the nucleotypes.
Yamakawa et al., (1994) reported the genetic relationship of the virulent Australian bluetongue virus serotype 23 with seven other serotypes of BTV. Complete nucleotide sequence of L2 dsRNA of BTV-23 and the predicted amino acid sequence of the protein was compared with the VP2 sequences of the five USA serotypes (BTV-2, -10, -11, -13 and -17) and an Australian isolate of BTV-1. The comparison revealed that the VP2 of BTV-23 is closely related to that of BTV-1, sharing 52% identical and 72% similar sequences. Also the VP2 of the two Australian serotypes (BTV-23 and BTV-1) are more closely related to that of BTV-2 than to the other four USA serotypes. Based on these observations, a phylogenetic tree was generated which revealed three main monophyletic groups, one include BTV-10, -11 and -17, other Australian BTV-1, -23 and US BTV-2 while BTV-13 stands apart.

Pierce et al., (1995) reported the presence of a neutralizing epitope common to BTV-10 and BTV-17 on VP2 suggesting that both the serotypes have a genomic relationship. Pritchard and Gould (1995) sequenced various regions of VP2 genes of different bluetongue virus serotypes and compared them phylogenetically. Multiple amino acid sequence alignment of BTV serotypes and other Orbiviruses over a proposed major neutralization site showed a highly variable region between 317-335 aa. Comparisons of the partial nucleotide sequences of genome segment- 2 of BTV serotype 1 isolates over the region 2370-2845 bp showed that BTV-2 US isolate was closely related to BTV-1 isolates. BTV-13 was found related to BTV serotype 3 isolates, and was having 65% nucleotide homology. BTV serotypes 10, 11,17,4 and 20 isolates formed one nucleotype, as did BTV serotypes 3, 13 and 16 isolates. These results were in
agreement with the data provided by Erasmus (1990), in which he postulated serological relationships among BTV serotypes using plaque-reduction tests. Dahiya et al., (2004) designed a specific set of primers using VP2 gene sequences which can amplify six Indian isolates of BTV-1. The PCR product of VP2 gene was cloned and the positive clones were sequenced. The partial VP2 gene sequence (1240-1844 bp regions) studies revealed that BTV-1 Indian isolates were having 89% and 75% homology with Australian and South African BTV-1 isolates respectively. Phylogenetically, three BTV Indian isolates formed one group which is closely related to BTV-1 AUS isolates followed by BTV-1SA, BTV-2, 9, 23,13,17,10 and 11 isolates from different parts of the world.

Maan et al., (2007) compared the full length cDNA copies of genome segment 2 (seg-2, which encodes VP2) from the reference strains of each of the 24 BTV serotypes after cloning and sequencing. These data demonstrated over all inter-serotype variation in seg-2 from 29% (BTV-8 and BTV-18) to 59% (BTV-16 and BTV-22) while the deduced aminoacid sequences of VP2 varied from 22.4% (BTV-4 and BTV-20) to 73% (BTV-6 and BTV-22). Ten distinct seg-2 lineages (Nucleotypes A-J) were detected, with greatest sequence similarities between those serotypes that had previously been reported as serologically ‘related’. Nucleotype is defined by <35% difference in seg-2 nucleotide sequences. Fig.2 depicts the comparison of segment-2 (VP2 gene) from all 24 serotypes of BTV.

2.18: Extraction of Viral dsRNA

A number of protocols had been described for isolation of dsRNA from BTV infected cell culture, clinical sample and culicoides vector. This dsRNA
thus extracted was employed further for cDNA synthesis, PCR amplification, cloning, sequencing and expression related studies. Verwoerd (1969) first isolated genome of bluetongue virus and characterized as segmented double stranded Ribonucleic acid. dsRNA was isolated after diluting the virus 10 times with 0.01M sodium acetate containing 0.001M EDTA and 1% SDS followed by extraction with equal volumes of phenol. Viral RNA was precipitated with 2 volumes of ethanol after adding 2% potassium acetate.

A rapid method for RNA isolation from small volumes of BTV infected cell suspension was described by Squire et al., (1983). The genomic RNA in phenol extract of the infected monolayer was precipitated with 2M LiCl. The precipitated fraction contained both cellular and viral RNA. Ritter and Roy (1988) separated ssRNA from total BTV infected cellular nucleic acid by precipitation with 2M LiCl and ethanol and addition of 4M NaCl at 4°C overnight resulted in separation of contaminating DNA.

Dangler et al., (1990) extracted BTV ds RNA from infected Vero cell monolayer by addition of 100mM sodium acetate and 1% SDS followed by phenol-chloroform and isoamyl alcohol (25:24:1) extraction then nucleic acids were precipitated in the presence of ammonium acetate and ethanol. Chomczynski and Sacchi (1987) used single step method of RNA extraction by acid guanidium thiocyanate phenol chloroform (Trizol™ LS reagent) available commercially. Roy et al., (1988) employed differential lithium chloride precipitation technique for the extraction of dsRNA from BTV infected cells harvested at 60% CPE. The total RNA was extracted by SDS lysis followed by phenol chloroform extraction. The ssRNA was removed by 2 M LiCl precipitations and dsRNA was precipitated by 4M LiCl precipitation overnight.
Infected cell culture fluid was suspended in TE buffer and treated with 1% SDS before extraction of RNA using phenol chloroform method and ds viral RNA was purified by differential precipitation with 2 Mol L\(^{-1}\) and 40 Mol L\(^{-1}\) (Bandyopadhyay et al., 1998). Trizol™ LS reagent was used for extraction of RNA from blood samples, embryonated chicken eggs and infected cell culture by Byregowda (2000) and Billins et al., (2001). Savini et al., (2004) extracted total RNA from BTV infected BHK-21 cell culture using Trizol reagent and ssRNA was removed by precipitation with 2M LiCl. Then the dsRNA was purified from supernatant using gel extraction kit obtained from Qiagen, USA. Mertens et al., (2007a) extracted viral RNA from cell free supernatant using QIA amp, viral RNA mini kit (Qiagen, USA). Maan et al., (2007) extracted dsRNA from BHK-21 infected cells using Trizol™ reagent. ssRNA was removed by precipitation with 2M LiCl followed by precipitation of dsRNA using Ammonium acetate (7.5M).

### 2.19: Electrophoretic Migration of BTV dsRNA Segments

The dsRNA profile of BTV genome had been studied both in 1% Agarose as well as in RNA polyacrylamide gel electrophoresis (RNA-PAGE). Due to segmented nature of its genome, all the 10 dsRNA could be separated efficiently (Squire et al., 1983). Knudson et al., (1982) stated that the profile of genomic segment migration could be used as an epidemiological or taxonomic marker for the virus. Squire et al., (1983) studied the genome profiles of 200 field isolates of BTV by PAGE and revealed distinct variation among the electrophoretic profiles of many isolates. Squire et al., (1986) compared the dsRNA genome profiles of BTV serotypes using 1.2% agarose gel system and reported that nucleic acid
segments 7 and 8 were not resolved and noticed similar migration patterns for different serotypes. Pedley et al., (1988) studied the genome profiles of *Orbiviruses* in PAGE and agarose gels and reported that genome profile varied in PAGE depending on the heterogeneity of the genome segments which could be due to variation in secondary structure, base composition, RNA sequence rather than variation in molecular weight. In contrast to PAGE the genomic dsRNA profiles in agarose gel electrophoresis were alike and no variation was detected in PAGE. They concluded that the patterns of *Orbivirus* genome segment migration in agarose gels were group specific. Prasad and Minakshi (1999) compared the sensitivity of RNA-PAGE and DIA (Dot Immunobinding Assay) and reported that RNA-PAGE and DIA had comparable sensitivity and both could detect a minimum virus titre of $10^5$ TCID$_{50}$ ml$^-1$.

### 2.20: Polymerase Chain Reaction

PCR is an *in vitro* enzymatic method for amplification of selected sequences of nucleic acid (Saiki et al., 1988). The discovery of PCR by Mullis (1987) revolutionized the whole field of molecular biology. The RT-PCR (Reverse transcription PCR) is a method of amplifying RNA sequences. PCR could amplify defined nucleic acid fragments from a single cell or tissue sample by $\sim 10^6$ fold in a matter of hours (Ou et al., 1988 and Saiki et al., 1988). PCR is highly sensitive, rapid and less labour, intensive assay for detection of BTV isolates (Gould et al., 1989). With the advent of powerful PCR technique and the information on BTV genome sequence, specific oligonucleotide primers could be designed for amplifying different genome segments. PCR was used not only to detect BTV RNA in cell cultures and clinical samples but also for serotyping of
viruses (Mertens et al., 2007). Primers derived from highly conserved genes which codes proteins such as VP3, VP6, VP7, NS1 and NS3 were used by different workers for serogrouping, while primers derived from VP2 gene sequences were used for serotyping of bluetongue virus (Mertens et al., 2007a). Dangler et al., (1990) used RT-PCR for detection of BTV in infected cell cultures using primers specific to gene segment 6 that codes for NS1 protein. The test could detect two femtograms of BTV RNA.

Wade Evans et al., (1990) developed a polymerase chain reaction for detection of bluetongue virus in blood sample from experimentally infected cattle employing primers derived from genome segment 7 and reported to yield best amplification result by identifying as few as 6 molecules of segment 7 dsRNA per sample. Akita et al., (1993) developed RT-PCR using primers derived from highly conserved genome segment 10 for the detection of bluetongue virus in cell cultures. The test could found to detect as low as 1 pg of BTV RNA which is equivalent to the amount of viral RNA from 50 virions. Later this protocol was standardized for detecting the bluetongue virus in clinical samples viz., infected whole blood, spleen and semen (Akita et al., 1993). For the detection of BTV in clinical samples the RT-PCR was found to be more sensitive than virus isolation. Wilson and Chase (1993) used nested and multiplex PCR for detection and typing of bluetongue virus in Culicoides variipennis using two different sets of primers based on NS1 gene for nested PCR and 5 different sets of primers based on VP2 genes of five US serotypes simultaneously for multiplex-PCR. Nested PCR was able to detect 1 pfu of virus in infected biting midges. The sensitivity of multiplex PCR was found less than nested PCR.
Katz et al., (1993) developed PCR procedure coupled with enzyme linked oligonucleotide sorbent assay (ELOSA) which relied on annealing of separate biotinylated probes to the amplified nucleic acid. The complexes were captured on streptavidin coated microtitration wells and were detected using a HRP labeled antifluorescein antibody conjugate. The sensitivity of this PCR-ELOSA was found to detect 0.001 TCID\textsubscript{50} of virus. MacLachlan (1994) used PCR and chicken embryo inoculation methods for detection of BTV from experimentally infected calf. PCR could detect virus nucleic acids 16 to 20 weeks after virus inoculation, whereas infectious virus detected only after 2 to 8 weeks by virus isolation in ECE. Blood samples which were positive for PCR but not by virus isolation ECE were found to be non infectious for sheep. Aradaib et al., (1998) developed sensitive nested PCR based on NS1 gene for the detection of BTV in cell culture and tissue sample. Its sensitivity was reported to be 0.1 fg of BTV RNA (equivalent to 5 viral particles). Bandyopadhyay et al., (1998) used NS1 gene specific RT-PCR for detection of BTV in infected cell cultures and blood samples from sheep. Later, Prasad et al., (1999) standardized single tube RT-PCR for detection of BTV employing NS1 gene.

Johnson et al., (2000) compared the mRT-PCR (multiplex reverse transcriptase polymerase chain reaction) to the standard virus neutralization test for serotyping of bluetongue virus isolates. mRT-PCR was found to be more reliable and less time consuming than VNT. OIE (2000) recommended PCR assay as one of the official test for detection of BTV in animals for international movement and transport. Mertens et al., (2007a) designed sets of primers targeting segment-2 of BTV 1, 2, 4, 8, 9 and 16 isolated from USA for rapid identification and typing by RT-PCR. The RT-PCR was found sensitive, specific
and the results depicted perfect agreement with the results of conventional virus neutralization test for typing of bluetongue virus. The method did not show cross amplification with multiple isolates of BTV. The RT-PCR could also differentiate vaccine strains and field isolates.

2.21: Cloning

Purdy et al., (1985) polyadenylated L2 RNA at its 3’ ends and cDNA copies were synthesized with reverse transcriptase using oligo (dT)$_{12-18}$ primer and cDNA product duplexes were cloned into pBR 322, clones representing L2 DNA were identified by colony hybridization and RFLP and sequenced the cloned product by Maxam and Gilbert method. Ghiasi et al., (1987) separated purified dsRNA by agarose gel electrophoresis and L2 gene was electroeluted and was polyadenylated. cDNA copies were undertaken using oligo (dt) 12-18 primer. The products were cloned into pBR 322 vector. After transformation the colonies containing viral nucleic acids were recovered and screened by colony hybridization and RFLP pattern and sequenced by method of Maxam and Gilbert.

Pritchard and Gould (1995) synthesized cDNA copies of dsRNA using random primers and AMV-RT. The cDNA copies were cloned into M13 bacteriophage vector and PCR amplification was carried out. The PCR product was cloned into pUC vector and the L2 and L3 segments of different BTV isolates were sequenced. Balumahendiran (2006) amplified L2 segment of BTV-2 using segment specific primers by RT-PCR. The amplified product was cloned into T/A cloning vector PTZ57R/T (Fermentas, USA) for analyzing the partial sequence of L2 segment of BTV-2. The cDNAs of L2 segments of 24 serotypes were synthesized using anchor primers and the cDNAs were amplified by PCR using
primers complimentary to anchor primers. Further, the full length PCR product was cloned into pGEM-T easy vector (Qiagen, USA) for sequencing full length L2 genes of 24 reference serotypes (Maan et al., 2007).

2.22: Molecular characterization of bluetongue virus

The full nucleotide sequence of all 10 segments of BTV-10 was published by Roy (1989). These initial studies were the basis for the cloning and sequencing of many BTV gene segments as well as for the expression of their respective recombinant proteins. The sequencing and hybridization studies were the basis for molecular epidemiological studies and also RT-PCR techniques (Dangler et al., 1990, Wade-Evans et al., 1990), which are used today for routine diagnostic detection of BTV nucleic acids.

The bluetongue virus with its 24 serotypes and high antigenic variation among the serotypes created a nasty confusion in controlling the disease since last two decades. The outstanding technique, amplification of DNA by a polymerase enzyme, invented by the Kary Mullis in 1983 an American scientist, provided a solution for most challenging problems of biology (Mullis and Faloona, 1987). With this technique, BTV became one of the most well studied viruses at molecular level. Hence now a days it became a hand tool to type a bluetongue virus isolate using a type specific primers against L2 gene of BTV which is more specific, sensitive and less time consuming when compared to serological methods.

The BTV genome consists of dsRNA. The RNA was amplified by Reverse Transcription polymerase chain reaction. Zientara et al., (2004) standardized both group specific RT-PCR and type specific RT-PCR for the
diagnosis of BTV. The type specific primers designed for BTV serotypes 1, 2, 4, 9 and 16 were tested for specificity. However no cross amplification was found with other serotypes. A new wild type isolates from outbreaks after vaccination with attenuated BTV-2 &9 vaccines and typed it as BTV-2. A set of primers were designed based on S10 genome sequence of BTV-2 which helps in differentiating between vaccine strain and wild types.

Dahiya et al., (2004) designed serotype specific primers for amplification of hyper variable regions of VP2 genomes of BTV-1 and BTV-23. They successfully amplified 1240-1844bp region of BTV-1 which consists of two hyper variable regions with no cross amplification of heterologous serotype of BTV. Similarly, they standardized RT-PCR assay for amplification of a part of VP2 gene of BTV-23 without non-specific amplification.

Mertens et al., (2007b) designed primers and standardized RT-PCR assays targeting the segment-2 of the various bluetongue virus isolates that effect the Europe. These primers were tested for their efficacy, sensitivity and specificity in typing of the bluetongue virus isolates. The results of these assays showed perfect agreement with VNT. Moreover there were no cross amplification of the type specific primers with the other BTV reference strains and vaccine strains. The serotype specific assays were used to group the homologous serotypes into various topotypes.

Maan et al., (2007a) described a new diagnostic tool for sequence independent synthesis, amplification and direct-sequencing of full-length cDNAs of dsRNA genes. They designed a self priming anchor-primer which ligated at 3′ end of the ssRNA strand and consequently initiate synthesis of full length cDNAs from multiple genome segments simultaneously. These cDNAs were amplified
using 5-15-1 primer. For generating sequence data from both termini and to skip off a cloning step they designed universal-sequencing primers (phased primers) which consists of the sequence of primer 5-15-1 plus terminal six conserved-nucleotides. The outgoing forward and reverse foot-print primers were designed for confirming the sequences of these conserved termini of the 5’ and 3’ end of the BTV genome.

Maan et al., (2007b) cloned and sequenced the full length cDNA copies of each of the 24 reference BTV serotypes. When compared with coding region, the near terminal non-coding sequence was highly conserved with 2.9-64% variation in the 3’ and 5.9-64% variation in the 5’ NCR. The full length nucleotide sequence of the seg-2 reveals variation among reference strains of all 24 BTV types by 29% to 59% with terminal hexa nucleotides as the fully conserved sequence and VP2 amino acid sequence variation is 22.7% to 72.9% between BTV types. They constructed neighbour-joining tree which indicate 9 evolutionary branching points that correlate with the 10 nucleotypes.

Two reverse transcriptase quantitative assays were developed: one for segment-1 and another for segment-5 of BTV by Toussaint et al., (2007). This RT-qPCR could be used to detect the BTV infection in infected animals even before the antibodies were detectable.

A novel tool, the new real-time quantitative reverse transcription-PCR assay was developed by Hofmann et al., (2009) for typing of the BTV serotypes 1, 6 and 8. they designed type specific primers based on the reference sequences of BTV belonging to western group which are labeled with 6-carboxyfluorescein at the 5’ end and black hole quencher 1 carboxylic acid at 3’ end. The results of the
amplification brings out 100% serotype specificity with no cross amplification with heterologous serotypes.

The outbreak of BT in cows in Netherlands during the year 2008 gave the first report of BTV-6 in Europe which undergone full length characterization by Maan et al., (2010). The BT positive was confirmed by real time RT-PCR targeting segment1. Further, it was typed as BTV-6 by experimental primers designed against 24 serotypes, in which case amplification was seen only with BTV-6 primers (Mertens et al., 2007b). Also the anti sera from the infected cow were tested in SNT against all 24 reference strains of BTV and neutralization was seen against BTV-6 and BTV-8 (vaccine strain). The full length cDNA sequence of segment-2 had close relationship (99.8/99.7%nt/aa identity) to reference strain of BTV-6 and vaccine strain of BTV-6 from South Africa.

BTV RNA was identified by real-time RT-PCR targeting genome segment -10, in blood samples of Netherlands. The virus was isolated from the Heeten sample (IAH ‘dsRNA virus reference collection’ (dsRNA-VRC) isolate number NET2008/05) and typed as BTV-6 by RT-PCR targeting seg-2. Sequencing confirmed the virus type, showing an identical Seg-2 sequence to that of the South African BTV-6 live-vaccine-strain. Although most of other genome segments also showed very high levels of identity to the BTV-6 vaccine (99.7 to 100%) Seg-10 showed greatest identity (98.4%) to the BTV-2 vaccine (RSAvvv2/02), indicating that NET2008/05 had acquired a different Seg-10 by reassortment. Although Seg-7 from NET2008/05 was also most closely related to the BTV-6 vaccine (99.7% nt/aa identity), the Seg-7 sequence derived from the blood sample of the same animal (NET2008/06) was identical to that of the Netherlands BTV-8 (NET2006/04 and NET2007/01). This indicates that the blood contained two
different Seg-7 sequences, one of which (from the BTV-6 vaccine) was selected during virus isolation in cell-culture. The predominance of the BTV-8 Seg-7 in the blood sample suggests that the virus was in the process of reassorting with the northern field strain of BTV-8. Two genome segments of the virus showed significant differences from the BTV-6 vaccine, indicating that they had been acquired by reassortment even with BTV-8 and another unknown parental strain (Maan et al., 2010).

2.23: Genome segment-7

Seg-7 of NET2008/05 is 1156bp long, encoding 349 aa of the major BTV serogroup specific antigen and core surface protein – VP7. The aa sequence of VP7 is significantly more conserved (73.6% identity) than the nt sequence (63.3%) reflecting large numbers of synonymous mutations in the third base position. Seg-7 was reported to form six distinct clusters: three of these are primarily from western origins (western 1, 2 and 3) and three from an eastern origin (eastern 1, 2 and 3). However, Seg-7 from the Chinese strain of BTV-12 groups within western group 1, BTV-6 in the Netherlands suggesting some movement of strains between geographic regions. Analysis of additional isolates from around the world also identified four additional clusters, as well as an isolate from Yunnan, China (AY 386682) in western group 4, and BTV-15 from Australia (Ac.No, L11723) within western group 2. BTV-25 (TOV) showed 70 to 79.1/79.9% to 93.4% nt/aa identity with other BTV strains, representing a further distinct group/topotype and forms a western group 7. The nt/aa sequence of Seg-7/VP7 of NET2008/04 (KC isolate) and NET2008/05 (KC1/BHK1 isolate) showed 70.5/99.7% nt/aa identity to other BTV strains, and as observed with the majority
of other segments (except Seg-10), highest levels of identity to the BTV-6 vaccine strains in ‘western cluster 3’. However, in contrast Seg-7 from NET2008/06 showed 79.3 to 79.9/94.5 to 94.8% nt/aa identity with other BTV-6 strains, but 100% nt/aa identity with BTV-8 from the Netherlands 2006/2007 (NET2006/04 and NET2007/01), within ‘western group 1’ (Maan et al., 2010).