CROSS PROTECTION STUDIES OF BLUETONGUE VIRUS - 23

BY

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CERTIFICATE

This is to certify that Ms. **TANGUTURI SWATHI (I.D No. RVM/2019 - 19)** has satisfactorily prosecuted the course of research and that the thesis entitled **"CROSS PROTECTION STUDIES OF BLUETONGUE VIRUS - 23"** submitted is the result of original research work done and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by her for a degree of any University.

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This is to certify that the thesis entitled **"CROSS PROTECTION STUDIES OF BLUETONGUE VIRUS - 23"** submitted in partial fulfillment of the requirements for the degree of **Master of Veterinary Science** of **P.V. Narsimha Rao Telangana Veterinary University** is a record of *bonafide* research work carried out by **Ms. TANGUTURI SWATHI (I.D No. RVM/2019-19),** under our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of investigations have been duly acknowledged by the author.

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LIST OF ABBREVIATIONS

Abbreviation		Full form
%	:	Percent
/	:	Per
=	:	Equals to
>	:	Greater than
<	:	Less than
µ	:	Micron
µg	:	Microgram
µL	:	Microliter
µM	:	Micromolar
mM	:	Millimolar
^{o}C	:	Degree Celsius
^{o}F	:	Fahrenheit
3’	:	3-prime
5’	:	5-prime
A	:	Adenosine
BHK	:	Baby Hamster Kidney
bp	:	Base pair
BT	:	Bluetongue
BTV	:	Bluetongue virus
C	:	Cytosine
cDNA	:	Complementary deoxy ribo nucleic acid
cELISA	:	Competitive ELISA
cm	:	Centimeter
CO_2	:	Carbon dioxide
CPE	:	Cytopathic Effect
C_t	:	Cycle-threshold value
dH_2O	:	Distilled water
DMEM	:	Dulbecco’s modified Eagle’s medium

dNTP	:	Deoxynucleotide triphosphate
ds-RNA	:	Double stranded Ribonucleic acid
e.g.	:	Exempli gratia (for the sake of example)
EDTA	:	Ethylene Diamine Tetra Acetic acid
ELISA	:	Enzyme linked immune sorbent assay
et al	:	Et alia (and others)
EtBr	:	Ethidium bromide
F	:	Forward
FBS	:	Foetal Bovine Serum
Fig.	:	Figure
G	:	Guanosine
h	:	Hours
I.U	:	International Unit
Kbp	:	Kilo base pair
KCl	:	Potassium chloride
KH_2PO_4	:	Potassium dihydrogen phosphate
MEM	:	Minimum essential medium
Mg	:	Milligram
$Mgcl_2$	:	Magnesium chloride
Min.	:	Minutes
mL	:	Milliliter
mm	:	Millimeter
MOI	:	Multiplicity of infection
Mol.wt	:	Molecular weight
Na_2HPO4	:	Disodium hydrogen phosphate
Nacl	:	Sodium chloride
NaOH	:	Sodium hydroxide
nm	:	Nanometer
No.	:	Number
NS	:	Non-structural protein

OD	:	Optical density
OIE	:	Office International des Epizooties
OPD	:	o-Phenylenediamine dihydrochloride
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PFU	:	Plaque forming units
Pg	:	Picogram
pH	:	Potential of Hydrogen
PI	:	Percentage inhibition
Pmol	:	Pico mole
qRT-PCR	:	Quantitative Reverse Transcriptase Polymerase Chain Reaction
R	:	Reverse
RBC	:	Red blood cells
RNA	:	Ribonucleic Acid
Rpm	:	Revolutions per minute
RT-PCR	:	Reverse Transcriptase Polymerase Chain Reaction
Sec	:	Seconds
SNT	:	Serum neutralization test
T	:	Thymine
T25	:	$25cm^2$ tissue culture flask
TAE	:	Tris acetate EDTA
$TCID_{50}$	:	Tissue culture infective dose $_{50}$
Tm	:	Melting temperature
VNT	:	Virus neutralization test
VP	:	Viral protein
w/v	:	weight/Volume

DECLARATION

I, **TANGUTURI SWATHI (I.D. No. RVM/2019 - 19)** hereby declare that the thesis entitled **"CROSS PROTECTION STUDIES OF BLUETONGUE VIRUS - 23"** submitted to P. V. Narsimha Rao Telangana Veterinary University for the degree of **MASTER OF VETERINARY SCIENCE** is a result of original research work done by me. It is further declared that the thesis or any part thereof has not been submitted for any otherdegree or diploma.

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ABSTRACT

Bluetongue (BT) is an infectious, non-contagious, haemorrhagic, *Culicoides* transmitted viral disease of domestic and wild ruminants caused by the bluetongue virus (BTV). Sheep are the most susceptible species where severe clinical disease is seen.

Currently, 35 different BTV serotypes have been reported to be circulating in the world. In India, 24 serotypes have been reported based on the presence of neutralizing antibodies and virus isolation. Bluetongue is controlled mainly by vaccination. In India, a pentavalent inactivated vaccine containing BTV-1, 2, 10, 16 and 23 serotypes is currently in use. In recent times, the prevalence of BTV-23 was found to be very less in the outbreaks. Therefore the current study aimed at cross-neutralization studies of BTV-23 for possible replacement in the vaccine. Bluetongue Virus-23 was plaque purified and hyperimmune serum (HIS) was raised in Deccani sheep. This serum was tested for cross-neutralization against BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 and 24 serotypes by serum neutralization test (SNT).

BHK adapted BTV-23 was grown in Vero cell line and plaque purified using agarose overlying method. Gel electrophoresis of extracted RNA from plaque purified virus revealed 10 segmented genomes. The molecular confirmation of plaque purified virus was done by RT-PCR using available BTV primers. The PCR product on gel electrophoresis showed amplification with BTV-23 primers and the size of the amplicon was 1370bp, no amplification was seen with other primes.

Plaque purified virus was inoculated into two BTV seronegative Deccani sheep with a dose of 2ml of $10^{4.11}$/100µL $TCID_{50}$ and the control animal was injected with plain media. The inoculated sheep developed mild clinical signs such as pyrexia on 6 dpi i.e., 104.2^{o}F and 104.3^{o}F, pyrexia maintained for 4 days. Animals developed nasal discharge on 10dpi. A booster injection is given on the 15th day with the same dose and no pyrexia was observed in animals. The control animal exhibited no clinical signs.

The inoculated animal developed BTV antibodies between 7th-15th dpi detected by SNT and remained seropositive throughout the study. Antibody titre detected from the serum collected on 28th dpi was 1/320 in both inoculated animals. In cross-neutralization studies, BTV-23 hyperimmune serum didn't cross neutralize with any available BTV serotypes (1, 2, 4, 5, 9, 10, 12, 16, 21 and 24).

Plaque purified BTV serotype was confirmed as BTV-23 by molecular confirmation, and the hyperimmune serum raised will serve as a major tool in future neutralization assays for BTV serotype confirmation and cross-neutralization studies.

INTRODUCTION

CHAPTER-I

INTRODUCTION

Bluetongue (BT) is an economically important, non-contagious arthropod transmitted viral disease of domestic and wild ruminants. The causative agent, bluetongue virus (BTV) is transmitted by culicoides biting midges (Maclachlan and Mayo, 2013 and Koenraadt *et al.*, 2014). Bluetongue virus is the type species of genus *Orbivirus*, family Reoviridae and subfamily Sedoreovirinae (Mertens *et al*., 1989). Sheep are the most susceptible hosts for BT, whereas cattle, buffalo and goats serve as reservoirs for disease transmission (Mellor and Wittmann, 2002 and Coetzee *et al.,* 2014).

Bluetongue virus infection causes direct and indirect economic losses. Direct economic loses includes high morbidity, mortality, stillbirths, abortions, foetal abnormalities, reduced milk yield and fertility rate, weight loss, fleece losses, etc. Indirect losses are due to restriction on animal trade movement and their products, disease control such as vaccination, diagnosis, vector control and treatment of affected animals (Rushton and Lyons, 2015; Grewar, 2016 and Gethmann *et al.*, 2020).

Bluetongue virus was first described in South Africa in 1876 when European sheep intensive farming was introduced in the region (Maclachlan *et al.,* 2015). In 1924, first BTV outbreaks were observed among sheep in Cyprus. Subsequently, after 1950 the disease has been reported in Asia, Europe, Australia, and North America. The disease is present in all continents except Antartica (Mellor *et al.,* 2009). In India first BTV was reported among sheep and goats in Maharashtra (Sapre, 1964). It has been recognized as an emerging transcontinental disease (Alkhamis *et al.,* 2020).

Bluetongue virus distribution depends on vector presence, and it is endemic in geographical regions located in latitude of approximately 40^{o}-50^{o} North and 35^{o} South (Balwan *et al.,* 2021). Bluetongue outbreaks are highly seasonal, occuring during the late summer and autumn. Bluetongue virus outbreaks occur throughout tropical, subtropical and temperate regions of the world where competent vector population exists. India is endemic for BTV, frequent outbreaks noticed in southern states of India (Rao *et al.,* 2016a).

The genome is composed of ten linear segments (seg-1 to seg-10) of double stranded RNA (ds-RNA) packed within a triple layered icosahedral protein capsid (Grimes *et al.,* 1998). It encodes seven structural proteins (VP1 to VP7) and five non-structural proteins (NS1-NS5) (Ratinier *et al.,* 2011 and Stewart *et al.,* 2015). The VP2 is the most exposed protein on the mature virion and is responsible for receptor binding, type-specific neutralizing antibodies response and Haemagglutinating (Huismans and Erasmus, 1981).

World organization for animal health (OIE) listed BT as a multispecies disease (OIE, 2021). Currently, 35 BTV serotypes were recognized worldwide (Ries *et al*., 2021 and Breard *et al.*, 2021). India is enzootic for BT and 24 BTV serotypes have been reported from India based on neutralizing antibodies and virus isolation (Thota *et al.,* 2021). Currently, in India, a pentavalent inactivated vaccine having BTV serotypes BTV-1, 2, 10, 16, and 23 is available (Reddy *et al.,* 2010 and Naresh *et al.,* 2020). Infection with multiple serotypes is common in endemic areas. During 2017-2019 prevalence of BTV-1, 2, 4, 5, 12, 16 and 24 was high (Thota *et al.*, 2021 and Naresh *et al.*, 2020). The prevalence of different serotypes varies from time to time and season to season. BTV-23 was rarely detected in the recent tested time.

The purpose of this study is to determine, possible cross neutralization and cross protection of BTV-23 serotype with other available circulating BTV serotypes for possible replacement of serotypes in the vaccine according to the prevalence.

The current study was designed with the following objectives.

1. Adaptation of BTV-23 to Vero cell lines.
2. Titration of virus and plaque purification of BTV- 23.
3. Molecular confirmation of BTV -23 with serotype specific qRT-PCR targeting seg-2.
4. Raising of hyper immune serum in sheep.
5. Cross neutralization studies of BTV- 23 with other BTV serotypes.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

2.1 BLUETONGUE

Bluetongue (BT) is an economically important infectious, non-contagious, arthropod transmitted viral disease of domestic and wild ruminants. It is caused by the Bluetongue virus (BTV) which is the type species of the genus *Orbivirus*, within the family Reoviridae, order Reovirales (Mertens *et al.*, 2004). It is mainly transmitted by biting midges of the genus *Culicoides*. Sheep are the most susceptible host, whereas cattle and goats are reservoir hosts.

2.2 EPIDEMIOLOGY OF BLUETONGUE

2.2.1 History

The disease was first reported in the late 18th century as ‘tong-sikte' by French biologist Francois de Vaillant in the Cape of Good hope between 1781 and 1784 (Gutsche, 1979). Later Dr. Hutcheon recorded some clinical features of the disease in 1880 and 1902 and mentioned as 'malarial catarrhal fever’ in sheep in the veterinary record (Hutcheon, 1902). Hutcheon (1902) and Spreull (1905) were the first to give detailed disease descriptions of BT. The name bluetongue was given by Spruell (1905) which is a translation of the word ‘Blaauwtong’, which was used by Afrikaans farmers, named after observing the cyanosis of tongue in affected sheep. BT is also called 'Bekziekte’, which means mouth sickness. Sir Arnold Theiler in 1906 mentioned the causative agent of bluetongue as a filterable virus (Henning, 1956). BT is listed as a multi-species disease by the Office international des epizootics (OIE, 2021).

2.2.2 Distribution

2.2.2.1 World: After the first report of BT in Africa, BT was thought to be confined to Africa, but the first outbreak of BT outside the African continent was reported in sheep from Cyprus (East Mediterranean) in 1943 (Gambles, 1949). Subsequently, BT was reported all over the world, Israel in 1949 (Komarov and Goldsmit, 1951), California in 1952 (Alexander, 1959), Pakistan in 1958 (Sarwar, 1962), India in 1963 (Sapre, 1964), Australia in 1977 (George *et al*., 1978) and China in 1979 (Zhang *et al*., 1996).

The global distribution of the Bluetongue virus (BTV) lies approximately between latitude 40^{o}-50^{o}N and 35^{o}S (Walton, 2004; Purse *et al.,* 2005 and MacLachlan and Osburn, 2006). BT has been reported in many regions of the world such as Africa, the Americas, the Middle East, the Indian subcontinent, China, South East Asia, northern Australia, and Europe except Antarctica (Mellor *et al*., 2009).

Two geographic regions of BTV have been described based on phylogenetic studies and they divide the strains into western and eastern "topotypes". Western topotype includes America, Africa, Europe, and the Middle East. Eastern topotype includes China, South Asia, South East Asia, and Australia. At present 35 BTV serotypes had been reported worldwide (Breard *et al.,* 2021 and Ries *et al.,* 2021).

2.2.2.2 India: In India, BT was first reported in Maharashtra in 1963, the disease was reported in exotic sheep, namely Southdown, Rambouillet, Russian Merino, and Corriedale (Sapre, 1964). Subsequently, severe BT was reported in the Dorset breed in Andhra Pradesh in 1974, but the disease was not reported in native sheep. Later on, diseases were reported in native sheep and outbreaks have been reported annually since 1981.

Bluetongue disease was reported in different states which include Himachal Pradesh (Uppal and Vasudevan, 1980), Haryana (Vasudevan, 1982), Karanataka (Srinivas *et al.,* 1982), Maharashtra (Singh *et al.,* 1982), Rajasthan (Lonkar *et al.,* 1983), Andhra Pradesh (Babu *et al.,* 1988) and Tamil Nadu (Janakiraman *et al.,* 1991).

Bluetongue disease is endemic in India. A total of 24 serotypes have been recognized, based on serology and/or virus isolation of which 15 serotypes *i.e.,* BTV-1, 2, 3, 4, 5, 6, 9, 10, 12, 16, 17, 18, 21, 23 and 24 have been isolated by researchers involved in the All India Network Program on Bluetongue (AINP-BT, 2012) and other research laboratories (Chand *et al.,* 2015; Ranjan *et al.,* 2015; Krishnajyothi *et al.,* 2016 and Reddy *et al.,* 2016). A higher incidence of BT was noticed in the southern region of India compared to the northern region. The high sheep population density, favourable climate conditions for vectors, and susceptible sheep breeds contributed to the high incidence (Rao *et al.,* 2016b).

2.2.3 Host

Bluetongue is primarily a disease of sheep (Erasmus, 1975), but it also affects other vertebrate host which includes both domestic and wild ruminants, such as goat, cattle, buffalo, white-tailed deer, antelope, sambar and camelid species such as camels and llamas (MacLachlan, 1994 and Patton *et al*., 1994). Cattle, buffalo and goats are sub-clinically affected and act as amplifying hosts, due to the presence of prolonged viremia and absence of clinical signs (Tweddle and Mellor, 2002). But a higher incidence of clinical disease has been observed in cattle and goats infected with BTV-8 in Europe (Dercksen *et al*., 2007).

Although domestic livestock is known hosts, wild ruminants may also serve as natural reservoir hosts of BTV (Wilson and Mellor, 2009) and antibodies have been detected in wild carnivores in Africa (Coetzee *et al.,* 2012). In wild ruminants, severe clinical disease was noticed in white-tailed deer, pronghorn and desert bighorn sheep (Rao *et al.,* 2016b and Saminathan *et al.,* 2020).

2.2.4 Transmission

The disease is mainly transmitted among susceptible hosts through the bite of hematophagous midges of the genus *Culicoides*, belonging to the order Diptera and the family Ceratopogonidae.

Du Toit (1944) experimentally infected sheep with homogenates of either midge fed on infected animals or wild-caught *Culicoides* midges. Consequently, the sheep developed BT, which confirmed that *Culicoides* was a vector of BT.

Among 1400 species of *Culicoides* were reported, 30 species were identified to transmit BTV (Ander *et al.,* 2012 and Maheshwari, 2012). In India, 60 species of *Culicoides* were reported (Maheshwari, 2012). Once *Culicoides* vectors get infected by feeding on the BTV-infected hosts they remain persistently infected for their entire life span (Belbis *et al*., 2017).

2.2.5 Other Routes of Transmission

2.2.5.1 Transplacental Transmission: The passage of BTV across the placenta is another mode of transmission. Backx *et al.* (2009) demonstrated the transplacental transmission of wild-type BTV-8 in cattle under experimental conditions. The highest rate of vertical transmission of BTV-8 in sheep was found at midterm gestation (Van der Sluijs *et al.,* 2011).

In 2013, Rasmussen and co-workers first time demonstrated transplacental transmission of BTV-2 in experimentally infected sheep. BTV-1 induced transplacental transmission with a higher incidence compared to BTV-8 (Van der Sluijs *et al*., 2013a).

2.2.5.2 Veneral Transmission: Transmission of BTV has been reported among ruminant hosts through infected semen and embryos. Bull semen can also transfer the virus, but it can be infected only when the bull is viraemic (Howard *et al.,* 1985 and Ward, 1994) and when semen contains red or white blood cells with which viruses are associated.

The studies on the re-emergence of BTV-8 in France in 2015 suggest that outbreaks occurred from contaminated frozen semen with BTV-8 of the previous outbreak (Pascal *et al.,* 2020 and De Clercq *et al.,* 2021).

2.2.5.3 Contact and Oral Transmission: Menzies *et al.* (2008) reported direct contact transmission of BTV-8 in cattle by the ingestion of BTV contaminated placenta. Oral transmission of BTV was reported in calves fed with infected colostrum (Backx *et al.,* 2009).

Horizontal transmission had been reported with BTV-8 and BTV-1 in experimental sheep which are maintained in a vector-free environment (Van der Sluijs *et al.,* 2011 and Van der Sluijs *et al.,* 2013b).

The direct transmission seen in some BTV serotypes and was experimentally confirmed are BTV-26 (Batten *et al.,* 2014), BTV-27 (Schulz *et al.,* 2016) and BTV-28 (Bumbarov *et al.,* 2020).

2.2.5.4 Mechanical Transmission: Some arthropods like sheep ked *Melophagus ovinus*, (Luedke *et al.,* 1965) and soft ticks *Ornithodoros coriaceus* (Stott *et al.,* 1985) can act as mechanical vectors for BTV.

Bouwknegt *et al*. (2010) demonstrated the transmission of BTV-8 in ticks by transstadial passage in hard ticks and transovarial passage in soft ticks and therefore act as a source of the potential vector.

Bluetongue can be transmitted mechanically (iatrogenic) by contaminated surgical equipment and needles containing infected blood (Sperlova and Zendulkova, 2011 and Darpel *et al.,* 2016).

2.2.5 Clinical Signs

Clinical signs of disease in sheep may vary in severity, influenced by the type or strain of the virus infecting and also by the breed affected (Verwoerd and Erasmus, 2004). In sheep, the disease is manifested as an acute, chronic, or sub-clinical condition.

The incubation period of BT in natural infection is about 7 days and artificial infection ranges between 2 to 15days. Clinical signs are characterized by pyrexia (41^{o}-42^{o}C), nasal secretions, oedema of lips, tongue, face and lymph nodes, haemorrhages of the mouth and tongue, ulcers of the oral cavity (such as dental pad) and inflammation of the coronary bands combined with lameness or difficulty in walking. In severe cases, cyanosis of the tongue can also occur, giving the disease its name (Verwoerd and Erasmus, 2004; Dal Pazzo *et al*., 2009 and Maclachlan *et al*., 2009).

In chronic conditions animals show weakness, prostration and torticollis as well as wool break. Sometimes animals may develop a secondary bacterial infection such as bacterial pneumonia (MacLachlan and Gard, 2009).

In pregnant ewes, an infection may cause abortions, fetal mummification and congenital defects seen are hydrocephalus, cerebral cysts, retinal dysplasia, etc. (Saegerman *et al*., 2011).

In goats decrease in milk production, oedema of the lips and head, nasal discharge and erythema of the skin and udder were observed (Sperlova and Zendulkova, 2011).

In cattle, clinical signs observed are ocular discharge, conjunctivitis, congested oral mucosa, ulcers and necrotic lesions development on the lips and tongue and later become oedematous (Sperlova and Zendulkova, 2011).

2.2.6 Bluetongue Virus

Bluetongue is a segmented double-stranded RNA virus of the genus *Orbivirus*, family Reoviridae, subfamily Sedoreoviridae (Van Dijk and Huismans, 1988 and Roy *et al.,* 1990).

The virus is non-enveloped with icosahedral symmetry which measures about 80-90nm in diameter. The complete genome of BTV is approximately~ 19.2 Kb in length (Pringle, 1999). The viral genome consists of 10 segmented linear double-stranded RNA (Mertens and Diprose, 2004). The size of genome segments ranges from 3954 to 822 bp in the order of decreasing molecular size on agarose gel from Seg-1 to Seg-10 (Bhanuprakash *et al.,* 2009). The genome segment encodes 7 structural (VP1–VP7) and 5 nonstructural proteins (NS1, NS2, NS3/NS3A, NS4 and NS5) (Ratinier *et al.,* 2011; Patel and Roy, 2014 and Stewart *et al.,* 2015).

The mature BTV particles comprise three concentric capsid layers. The outer capsid protein layer (VP2 and VP5) has 60 trimers of VP2 (encoded by seg-2) which forms a triskeleton structure that is interspersed between 120 trimers of VP5 molecule (encoded by seg-6). The VP2 protein is a major determinant of the BTV serotype and is responsible for the stimulation of serotype-specific neutralizing antibodies and hemagglutination (Ranjan *et al*., 2015).

The innermost 'sub-core' layer is composed of VP3 protein and transcriptional complexes such as VP1, VP4 and VP6 are involved in viral replication and transcription. The outer surface of the subcore is composed of VP7. It provides strength and rigidity. The VP7 is a major group-specific antigen determinant and it is commonly used in a c-ELISA assay for the detection of anti-BTV antibodies (Rao *et al*., 2017).

The non-structural (NS) protein is seen in infected host cells. NS1 protein forms tubules for the translocation of progeny virus particles to the cell membrane of the infected host cell. The ssRNA-binding NS2 protein has nucleotidyl phosphatase activity and forms an inclusion body in the cell cytoplasm (Roy, 2017). NS3 and NS3a are produced in insect cells for the release of virion particles from infected cells. NS4 is involved in virus-host interaction and antiviral immune response (Saminathan *et al*., 2020).

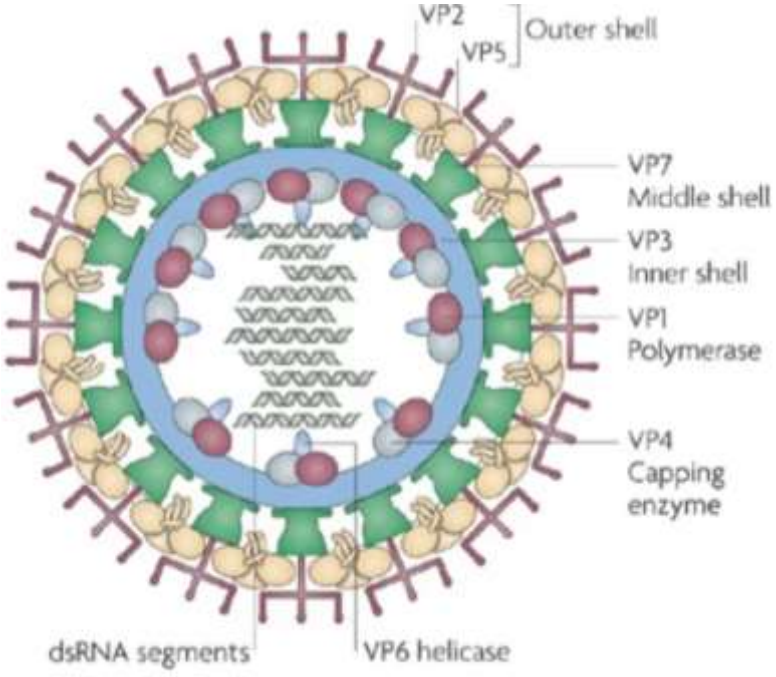


Fig 2.1: Bluetongue Virus structure

Schematic diagram of BTV representing structural proteins and ds-RNA segments (Roy *et al.*, 2009).

Table 2.1 Bluetongue Virus genome segments, the encoded proteins and their characteristics and functions

Genome segment	Segment size (bp)	Proteins encoded	Characteristics/functions
Seg-1	3954	VP1	RNA dependent RNA polymerase
Seg-2	2926	VP2	Cell attachment, entry Haemagglutination Virus neutralization antigen determines serotype
Seg-3	2770	VP3	T=2 icosahedral symmetry Scaffold for addition to the core surface layer, Outer coat proteins, Localizes transcriptase complex on an internal surface of the capsid, at 5-fold axes
Seg-4	1981	VP4	Capping enzyme-nucleoside phosphohydrolase, guanylyltransferase, transmethylase
Seg-5	1769	NS1	Abundant in the infected cell cytoplasm Forms tubules, with unknown function Aids virus released from insect cells Viral protein translation enhancer Co-localizes with the centrosomes- may play a role in disruption and blocking of cell division in mammalian cells.
Seg-6	1638	VP5	Has coiled-coil domain – induces membrane

Table 2.1 (cont.).

			permeabilization during initiation of infection and causes syncytia formation Affects the specificity of virus neutralization
Seg-7	1156	VP7	Core structural protein T=13 icosahedral symmetry Receptor binding for culicoides cells Group / virus-species-specific antigen
Seg-8	1124	NS2	Forms inclusion bodies Aids in early morphogenesis Binds mRNA Phosphorylated by protein kinase Blocks spindle formation and cell division
Seg-9	1046	VP6	Helicase, binds ss/ds RNA, ATPase
		NS4	Imparts viral fitness to IFN response
Seg-10	822	NS3	Glycoprotein Aids virus release Localizes to cell membrane late in infection-interacts with lipid droplets in infected cell
		NS3A	The glycoprotein binds cellular exocytotic components Viroporin - aids virus release from insect cells
		NS5	Localizes to nucleus

(Rao *et al.*, 2017)

2.3 PROPAGATION AND ISOLATION OF BLUETONGUE VIRUS

Haig *et al.* (1956) observed the cytopathogenic effect of the bluetongue virus in primary lamb kidney cells. Five different strains of bluetongue virus were employed in the study Cyprus strain, Estantia strain, Ermelo strain, Vlak strain and California II strain obtained from sheep. All virus strains were adapted to propagate in embryonated eggs. 0.2 mL of 10^{-2} suspension virus was inoculated in primary lamb kidney cell culture. Cytopathogenic changes were noticed from the third day of incubation in cells such as swollen, granular, shrinkage and later cells detached.

Mc Phee *et al.* (1982) assessed the replication of Australian BTV in eight continuous cell lines (BHK-21, Vero, LLC-MK2, CV-1P, CSL-503, L929, SVP, and *Aedes albopictus* cells) and 11-day old embryonated chicken egg (ECE) at different multiplicities of infection. Maximum extracellular virus yields were attained from BHK-21 cells at both high and low multiplicity of infection than other cell lines tested. No cytopathogenic effect (CPE) was seen in *Aedes albopictus* cells.

Blanchard-Channell and Stott (1989) propagated BTV serotype 17 in Vero cells in two different media, MEM and RPMI1640 supplemented with serum extenders to determine a particular medium formulation capable of supporting better virological assays as well as cell growth. Cells grown in RPMI1640 showed better growth and a high virus titer was obtained than MEM media used.

Chung *et al.* (1991) inoculated the blood sample of a ram to 11-day-old embryonated chicken eggs by intravenous route. All the egg embryos which were injected with blood samples died within 7 days after inoculation. The embryos were homogenized and inoculated onto Vero cells and the cytopathic effect was observed. The serotype was identified as serotype 11 by serum neutralization tests.

Roy and Mehrotra (1999) adopted BTV isolates to BHK-21 cells. Evidence of cellular destruction was observed from 24 hours post-infection. The H&E stain of the infected BHK-21 monolayers by 48 hours of post-infection revealed intracytoplasmic eosinophilic, perinuclear inclusion bodies, vacuolation of cytoplasm and giant cell formation.

Clavijo *et al.* (2000) collected blood samples and tissue samples from the animals and inoculated them into 10 days old embryonated chicken egg (ECE) by the intravenous route (IV). The embryo died between day 3 to day 6 after inoculation and the infected embryo had a characteristic cherry-red discoloration due to hemorrhages

and oedema. Tissue is homogenized and used as inoculum for BHK-21 cells infection. After infection BHK-21 cells produced a distinct cytopathic effect (CPE) at 3–5 days post-infection. The CPE observed was the appearance of foci of rounded and refractile cells, foci rapidly enlarge and rupture, whereupon they detach and float freely in the culture media.

Billinis *et al.* (2001) reported that the duplex RT-PCR method is specific and more sensitive for BTV detection. Twelve day old embryonated chicken eggs were inoculated with 0.1 mL washed, packed and ultra-sonic treated blood cells by intravenous route. Bluetongue virus positive samples caused haemorrhages in the embryo and resulted in embryo death. BHK-21 cell monolayers infected with 1 mL of a homogenized mixture of dead embryos. Positive samples showed a cytopathic effect. Virus identification was carried out by indirect immunofluorescence. Among 36 blood samples were collected and examined, 17 samples showed were detected positive for BTV by RT-PCR, whereas only 13 were detected by virus isolation in embryonated chicken eggs and 9 samples by cell culture assays.

Sekar *et al.* (2009) compared the susceptibility of BHK-21 and Vero cell lines to BTV infection. Both cell lines were maintained in MEM supplemented with 10 % FBS. BTV infected blood samples collected during the high febrile period, were processed and infected both monolayers. CPE characterized by swollen, rounding of cells, syncytia formation, giant cell formation, and finally detachment from the attached surface was observed from 36 hours post-infection in both cell types. But high virus titre was obtained in the Vero cell line ($10^{10.3}$- $10^{10.8}$) than in BHK-21 ($10^{8.1}$- $10^{8.8}$). They concluded that maximum infectivity titre was observed in the Vero cell line than BHK-21 cell line.

Maan *et al.* (2015) propagated BTV in BHK-21 clone -13 cells in Dulbecco's minimum essential medium (DMEM) at 37^{o}C supplemented with antibiotics and glutamine. After infection, cell cultures were incubated at 37^{o}C which produced cytopathic effect (CPE) at 48-72 hours post-infection. The cell culture fluid was harvested 3-4 days post-infection and used for viral genome extraction.

Lean *et al*. (2019) evaluated the pathogenesis of BTV *in-ovo* with a pathogenic isolate of BTV-3, a blood sample collected from an infected sheep. Compared the virus propagation and isolation by two methods: 1) ECE (one passage), followed by *Aedes* cells (C6/36) (one passage) and then BHK-BSR cells (three passages), (2) KC cells (one passage), followed by BHK-BSR cells (three passages). Vero cells infected with KC passaged virus produced CPE in 12 h of post-infection (HPI) and ECE passaged virus didn't produce CPE until 24 HPI. They observed more infectious virus obtained KC passaged virus than ECE passaged virus.

2.4 PLAQUE PURIFICATION

Howell *et al.* (1967) plaque purified tissue culture adapted and egg adapted strains of BTV in mouse fibroblast cells. They followed the agarose overlaying method, cells were maintained in a modified eagle's medium with 10% FBS. Plaques are formed by tissue culture and egg-adapted strains but they differ morphologically in size, shape and perimeter. Plaques formed earlier in Egg adapted strains than in tissue culture-adapted strain. Plaques were observed from the 3rd day onwards. They also observed that the size and number of plaques developed depend on the agarose concentration and depth. They demonstrated one virus particle produces each plaque.

Blanchard-channell and Stott (1989) demonstrated plaque assay for BTV serotype 17 prototypes (62-45S) in Vero cells using two different media, MEM and RPMI 1640 supplemented with different varieties and combinations of serum extenders to study the effect of medium and serum variations on producing plaques morphology. During their study observed both basal medium and serum supplements were important for the maintenance of cell quality under agarose overlay. MEM showed better results than RPMI 1640. Cells supplemented with 3.5% FBS/SerXtend developed larger plaques while 10% FBS usage result in small plaques. Vero cells maintained in RPMI 1640 with either 3.5% or 5% FBS/SerXtend showed well visible plaques and poorly visible plaques seen in cells maintained in MEM with 10% FBS or 5% FBS/SerXtend.

Pullinger *et al.* (2016) plaque purified BTV-1 and BTV-26 isolates which were obtained from infected sheep blood. Plaque purification was done in BSR cells maintained in DMEM with 5% FCS. Viral dilution prepared in DMEM media with 5% FCS, 0.5 mL of diluted virus added to confluent monolayers of BSR cells in 12 well plates. After 90 min at 37°C with 5% CO_2, the diluted virus was removed and replaced with 3 mL of MEM containing 3% FCS and 1.5% melted agarose overlayed. Plates were incubated at 37°C with 5% CO_2 until plaques were visible. These were picked into DMEM media with 5% FCS, before passage on BSR cells. The plaque purified viruses were used to make a series of reassortant strains containing individual genome segments from BTV-26, which were generated within a BTV-1 genetic 'backbone'.

Srikanth Reddy (2018) carried out a plaque purification assay of BTV serotype 4 isolates in the Vero cell line. Plaque purification was carried out three times using the agarose overlaying method in 6 well plates, where the stock virus diluted from

10^{-2} to 10^{-6}. Plaques were observed from the 3rd day of infection and became prominent on the 4thday of infection, plaque size is approximately 1mm. Plaques were picked and grown in a T25 flask and the cell culture fluid was used for RNA isolation and confirmed through RT-PCR where only the BTV-4 serotype is seen and no other serotype was detected. The hyperimmune serum is raised in sheep against the plaque purified virus BTV-4.

Srinivas *et al.* (2018) plaque purified of BTV-12 isolates was done. Bluetongue Virus-12 isolates adapted to the Vero cell line had a titre of virus $10^{6.116}$/mL. The stock virus was diluted from 10^{-2} to 10^{-6} and plaque purification was done three times in 6 well plates by using the agarose overlaying method. Plaques were picked and subsequently grown in a T25 flask and Plaque purification was confirmed by RT-PCR amplified only a product of 750 bp with BTV-12 specific primers. And it was also verified with another primer specific for BTV-1, 2, 4, 9, 10, 16, 21, 23 and 24, but no amplification was seen with these primers. The kinetics of infection and cytopathic effect of BTV-12 was studied in the Vero cell line by using H&E staining of the infected Vero cell layer. The infected cell was observed at 24, 36, 48 and 72 h of post-infection.

Rahman *et al*. (2020) reported the presence of a calcium-binding region in non-structural protein 2 (NS2), calcium-sensing triggers viral inclusion bodies (VIB) assembly and helps in virus replication and assembly. Mutations were introduced in the BTV genome by site-directed mutagenesis, targeting three sites (A1, A2, A3) of the Ca^{+2} binding region in NS2 protein and recombinant NS2 protein produced. Plaque assay was performed for mutant BTV and wild-type BTV in BSR cells (BHK-21 subclone) and BS8 cells. Cells were cultured in DMEM, supplemented with 5% FBS at 35^{o}C in 5% CO_2. The monolayer was overlaid with 1% agarose and incubated

for 3 days at 35^{o}C in 5% $CO_{2.}$ They observed A1 mutant failure to recover infectious virus and no plaque formation and plaques formed with wild-type BTV. They explained the importance of Ca^{+2} for the production of infectious viruses.

2.5 TYPING OF BTV ISOLATES

For effective control of bluetongue disease, an early diagnosis and effective prophylactic measures like vaccination are required. Early diagnosis of BTV and its characterization up to the serotype is essential which can be fulfilled by various typing methods like serological typing by serum neutralization test, plaque neutralization test, fluorescence inhibition test (Blacksell *et al.,* 1993) and molecular methods using PCR technique (Mertens *et al.,* 2007).

2.5.1 Molecular Typing

Subhadra *et al.* (2014) evaluated a SYBR green-based qRT-PCR assay to detect BTV. They designed primers based on the NS3 gene and melt curve analysis indicated the absence of non-specific amplification (R2 = 0.987). In this assay, they compared the sensitivity of conventional PCR and qRT-PCR. Examined 32 blood samples and 24 were tested positive for BTV RNA. They observed that few samples which are shown negative through conventional PCR, were tested positive by the real-time PCR. They concluded real-time PCR assay was specific, sensitive and rapid in the detection of BTV in blood samples.

Feng *et al.* (2015) developed a BTV serotype-specific qRT-PCR assay with TaqMan MGB probes based on the VP2 gene. This assay proved a rapid, sensitive, and reliable method for the identification, differentiation and quantification of 22 BTV serotypes.

Bumbarov *et al.* (2016) detected BTV contaminations in commercial vaccines of Lumpy skin disease (LSD) and Sheep pox (SP) using qPCR for VP1 and NS3 genes. The Sheep pox vaccine-derived sample further propagated in ECE and Vero cells. Sequence analysis of the LSD vaccine showed 98-99% similar to BTV-9 and analysis of the six genomic segments from the SP vaccine-derived isolate showed 66.3-97.8% similar to BTV-26.

Maan *et al*. (2016) developed a quantitative real-time type-specific RT-PCR assay for the detection and typing of BTV based on the TaqMan fluorescence probe. The real-time RT-PCR assay targeting Seg-2 (VP2) is considered more sensitive than the conventional RT-PCR assay for typing BTV RNA**.**

Krishnajyothi *et al.* (2016) applied an RT-PCR assay for confirmation of BT during the 2010 outbreak in the Telangana State of India from infected sheep blood. The BTV was confirmed by Seg-5 and Seg-7 based on conventional RT-PCR. Serotype-specific RT-PCR targeting Seg-2 was carried out initially with primers of common circulating serotypes in India, followed by primers of 26 worldwide serotypes of BTV. They found samples were amplified with BTV-24 primers only and subsequent sequencing analysis proved BTV-24 in the sample.

Lakshmi *et al.* (2018) standardized qPCR and determined the limit of detection (LoD) for virus-spiked culture medium and blood as 10^3 $TCID_{50}$/mL and 10^4 $TCID_{50}$/mL with RT-PCR and 10^0 $TCID_{50}$/mL and 10^2 $TCID_{50}$/mL with real-time PCR, respectively. The study suggests that real-time PCR stands out as a very sensitive and reliable assay for the detection of BTV present in different types of samples.

Typing of BTV isolates obtained from field outbreaks of BT during 2016-18 from the states of Telangana and Andhra Pradesh was done by using cell culture and molecular diagnostic techniques (Sharanya, 2018). Molecular confirmation of group-specific BTV done by RT-PCR targeting primers specific for NS3 and subsequent molecular typing of positive sample done with real-time PCR using primers specific to VP2 gene of 29 known serotypes.

Thota *et al.* (2021) reported VP2-based molecular detection of BTV from the blood samples collected in clinically infected sheep. They detected major circulating serotypes of BTV during 2014-2018 in Telangana and Andhra Pradesh. A total of 272 blood samples were collected and screened for the BTV serotypes through conventional RT-PCR and the serotype circulating are eastern topotypes (BTV-1, BTV-2, BTV-4 and BTV-16) and Western topotypes (BTV-16 and BTV-24).

Saminathan *et al.* (2020) studied the transplacental transmission of BTV serotype-1 in IFNAR1 blocked mice during early and mid-gestation. Viral loads from blood and tissue sample were quantified by real-time PCR. BTV RNA was detected in blood, uterus and placenta. The highest copy number was detected on early-gestation and mid-gestation.

2.5.2 Serological Typing

Serum neutralization test (SNT), considered as gold standard ‘for serotyping’ as used to evaluate serotype-specific neutralizing antibodies. The highest serum dilution showing complete inhibition of CPE in monolayers was taken as neutralizing antibody titre.

Sreenivasulu and Subbarao (1999) conducted a serological survey in Andhra Pradesh. A total of 976 sera were collected from different species of healthy animals and screened by c-ELISA. The study revealed the presence of BTV antibodies in 45.71% of sheep, 43.56% of goats, 33.11% of cattle and 20% of buffaloes. Region-wise seroprevalence was reported as 46.75% in Rayalaseema, 46.05% in Telangana and 33.54% in coastal regions of Andhra Pradesh.

Sairaju *et al.* (2013) conducted a study to determine the seroprevalence of BTV in Andhra Pradesh during 2005-2009. Serum samples were collected from unvaccinated sheep between the ages of 0.5-3 years. Group-specific detection of BT done by c-ELISA, 52 sera samples were positive for BTV subjected to type-specific neutralization with available serotypes- 1, 2, 9, 10, 21 and 23. A total of 52 samples tested 50%, 44.23%, 21.15%, 26.92%, 0% and 15.38% were neutralized by BTV serotypes 1, 2, 9, 10, 21 and 23, respectively and 36.92% of sera samples didn't neutralize with any available serotypes, suggesting the prevalence of other serotypes.

De Campos Nogueira *et al.* (2016) studied seroprevalence of BTV serotype 4 in cows older than 1 year age in Brazil. A total of 1716 serum samples were collected and a Virus neutralization test (VNT) was performed in Vero cell lines maintained in MEM. Serum samples were used in 1:10 to 1: 640 serial dilutions to detect antibodies against BTV-4. They found 86% of serum samples positive for BTV-4.

De Ankan *et al.* (2019) conducted a study to investigate the prevalence of BT and type-specific neutralizing antibodies in goats. Neutralizing bodies in the selected serum sample were detected by SNT against six BTV serotypes. BTV-1 was found to be more predominant (65.27%), BTV-16 (26.38%), BTV-10 (20.83%), BTV-9 and 23 (13.88%) and BTV-2 (6.94%).

Putty *et al.* (2020) carried out serological typing of BTV in reservoir hosts such as cattle and buffaloes of different age groups in Telangana and Andhra Pradesh where the disease is major prevalent. A total of 321 blood samples were collected and tested for group-specific seroprevalence by c-ELISA and type-specific seroprevalence by serum neutralization test (SNT). Few samples neutralized against BTV-1, 2, 4, 5, 9, 12, 16 and 24 serotypes, and some samples could not neutralize any of the tested BTV serotypes. Most of the sera neutralized more than one serotype.

Naresh *et al*. (2020) studied the seroprevalence of BTV in sheep (6 months to – 1-year-old) from six districts of Telangana district during 2018-2019. Group-specific serotyping was done by c-ELISA and type-specific seroprevalence was detected by neutralization assay. The study was conducted to monitor the circulating serotype of BTV. They found highest seroprevalence in Khammam district and seroprevalence of BTV- 1, 2, 4, 5 9, 10, 12, 16, 21, 23 and 24 were reported as 16.66%, 11.66%, 31.66%, 11.66%, 5%, 6.66%, 16.66%, 8.33%, 13.33%, 6.66% and 16.66%, respectively. Among them, the prevalence of BTV-4 was found to be predominant.

Studies were conducted to investigate neutralizing antibodies to different BTV serotypes in the goats. 36 samples out of 186 serum samples tested positive by indirect ELISA. Type-specific neutralization was done with available serotypes BTV-1, 2, 9, 10, 16 and 23. Among them, 15 samples of neutralizing antibodies were detected and the rest of the serum samples couldn't neutralize with the available serotypes. Neutralizing antibodies to BTV-10 were found in more numbers followed by BTV-1, 2, 23, 9 and 16 serotypes. They found most of the sera neutralized more than one BTV serotypes indicating superinfection by multiple BTV serotypes in goats Ain *et al*. (2020).

2.6 CROSS-NEUTRALIZATION STUDIES

Umeshappa *et al*. (2010) conducted a study on cell-mediated immune response and cross-protective efficacy of binary ethylenimine-inactivated (BEI) BTV-1 vaccine in sheep. Sheep between age group 1.5-2yrs screened for BT antibodies by c-ELISA and seronegative animals were selected. BTV-1 vaccine at a titre of 1.3×10^6 pfu/mL was given and the second dose was given on the 21st day. After 3 months of vaccination, an animal is challenged with the BTV-23 serotype and found protection against the virus.

Balam (2011) studied the antigenic relationships among BTV-2, 9 and 15 serotypes. Viruses purified by the PEG precipitation method and hyperimmune serum were raised in New Zealand white rabbits. The reciprocal cross-neutralization test was done between BTV- 2, 9 and 15 serotypes to determine the R% value which indicates antigenic relatedness. The R% value obtained was less than 10% indicates weak antigenic relationship that is 2.8, 3.53 and 2.8 for BTV-2 & 9, BTV-2 & 15 and BTV-9 & 15 respectively.

Zulu and Venter (2014) evaluated cross-protection of BTV serotype 4 with other serotypes in sheep (*in-vivo*) based on previously reported cross-neutralization of specific BTV serotype *in-vitro* studies. Bluetongue Virus serotype-4 at a titer of 4.2×10^6 log10 $TCID_{50}$/ mL of 2 mL injected in sheep. Serum was collected at different intervals 0th, 14th and 28th days. After the 28th-day animal is challenged intravenously with different serotypes, namely BTV-1, 4, 9, 10 and 11. Animal monitored for clinical signs and serological cross-neutralization was assessed by using a serum neutralization test (SNT). Cross - protection was estimated by observing

clinical signs and by SNT assay against the challenged type. Good protection was noticed in animals challenged with BTV-9, 10, 11 viruses, whereas 20% cross-protection was seen with BTV-1 virus and 80% of animals' clinical signs were observed.

Reddy *et al*. (2018) reported that in their study 1mL containing $10^{6.46}$ $TCID_{50}$ of plaque purified BTV-4 virus was inoculated to sheep via intradermal route and subcutaneous routes on the 0^{th}day and 15^{th}day. After 28 dpi serum samples were collected from sheep and then cross-neutralization was tested against all available plaque purified serotypes (BTV-1, 2, 9, 10, 12, 16, 21, 23 and 24). After cross-neutralization, they observed BTV-9 and BTV-10 serotypes were neutralized with BTV-4 serum and thus revealed heterotypic cross-neutralization, but didn't show any cross-neutralization with other available plaque purified serotypes.

Srinivas (2018) studied the cross-neutralization assay of BTV-12 in Vero cells. Plaque purified BTV-12 serotype and raised HIS in sheep against the virus. Serum samples were collected at different intervals and SNT was done with serum samples collected on the 28^{th} day. Cross neutralisation were done with all available BTV serotypes BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 and 24. They found HIS could neutralize BTV-1.

Kesavulu Naidu (2019) performed plaque purification of BTV- 24 serotype in Vero cells and purified virus injected into sheep to raise HIS. 1 ml of $10^{5.72}$/ mL $TCID_{50}$ inoculated into sheep in two different sites such as 0.5 mL subcutaneous route and 0.5 mL intradermal route. Serum samples were collected at different time intervals and detected for the presence of BTV antibodies by c-ELISA. At 28 Days P.I

antibody titre was found to be 320, SNT has performed with 28th Day serum with available serotypes such as BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 and 24. They reported that BTV-24 HIS couldn't neutralize with any other BTV serotype and found no cross-protection with other serotypes.

Martinelle *et al.* (2018) assessed the cross-protection induced by a BTV serotype 8 vaccine towards other BTV serotypes, namely BTV-1, 2, 4, 9 and 16 in calves (5.5 - 6 months age). For each tested serotype, two groups of Holstein's calves were used: one group vaccinated against BTV-8, and the second vaccine injection given after 33days interval before the challenge, the other group of an animal was non-vaccinated. Animals were injected with BTV serotype 2.5 to 4mL of inoculum to a titre of 10^6 $TCID_{50}$/animal. Clinical signs, rectal temperature monitored and serological relationship was assessed by SNT. In the cross-neutralization study, a partial serological cross-reactivity was seen between BTV-8 and BTV-4 and between BTV-1 and BTV-8.

Jyothi *et al*. (2020) developed a subunit vaccine for three different BTV proteins (VP2, VP5 & NS1) to provide broad-spectrum protection against all multiple serotypes. BALB/c was immunized with these proteins and developed antibodies. Cross-neutralization done against different BTV serotypes was determined by serum neutralization test. High neutralizing antibody titer developed with the recombinant protein (subunit vaccine) in mice. Cross-neutralization was observed with BTV-1, 2, 4, 9, 10, 16, 21 and 23. No neutralization antibodies were observed for BTV-5, 12 and 24.

2.7 ANIMAL EXPRIMENTATION STUDIES

Ghalib *et al.* (1985) studied virological, clinical and serological responses caused by BTV- 10, 11, 13 and 17 serotypes in 3-6 months old sheep. A 2 mL of purified BTV with a titre of 10^7 pfu/mL was injected into sheep via different routes, 1 mL intravenous, 0.5 mL each by intradermal and subcutaneous routes. After 2-3 days of post-inoculation (P.I) developed viremia and reached the peak by Day 7 P. I lasted viremia for 2-3 weeks. Animals showed mild clinical responses such as pyrexia, salivation, dyspnoea, and leukopenia, and no mouth lesions were observed. Antibodies started to appear from the 10^{th} of P.I and reached maximum by Day 28 P.I.

Chander *et al.* (1990) studied clinical and immune responses in crossbred sheep with reference and local strain. Each animal was injected with a 10 mL BTV-1 virus of 10^6 $TCID_{50}$/ mL titer via subcutaneous and intradermal routes. Animals infected with local strain developed mild clinical signs and animals infected with reference strain developed respiratory signs. All animals developed pyrexia (40^oC – 41.1^oC) between 7-13 dpi. Neutralizing antibodies developed in animals infected with local and reference strains. Serum neutralization (SNT) mean antibody titre was 1/60 and 1/65 on 21 dpi, but peak titre was calculated as 1/160 and 1/120 on 28 dpi infected with local and reference strains, respectively.

Umeshappa *et al.* (2011) compared the intradermal (ID) and intravenous (IV) inoculation of BTV-23 serotype in sheep. Animals were injected with 6 mL of viral inoculum containing a titer of $10^{5.5}$ $TCID_{50}$/mL. Compared to IV inoculation, ID inoculation resulted in relatively increased clinical signs and lesions, found more efficient in disseminating and spreading the virus to systemic organs. Intradermal inoculation resulted in a higher antibody titre and it reproduced many aspects of the natural route of infection.

Batten *et al.* (2012) studied BTV serotype-26 pathogenesis and infection kinetics in goats. Goats were inoculated subcutaneously with 1 mL of KUW2010/09 BTV-26 BHK2 at a titre of $10^{6.58}$ $TCID_{50}$/mL. The animals are daily monitored for temperature and clinical signs up to 14 days post-infection (dpi). They detected a high level of viral RNA and virus isolated from the blood of infected animals, but the animals did not develop clinical signs of BTV. Antibodies against BTV were detected between 7 and 11 dpi in all infected animals. They concluded that BTV-26 replicates to high levels in goats, causing no clinical disease, suggested goats may be the natural host for this virus.

Schulz *et al.* (2018) studied experimental infection of BTV-4 field strain from Bulgaria in sheep, goats, and cattle. Animals were inoculated with 2-4mL of 10^6 $TCID_{50}$/mL and observed clinical signs. Mild clinical signs developed in sheep and goats such as increased temperature (39.7-41.8^oC) and nasal discharge. In cattle, only an increase in temperature was noticed and no clinical signs were observed. Neutralizing antibodies developed 10 Day P.I, reached a peak at 28 Day P.I. and in cattle, viremia lasts longer.

Reddy *et al*. (2018) experimentally inoculated BTV-4 in Deccani sheep between the age group of 6-12 months. 1mL of $10^{6.46}$/mL $TCID_{50}$ virus was inoculated to sheep by two routes 0.5mL intradermal region and 0.5mL subcutaneous at shoulder region and on the 15th day, 2nd injection was given with the same dose. Sheep had developed mild pyrexia of 103.6^oF on 6 dpi, which persisted for 4 days along with nasal discharges, oedema of the buccal area and lips between 10-11 dpi. Both animals developed a humoral immune response on the 7th day of post-inoculation, which was observed to maintain till the end of the experiment (28 dpi).

Srinivas (2018) plaque purified BTV-12 and raised hyperimmune serum in Deccani sheep. One mL of $10^{6.116}$/mL virus was inoculated to 6-8 months old Deccani sheep by two routes i.e., 0.5 mL via intradermal and 0.5 mL via subcutaneous routes at the shoulder region. Animal exhibited mild clinical symptoms such as pyrexia on 6 dpi (103.8^{o}F-105.1^{o}F) which persisted for 4days and nasal discharges developed on 13dpi.

Kesavulu Naidu (2019) studied plaque purification and characterization of BTV-24 in sheep and raised hyperimmune serum against BTV serotype 24 in sheep. In this study, BTV-24 was adapted and plaque purified in the Vero cell line. After plaque purification, 1 mL of $10^{6.46}$ $TCID_{50}$ of BTV-24 Virus was inoculated to sheep via intradermal route and subcutaneous routes on 0th day and 15th day with the same dose. Sheep had developed pyrexia of 105.4^{o}F on 6 dpi, which persisted for 4 days along with nasal discharges developed on 12 dpi. Both animals developed a humoral immune response on the 7th day of post-inoculation, which was observed to maintain till the end of the experiment (28 dpi).

Bumbarov *et al.* (2020) carried out animal experimental studies of BTV serotype-28 in sheep (6 months old ewe). Sheep were injected with Vero-cultured BTV-28 with an infectious titre of $10^{6.3}$ $TCID_{50}$/50 μL given at different sites subcutaneously, intradermal and intravenously of 2 mL viral suspension. After 4–6 days post-injection (dpi), infected groups showed an elevation of the body temperature (39.5–41.5°C), lasting for 2–8 days, after which the temperature decreased to the normal range. Mild to medium clinical signs were seen such as conjunctivitis, nasal discharge, coronitis, and mild tongue cyanosis, clinical signs lasted from 2 to 16 dpi, after which the animals recovered.

MATERIALS AND METHODS

CHAPTER-III

MATERIALS AND METHODS

3.1 GENERAL LABORATORY MATERIALS

3.1.1 Glassware

Glassware manufactured by Borosil and Schott Duran was used in the present study.

3.1.2 Plasticware

Plasticware of different suppliers *i.e.*, Tissue culture flasks T75 (75cm^2), T25 (25 cm^2) used in cell culture study were obtained from 'TPP'. Disposable pipettes (2 mL, 5 mL, 10 mL) obtained from 'Corning' were used. 'Axygen' and 'Tarsons' brand centrifuge tubes (15 mL, 50 mL), microfuge tubes (2 mL, 1.5 mL), PCR tubes (0.2 mL), and micropipette tips (1 mL, 200 µL, 10 µL) were used in the study. MicroAmp® Fast 96-Well Reaction Plate (0.1 mL) (Part No.4346907) from 'Applied Biosystems' and TM ThermalSeal RT (TSRT2100) from 'Excel Scientific' were used in the molecular study.

3.1.3 Chemicals and Reagents

Chemicals used for cell culture work in the present experiment were of 'AnalaR' or 'ExcelaR' grade from Gibco, HiMedia, Qualigens, Sisco Research Laboratories (Mumbai, India) and Sigma Aldrich (St. Louis, USA). All solutions and buffers were prepared by using molecular-grade reagents. The reagents and labware were availed from suppliers including Merck (Mumbai), Qualigens, Sigma Aldrich (Hyderabad) and Fischer Scientific. Chemicals used for molecular work in the present study were of molecular grade from TaKaRa, HiMedia, New England BioLabs, Sigma (USA) and Life Technologies.

3.2 STERILIZATION OF LABWARE

3.2.1 Glassware

Glassware was soaked in neutral detergent (Labolene) for one hour and scrubbed. Washed them three times in tap water or till all traces of detergent were removed. Finally, they were rinsed with double-distilled water and kept inverted for air drying. Dried glassware was packed and sterilized in a hot air oven at 160^{o}C for 1h 30 min.

3.2.2 Plasticware

Plasticware (like filter assembly, screw caps) was soaked in lukewarm water with added detergent for 30 min and rinsed thoroughly in tap water followed by double-distilled water. The washed material was inverted for air drying on blotting paper. Screw caps were wrapped initially with aluminium foil and then with brown paper for sterilization. Filter assemblies were loosely tightened by placing a 0.22 μm membrane filter (Catalogue No. GVWP04700; M/s. Millipore) and packed in brown paper. Packed filter assembly, screw caps, micropipette tips, microfuge tubes and PCR tubes were sterilized by autoclaving at 121^{o}C/15 psi for 15 min.

3.2.3 Aqueous Solutions

Double-distilled water was used for the preparation of all aqueous solutions. Wherever necessary, solutions were sterilized by autoclaving at 121^{o}C/15 psi/15 min or by membrane filtration using a 0.22 μm membrane filter.

3.2.4 Liquid Material

Liquid materials like cell culture media and trypsin were sterilized by filtration through 0.22 μ membrane filters.

3.3 PREPARATION OF ESSENTIALS

3.3.1 Media

A sachet content of minimum essential media powder (GIBCO, Cat No. 41500-067) was dissolved in 1000 ml of autoclaved millipore water in a conical flask gradually with constant mixing. After all the constituents were dissolved completely, 2.2 gm of $NaHCO_3$ was added (as per manufacturer). To that 100 IU/mL of Benzyl Penicillin, 100 μg/mL of Streptomycin sulphate, 5 μg/mL of Ciprofloxcin and 2 μg/mL of Amphotericin B were added. Medium was filtered through 0.22 μm membrane filter using filter assembly. Then the media was inoculated onto BHI agar and SDA agar plates to check for any bacterial or fungal contamination. Plates were sealed with paraffin film and kept in an incubator at 37^{o}C for one week. After sterility check, the prepared media was properly sealed and stored at 4^{o}C and brought to room temperature before use.

3.3.2 Phosphate Buffer Saline (PBS)

For preparation of PBS, 8 g of NaCl, 0.2 g of KCl, 2.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 were added in a glass bottle to make the volume up to 1000 ml to give a final concentration of 1X. The pH of the solution was adjusted to 7.2 and finally, this solution was sterilized by autoclaving.

3.3.3 Trypsin Versene Glucose (0.2%)

Trypsin from porcine pancreas (T4799-10G; Sigma-Aldrich) powder of 0.2 g, 0.2 g of EDTA, 0.05 g of glucose, and 0.03 g of $NaHCO_3$ were added in a sterile glass bottle containing 100 mL of 1X PBS. It was stirred overnight in a refrigerator for proper mixing into a uniform solution. The next day, the prepared solution was sterilized by filtering through a 0.22 μm membrane filter and stored at 4ºC.

3.3.4 Freezing Medium

The freezing medium was prepared by adding 10% cryoprotectant Dimethylsulphoxide (DMSO) (042982; SRL) and 20% FBS in DMEM. This solution was mixed with gentle swirling. The prepared solution was sterilized by filtering through a 0.22 μm syringe filter.

3.3.5 10% Formalin

Ten mL of 37% (w/v) formaldehyde solution (Qualigens) was dissolved in 90 ml of deionized water to give a final concentration of 10% formalin.

3.3.6 1% Crystal Violet Solution

1 g of crystal violet powder was dissolved in 100 ml of 70% methanol.

3.3.7 Trypan Blue (0.4%)

For preparing Trypan blue (0.4%), 0.2 g of Trypan blue was dissolved in 50 ml of PBS (pH - 7.2) and stored at room temperature. This was used to count viable cells before seeding.

3.3.8 Sea Plaque Agarose

2.4 gm of Sea plaque agarose powder (Cambrex BioScience, Cat. No.50100) was dissolved in 50 ml of millipore water and sterilized by autoclaving at 121^{o}C, 15lb/20 minutes, and then stored at room temperature.

3.4 CELL LINES

Vero cell lines were obtained from ELLA Foundation, Shameerpet, Hyderabad, and maintained at the Department of Animal Biotechnology, C.V.Sc., Rajendranagar.

3.4.1 Cryopreservation of Cells

3.4.1.1 Freezing of Cells: The cell layer at the late log phase of growth was processed by decanting the spent medium and washing the monolayer with 2 mL of 1X PBS gently. After decanting PBS, 1 mL of 0.2% trypsin was added ensuring that the monolayer is completely covered. All but a few drops of the trypsin were removed gently by tilting the flask. Then the flask was incubated at 37ºC until the cells round up. Two mL of growth medium was added immediately and the cells were dispersed by repeated pipetting. Cell count was carried out by trypan blue exclusion assay. Then cell pellet was obtained by centrifugation at 1000 rpm/2 min.

Cells at the concentration of 2 X 10^{6} cells /mL were suspended in the Freezing medium and aliquoted into cryovials. Then the sealed vials were placed in a pre-cooled freezing container containing Isopropanol and allowed to freeze at the rate of 1^{o}C/min by placing at -70^{o}C for 3 h. Then the vials were immediately transferred into a liquid nitrogen container (-196^{o}C).

3.4.1.2 Thawing of Cryopreserved Cells: A vial containing cryopreserved cells was immediately transferred from a liquid nitrogen container to a water bath at 40^{o}C for 5 min allowing rapid thawing. Then cell pellet was obtained by centrifugation at 3000 rpm/10 min. The freezing medium was discarded and cells were washed with a plain medium at 3000 rpm/10 min. Fresh growth medium was added to the obtained cell pellet. Then based on the cell viability count by trypan blue assay, seeding of cell culture flask was done.

3.4.2 Maintenance of Cell Lines

3.4.2.1 Subculturing of VERO Cell Line: Vero cells were maintained in a tissue culture flask with growth medium (GIBCO^{TM} Minimum Essential Medium, LOT.No.1708583) with 5% Fetal bovine serum (HiMedia LOT.No. 0000172798). After the formation of a healthy 80-90% confluent monolayer, the growth medium was decanted completely and washed with 1X PBS to remove any dead cells present and ensure that serum is not present as it contains trypsin inhibitors. After washing, 0.2% pre-warmed trypsin (37^{o}C) was added to the cell monolayer and incubated at 37^{o}C for 3-5 min allowing cells to detach. Then, 2 ml of growth medium with 5% fetal bovine serum was added to the flask to inhibit the action of trypsin, and splashing with gentle pipetting was done until there was a uniform distribution of the cells without any clumps. Later the cells were distributed into a T25 flask with a split ratio of 1:2 and a sufficient amount of growth medium was added i.e., around 5-7 ml so that the whole cell surface of the flask is covered with the medium. All the flasks were incubated in a CO_2 incubator at 37^{o}C with 5% CO_2 and cells were observed for every 24 h to check for confluent monolayer. Cell count was carried under an inverted microscope using a hemocytometer by trypan blue exclusion assay.

3.4.2.2 Cell Count-Trypan Blue Exclusion Assay: The cell suspension was mixed thoroughly to take 50 µL of a sample. This cell sample was added to 450 µL of 0.4 % trypan blue solution and mixed gently. Neubauer chamber with glass cover was placed on the microscope stage. Then 20 µL of the mixture was loaded into the chamber of the hemocytometer slide, by capillary action. Live cells were counted by 10X objective, in the central 1mm² area of the grid. The cell concentration was calculated using the formula given below.

$c = n \times 10^4 \times$ dilution factor

Dilution factor = 10

c - Cell concentration (cells /mL)

n – Number of cells in the area

3.4.2.3 Cell Seeding: Cells were seeded at the concentration of 2×10^4 cells per mL into tissue culture flasks (T25) and incubated at 37ºC in a 5% CO2 incubator until a complete monolayer was formed.

3.5 ADAPTATION OF BTV-23 TO VERO CELL LINE

BHK-21 cell line adapted BTV-23 serotype was passaged 4 times in Vero cells to show cytopathic effect (CPE) within ideal time. At 80-90% confluence of the monolayer in a tissue culture flask, the growth medium was discarded and washed with 1XPBS. Then, 2 mL of 1% MEM with FBS was added along with 50 µL of stock virus and incubated at 37ºC in a 5% CO_2 incubator. After 1h adsorption, 4 mL of 1% MEM with FBS was added and kept in a CO_2 incubator along with a control flask in which only maintenance medium was added without virus. Thus infected flasks were subjected to microscopic examination every 18-24 h. The infected flask

was compared with the control flask for CPE in Vero cells which was characterized by vacuole formation, rounding, and clumping of cells and eventually thinning of the monolayer.

3.6 PLAQUE PURIFICATION OF BTV

6-well tissue culture plates were used for plaque purification in which each well was seeded with $1X10^6$ Vero cells in a 10% growth medium and incubated at 5% CO_2 level at 37ºC. BTV-23 was serially diluted 10^{-1} from 10^{-7} in plain medium, in six wells, a monolayer of five wells was infected with 1ml of different virus dilution starting from 10^{-3} to 10^{-7} and 6^{th} well was kept as cell control. This plate was kept in an incubator with 5% CO_2 at 37ºC for 1h. Involving swaying for every 10-15 minutes to ensure virus adsorption. During this incubation time, already prepared 2.4% sea plaque agarose was liquefied in the microwave and maintained at 42^oC in the water bath. After incubation, the inoculum was drained off completely with the help of a 1 mL micropipette to remove unadsorbed virus without disturbing the monolayer and immediately overlayed with 10 mL of 2X MEM with 3.5% FBS; 2.4 % sea plaque agarose mixture in a 1:1 ratio. This draining off and overlaying was carried out for one well for each time instead of discarding all wells at a time to counter drying of monolayer as overlaying was done slowly.

Finally, overlayed plates were allowed to solidify and then transferred to an incubator. The plates were observed daily for plaque formation. Plaques that are more distinct and isolated from others were collected with the help of a micropipette in 200 µL of 10% MEM. 5 plaques per plate were collected and infected to Vero cells in a 12-well plate for virus propagation.

3.6.1 Confirmation and Selection of the Plaques

Vero cells were seeded in 12 well tissue culture plates at a concentration of 2×10^5 cells per ml. 2ml of the cell suspension was added per well and incubated at 37^oC in a CO_2 incubator at 5% CO_2. The monolayer in each well was infected with 200 µL 10% MEM in which plaque was dissolved. To each well 1.5 mL of 1% growth medium was added and incubated at 37ºC in a CO_2 incubator at 5% CO_2. Plates were observed under an inverted microscope at 18-24 h intervals and resultant cell culture fluid showing CPE was collected and used in the next round of plaque purification. The same procedure was carried out another two times. For each successive purification, the previous round plaque which was more independent from others was selected.

3.6.2 Molecular Confirmation of Plaque Purified Virus

After plaque purifying the putative virus three times, one plaque was infected to T25 tissue culture flask to get sufficient amount of cell culture fluid for molecular confirmation and to calculate $TCID_{50}$ titre to inoculate selected animals to raise hyperimmune serum (HIS) against this serotype.

3.6.2.1 Freeze Thawing of Cell Culture Fluid: At 90% CPE in a T25 flask, cell culture fluid was freeze-thawed three Times. Freeze-thaw involved rapid freezing of cell culture fluid at -70^oC for 20 min. and rapid thawing to 37^oC in the water bath.

3.6.2.2 RNA Isolation: Bluetongue virus genome i.e., double-stranded RNA virus (ds-RNA) was extracted from the cell culture fluid by Acid Phenol method using TRIZOL^R reagent.

1. 15 mL of infected Vero cell culture supernatant was centrifuged at 4000 rpm for 10 min to obtain a pellet.

2. To the pellet, 750 µL of TRIZOL^{R} was added.

3. The mixture was vortexed for 1 min for proper mixing and incubated at room temperature for 5 min.

4. To the sample, 250 µL of chloroform was added, mixed, and incubated at 4^{o}C for 10 min.

5. The mixture was centrifuged at 13200 rpm for 20 min at 4^{o}C.

6. Aqueous phase was collected carefully and transferred to another RNase-free 1.5mL eppendorf tube without touching the protein layer.

7. Equal amount of ice-cold isopropyl alcohol was added to the collected aqueous phase.

8. This mixture was incubated at -20^{o}C overnight.

9. The following day this mixture was centrifuged at 13200 rpm for 20 min at 4^{o}C

10. Supernatant was discarded leaving the small pellet.

11. 1ml of 70% ethanol was added to the pellet and centrifuged at 13200 rpm for 10 min at 4^{o}C.

12. Supernatant was discarded and the pellet was air-dried.

13. The pellet was dissolved in 30 µL of nuclease-free water and stored at -20^{o}C.

3.6.2.3 Agarose Gel Electrophoresis of ds-RNA: Gel electrophoresis was carried out for confirmation of the segmented ds-RNA pattern of the virus.

1. One g of Agarose (MB002-500G; HiMedia) powder was dissolved in 100 mL 1X TAE buffer and heated in a microwave oven till melted completely.

2. After letting it cool down to 50^{o}C, Ethidium Bromide (EtBr) (E8751-10G; Sigma-Aldrich) was added to a final concentration of 0.5 μg/mL. The content was swirled gently to mix the reagents.

3. The molten agarose was poured into a casting tray and after positioning the required comb; it was allowed to solidify for at least 20 min.

4. The comb was removed gently and the gel tray was positioned in the electrophoresis tank and buffer was added so that the gel was just submerged within the buffer.

5. Five μL of each RNA sample with 1 μL of Gel Loading Dye, Purple (6X), no SDS (Cat.No.B7025S; New England BioLabs) and 1Kb DNA ladder (Cat.No.N3232L; New England BioLabs) were loaded in wells.

6. Electrophoresis was carried out at 75 volts for 1h and 30 min.

7. The gel was transferred to gel documentation system (Gene flash, Syngene bioimaging) having UV trans-illuminator and observed under medium wavelength for the desired bands.

8. The expected size of viral dsRNA genome segments was estimated by comparison with that of a standard DNA marker.

3.6.2.4 Reverse Transcription-Polymerase Chain Reaction (RT-PCR): RT-PCR was done in two steps. First cDNA synthesis was carried out and the synthesized cDNA was used for PCR.

3.6.2.4.1 Reverse Transcription: The reaction was carried out using PrimeScript™ 1st strand cDNA Synthesis kit (Cat No.6110A; TaKaRa). A 20 μL of RNA of each sample was converted to 40 μL of cDNA, in the following steps.

1) The reagents were allowed to thaw completely. Then were mixed gently and spun briefly.

2) The following mixture was prepared in a microfuge tube. The master mix was prepared by scaling up based on the volumes listed below to the desired number of reactions.

Reagents	**Volume**
Random hexamers	2 µL
dNTP Mixture	2 µL
Template RNA	20 µL
RNase free dH_2O	6 µl
Total	30 µL

3) This Template RNA-Primer Mixture was incubated for 5 min at 65°C and then snap cooled on ice.

4) The following reaction mixture was prepared.

Reagents	**Volume**
Template RNA-Primer Mixture	30 µL
5X PrimeScripit Buffer	8 µL
RNase Inhibitor	1 µL
PrimeScript RTase	1 µl
Total	40 µL

5) This reaction mixture was added into the processed template RNA-Primer Mixture by mixing gently and then incubated at the following conditions in a thermal cycler (Prima-Duo, HiMedia).

25^0C	10 min
42^0C	60 min
70^0C	10 min (for enzyme inactivation)
4^0C	∞

3.6.2.4.2 Polymerase Chain Reaction (PCR): The polymerase chain reaction was carried out using EmeraldAmp® GT PCR Master Mix (RR310A; TaKaRa) with a set of forward and reverse primers, which amplify a 98 bp region in the *NS3* gene segment (Table 3.1). The primers were obtained in lyophilized form and were reconstituted with nuclease-free water to obtain 100 pmol/μL stocks. Stocks were made into a 10 pmol/μL working solution and used for PCR.

Table 3.1 *NS3* gene-specific primers (Hoffmann *et al*., 2008)

Primer code	**Primer sequence (5′-3′)**
Seg10_F	TTGGAYAAAGCRATGTCAAA
Seg10_R	ACRTCATCACGAAACGCTTC

R=A/G and Y=C/T

1) The reagents were allowed to thaw completely, mixed gently, and spun briefly. The PCR master mix was prepared by scaling up based on the volumes listed below to the desired number of PCR reactions.

2) Using the cDNA synthesized, the PCR mixture was prepared as follows.

Reagent	**Volume**
EmeraldAmp GT PCR Master Mix (2X Premix)	6.25 μL
cDNA	2 μL
Forward Primer (10 μM)	0.5 μL

Reverse Primer (10 μM)	0.5 μL
dH_2O (Sterile distilled water)	3.25 μL
Total	12.5 μL

3) The above-mentioned contents were transferred to a 0.2 mL tube on ice. The PCR mixture was mixed thoroughly by using the pipette and was spun briefly.

4) The tube was placed in a thermal cycler and the following cycling conditions were followed.

Initial denaturation	98^oC/3 min	
Denaturation	98^oC/10 sec	35 cycles
Annealing	55^oC/30 sec	
Extension	72^oC/15 sec	
Final extension	72^oC/5 min	
Hold	$4^oC/\infty$	

5) Then gel electrophoresis was carried out and the specificity of the RT-PCR product was confirmed by comparison of migration distances with 100 bp DNA ladder (Cat.No.N3231S; New England BioLabs), along with positive template control and no template control (NTC), using gel documentation system.

3.6.2.5 Quantitative RT-PCR / Real-time RT-PCR: The real-time polymerase chain reaction was carried out using Power SYBR® Green PCR Master Mix (Cat. No.4367659; Applied Biosystems) with serotype-specific primer set obtained in lyophilized form Bioserve (Table 3.3), in StepOnePlus Real-time PCR system (instrument) from Applied Biosystems. Primers were reconstituted with nuclease-free water to obtain 200 pmol/μL stocks. Stocks were made into a 10 pmol/μL working solution and used for PCR.

Power SYBR® Green PCR Master Mix (2X concentration) contains SYBR® Green I Dye, AmpliTaq Gold®DNA Polymerase, dNTPs, Passive reference, and Optimized buffer components. The SYBR Green dye binds to double-stranded (ds) DNA, thus providing a fluorescent signal that reflects the amount of ds-DNA product generated during PCR (Fig 3.1).

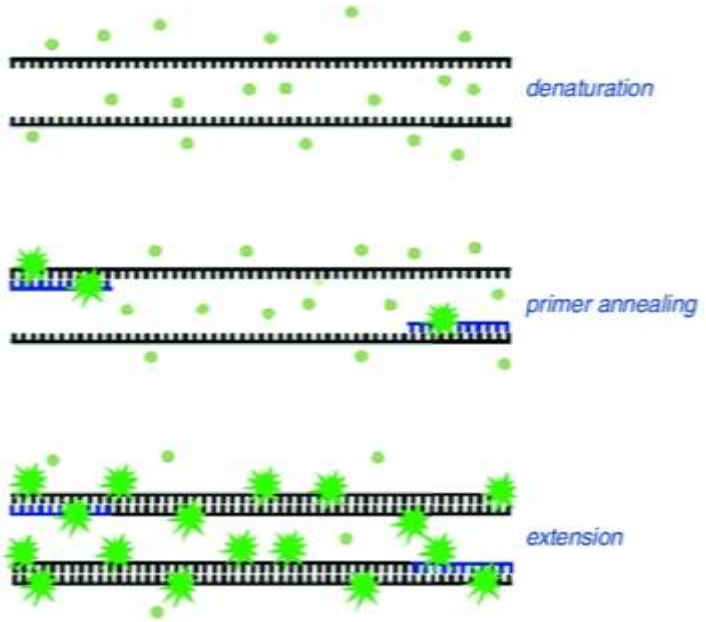


Fig 3.1: SYBR Green dye based qPCR detection

1) The Power SYBR® Green PCR Master Mix and the working solution of the primer set were allowed to thaw completely. Then mixed gently and spun briefly.

2) PCR master mix was prepared by scaling the volumes listed below to the desired number of PCR reactions.

Reaction Component	**Volume**
Power SYBR® Green PCR Master Mix (2X)	5 µL
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL
cDNA	1 µL
Nuclease-free water	3 µL

Total 10 µL

3) The PCR mixture was mixed gently and spun briefly.

4) Then PCR reaction plate was prepared by distributing 9 µL of PCR mixture into each well of MicroAmp® Fast 96-Well Reaction Plate, to that 1 µL of a template (cDNA) was added, according to template sheet including no template control.

5) The loaded reaction plate was sealed with ThermalSeal RTTM sealing foil and was centrifuged for 2 min at 1500×*g* by placing the multiwell plate in a standard swing bucket centrifuge containing a rotor with a suitable adaptor for multiwell plate and balancing it with a suitable counterweight (eg: another multiwell plate).

6) The Multiwell Plate was transferred into the plate holder of the StepOnePlus Real-time PCR system Thermal Cycling block. Then the programmed plate document was run according to the following cycling conditions for amplification and melt curve generation (Table 3.2).

Table 3.2 PCR conditions followed in Real-time PCR

Enzyme Activation	**PCR**		**Melt Curve Stage**		
Hold	Cycles (40 cycles)				
	Denaturation	Annealing/Extension			
10 min	15 Sec	1 min	15 sec	1 min	15 sec
95°C	95°C	60°C	95°C	60°C	95°C

Table 3.3 Sequence of primers used for molecular typing (Maan *et al*., 2016)

Oligo Name	Oligo Sequence (5'-3')
BTV-1F (E)	GCYAAATTRCGAATCAARCATRGYG
BTV-1R (E)	GTTARCCTCTGCAAYACAATAGG
BTV-2F(E)	GAGCATTTGTTGAAARGTTATG
BTV-2R(E)	GATATCRAAYGCGTACATYTCTG
BTV-4F(W)	GAACACGAAGATATCGCAG
BTV-4R(W)	GCATARAGAAGCTARATGTATCTTCA
BTV-5F(W)	GCTTCTCAGGATGGATGAG
BTV-5R(W)	CARRTCRAYCTTAAYRTCRTAYC
BTV-9F(E)	GTATGATACCAGGCCAGCG
BTV-9R(E)	GTTCATTTTGAGGATCATCCA
BTV-10F(W)	TATTRACWACWGAACCAAACCT
BTV-10R(W)	GYGARTTRATCCRTTTGTCAT
BTV-12F(W)	ATACAATTCAGGCTATCCRGA
BTV-12R(W)	CAATGATYGTTCCTCGTAAGC
BTV-16F(E)	GACCTGAATATAAACCGCGAG
BTV-16R(E)	ATTAATCAATTCGTACTCCCAGTG
BTV-21F(E)	GCCAGATTAAAGATAACGCA
BTV-21R(E)	GTAAATCGATAGGGTCCG
BTV-23F(E)	GCGGARYTGTTAGATGGCTATG
BTV-23R(E)	GGAATTTGWGYRACRTCATGACG
BTV-24F(W)	GAACTAYGAGAAGCTTAYR
BTV-24R(W)	GCGAAAARTCYYTCATATCTA

R=A/G and Y=C/T

3.6.2.6. Conventional PCR Setup:

Polymerase chain reaction (PCR) for plaque purified virus was carried out with primers (IDT-DNA) specific for available bluetongue virus serotypes (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23, and 24) and their 5'-3'sequences were mentioned (Table 3.4). Each PCR reaction (using Emerold's kit) final concentration was prepared for 12.5 µl as given below.

Reaction Component	**Volume**
EmeraldAmp GT PCR Master Mix (2X Premix)	6.25 µL
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL
cDNA	2 µL
Nuclease-free water	3.25 µL
Total	12.5 µL

Table 3.4 Segment 2 specific Primers sequence (5′-3′) used for PCR

Oligo Name	**Oligo Sequence (5'-3')**
BTV-1EF	TGT CGA GCC GAT TGA AGA TCC GTC
BTV-1ER	ATC GTC ATT CCG TCG TTG TGC G
BTV 2EF	TAC GCA CCT CGT GAG AGA GA
BTV 2ER	GTT GGA GGA ACC AAC TTC CA
BTV 4P2F	GTT GGA TCT GAG AAA TGG GT
BTV 4P2R	AAG ACA CGG ATA AGG ATT CG
BTV 5 F	GTCAAACACTGTCACCACGC
BTV 5 R	TTGCGAAGAGCTTGCCCATA
BTV 9EF	GAT GGA ACG GCT AAA CCA AA
BTV 9ER	TGG ATA TTT GAC ACG AGC GA
BTV 10F	TGT ATC GTT AAG GCG AGG TCA GCA

Table 3.4(Cont.).

BTV 10R	TGT CTT CTA ACG GCC TCT CAC G
BTV 12F	TTT AGG TGA CCA TGT GGA GAC G
BTV 12R	CAA CGC ACT TTC GCA AAA CC
BTV 16EF	TCG AGG AAA GCG GAT ACC ACG T
BTV 16ER	CGT TGC GCT AAC TCG ACT TCG C
BTV 21F	GCA GAT TCG TAC AAC CAA CGG CC
BTV 21R	TTG GGA TTT GCG AGGCGC GA
BTV 23F	GCG TTG CGA TGG ATG AGT TAG CA
BTV 23R	GGT GGT CAT CTC TTC ATC TTC GGG G
BTV 24P3F	GTT TCA TGA ATT TGA AGG ACG
BTV 24P3R	ACC TTG TGA AAT CTT AGT YTT TG

(Reddy *et al*., 2016)

Table 3.5 PCR conditions followed in thermo cycler:

For BTV-1,2,4,5,9,10,12,21,23,24	**For BTV-16**
1.Initial denaturation -98^{o}C/3 min	94^{o}C/3 min
2.Denaturation -98^{o}C /10 sec	94^{o}C/30 sec
3.Annealing -55^{o}C/30 sec	50^{o}C/30 sec
4.Extension-72^{o}C/1 min 30 sec	72^{o}C/1 min
5.Final extension-72^{o}C/5 min	72^{o}C/10 min
Hold at – 4^{o}C	4^{o}C
No of cycles (for 2,3,4)-35	35

PCR thermo cycler was set to hold at 4^{o}C after completion of reaction**.**

3.7 TITRATION OF THE VIRUS

After confirmation by PCR, the virus titre was calculated to know the $TCID_{50}$ after plaque purification. Titre of putative BTV-23 serotype was determined in terms of tissue culture infective dose 50 ($TCID_{50}$) in 96 well tissue culture plates. Cells were seeded at the rate of $1x10^4$ per well in MEM with 5% FBS and incubated in a CO_2 incubator at 37°C. At 80-90% confluence, cells were infected with 100 µL of 10^{-1} to 10^{-8} serially diluted virus in 1% growth medium, each dilution in a separate row in replicates of eight along with two cell control rows and incubated in a CO_2 incubator (Table 3.6). Plates were observed at 12-18 h intervals for characteristic CPE under an inverted microscope and final readings were taken on the 5th day of infection. Virus titre was calculated according to Reed and Munch's method (1938). Different dilutions were taken as shown below.

Table 3.6 Infection of 96-well plate for $TCID_{50}$

CC	CC	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	CC	CC
CC	CC	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	CC	CC
CC	CC	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	CC	CC
CC	CC	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	CC	CC
CC	CC	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	CC	CC
CC	CC	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	CC	CC
CC	CC	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	CC	CC
CC	CC	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	CC	CC

CC: Cell control

3.7.1 Protocol for Crystal Violet Staining

After titration of virus in 96 well tissue culture plates, wells were stained with crystal violet stain.

1. The medium was discarded from the plate and the cells were washed with 1X PBS (200 µL/well).

2. The cells were fixed in 10% formaldehyde (200 µL/well) and incubated for one hour at room temperature.

3. Formaldehyde was discarded and the plate was washed twice with 1X PBS.

4. Crystal violet (1%) solution (200 µL/well) was added and kept on a shaker for 1 h. Crystal violet solution was discarded and the cells were washed thrice with 1X PBS and dried at room temperature.

3.8 ANIMAL INOCULATION

Plaque purified virus was given to sheep to raise hyperimmune serum (HIS).

3.8.1 Selection of Animals

For antibody production, BTV seronegative sheep were selected based on competitive enzyme-linked immune sorbent assay (c-ELISA) results. For this test, blood samples from each sheep were collected separately in serum vacutainers and kept at 4ºC. Serum was separated by centrifuging the blood at 4000 rpm, 4^{o}C for 15 min, and the serum was aliquoted and stored at -20ºC.

c-ELISA was done using the bluetongue antibody test kit supplied by the M/s Veterinary Diagnostic Technology, Inc., USA, according to the kit protocol (Afshar *et al.*, 1989).

Procedure

1. Diluting and washing buffers were prepared in 1X concentration in deionized water.

2. Control and test serum were prepared by diluting them at 1: 5 in dilution buffer.

3. Precoated plates were washed with wash buffer twice.

4. 50 µL of test and negative, weakly positive, strong positive controls were added to wells.

5. After adding test and control sera, immediately 50 µl of the diluted (1:100) monoclonal antibody was added to each well.

6. The plates were incubated at room temperature for two hours covered with a lid.

7. Just before the incubation period ends, 1: 300 dilution of the conjugate was prepared in a 1X diluting buffer.

8. At the end of the incubation period, the reactants were discarded from the wells and washed with wash buffer thrice.

9. Diluted conjugate (100 µL) was added to each well and incubated at room temperature for one hour.

10. Buffer substrate solution was prepared by dissolving the contents of the buffer substrate capsule in 100 mL of deionized water.

11. In the OPD buffer substrate solution, the wells were washed 5 times with washing buffer. Two mg of OPD (1 mg/2.5 mL) tablet is dissolved in 5 mL buffer substrate solution after preparing.

12. Now 100 µL of substrate OPD solution was added to all wells and the plate was incubated for 10 min in dark.

13. After 10 min, 50 µL of stop reagent (3N sulfuric acid) was quickly added to all the wells and OD values for each well were taken at 490 nm wavelength using an ELISA

microplate reader. The percent inhibition (PI) of binding of the monoclonal antibody by a test serum was calculated by the formula. Sera showing less than 50% inhibition were considered seronegative whereas the sera showing >50% inhibition was considered as seropositive.

3.8.2 Housing of Experimental Animals

Three BTV seronegative Deccani sheep (6-8months) were selected for use in the present study and were shifted to the insect-proof experimental animal house at the College of Veterinary Science, Rajendranagar, Hyderabad. All the animals were dewormed and held for one week before the start of the experiment for acclimatization. Animals were provided with feed, fodder and water ad libitum throughout the study for 2 months.

3.8.3 Dose and Route of Inoculum

After deworming and acclimatization, 2 mL of $10^{4.11}$/100 µL $TCID_{50}$ plaque purified confirmed BTV-23 virus was inoculated to sheep by two routes i.e., 1 mL via intradermal; 1 mL via subcutaneous routes at the shoulder region. For the control animal, 1 mL of plain MEM was given to compare results. Before inoculation animals were examined for their general health status i.e., temperature and physical appearance. The selected site for infection was shaved and sterilized with 70% ethanol. On the 15^{th} day of infection, one more injection of the same dose was given to both animals. From the day of inoculation, animals were examined regularly for mild clinical signs. Temperatures were recorded twice daily and serum samples were collected in sterile serum vacutainers (BD Vacutainer® Ref.365152) on 0, 7, 15 and 28 days post-inoculation (dpi).

3.9 CROSS-NEUTRALIZATION STUDIES

Serum neutralization study was carried out with BTV-23 using HIS at different dilutions (1/20, 1/40, 1/80, 1/160, 1/320, 1/640 and 1280) and cross-neutralization studies with available plaque purified types (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 and 24).

3.9.1 $TCID_{50}$ Calculation

$TCID_{50}$ was calculated for BTV-23 after plaque purification used in homologous serum neutralization tests and for cross-neutralization studies, $TCID_{50}$ was calculated for available types (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 and 24).

3.9.2 Neutralization Studies

96-well plates were seeded with Vero cells at the concentration of 1×10^4 cells per well in 5% MEM and incubated at 37^{o}C with 5% CO_2. On the second day, after observing monolayer in 96-well plate, serum raised against BTV-23 was serially diluted (1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640 and 1/1280) and 50 µL each of the diluted serum was incubated with equal quantity of 100 $TCID_{50}$/50 µL virus in the deep well plate for 1 h at 37^{o}C. A hundred µL of this mix was used to infect each well in quadruplicate. Plates were observed under an inverted microscope at 12 h interval for CPE and the final titer was taken on day five.

RESULTS

CHAPTER IV

RESULTS

4.1 MAINTENANCE OF CELL LINE

Within 24-48 h of seeding, under optimum conditions (5% CO_2 at 37^{o}C) tissue culture flasks showed about 90% of confluence (Fig 4.1a). Change in medium color to yellow was the indication for the exhaustion of medium and acidic pH. In such conditions, the medium was replaced with a maintenance medium containing 1% FBS to maintain a fully grown monolayer. These cell culture flasks were used for infection purposes.

4.2 ADAPTATION OF BLUETONGUE VIRUS SEROTYPE-23 TO VERO CELL LINE

Stock virus grown in BHK-21 cells was adapted to the Vero cell line by serially passaging four times to observe the CPE between 36-48 h.

In the first two passages, the CPE was seen by the 4th day of infection and complete CPE was observed by 6-7 days. In the next two passages, CPE was observed between 36-48 h as rounding. Clumping of dead cells and complete peeling off of monolayer (Fig 4.1b) was observed within 72 h of infection while the control flask didn‘t show any CPE (Fig 4.1a).

Fig 4.1a

Fig 4.1b

Fig 4.1 VERO CELL MONOLAYER. Fig 4.1a uninfected (10X) confluent layer; Fig 4.1b Infected with BTV-23 (10X) Rounding and clumping of cells 4dpi.

4.3 PLAQUE PURIFICATION OF BTV

Sea plaque agarose overlayed on a monolayer of 6-well plate solidified within 30-45 min which was then returned to the incubator.

On regular microscopic observation, plaques were identified from the 4^{th} day of infection as CPE but restricted to areas showing dead cells scattering light surrounded by a healthy monolayer. Plaques were best identified in low power magnification (Fig 4.2a) and plaques in different sizes; different magnifications are shown (Fig 4.2a. to 4.2d). Plaques of approximately not more than 1mm in diameter were visible to the naked eye against light source as pale areas in monolayer.

The number of plaques was observed to decrease as dilution of virus increased which could be observed best at a glance after staining with 1% crystal violet solution (Fig 4.3a & 4.3b). Among wells infected with 10^{-3} to 10^{-7} virus dilutions, only 10^{-3} to 10^{-4} dilutions produced more number of plaques whereas in monolayer infected with 10^{-5} and 10^{-6} dilutions, few plaques are seen. For collection, plaques were marked under the plate by observing through a microscope and then collected by using a 50 µL micropipette in 200 µL of MEM with 10% FBS. The infected plate was also stained with 1% crystal violet solution to observe plaques. Plaques remained as white spots in the stained monolayer.

The plaques were collected from 6-well plate and infected into 12-well plate and the CPE was observed within 3 days of infection. Among these, CPE showing cell culture fluid of respective plaque, particularly which is more isolated from others in a monolayer of 6-well plate was used for a successive round of plaque purification. Finally, a selected plaque from the third purification was grown in a T25 flask and the resultant cell culture fluid was used for molecular confirmation and characterization.

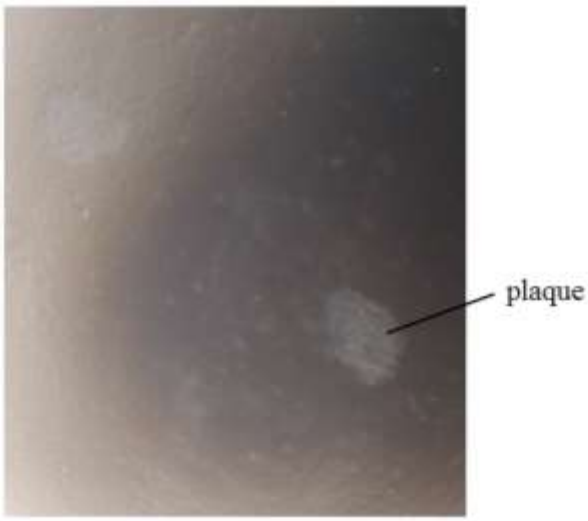


Fig 4.2a

Fig 4.2b

Fig 4.2c

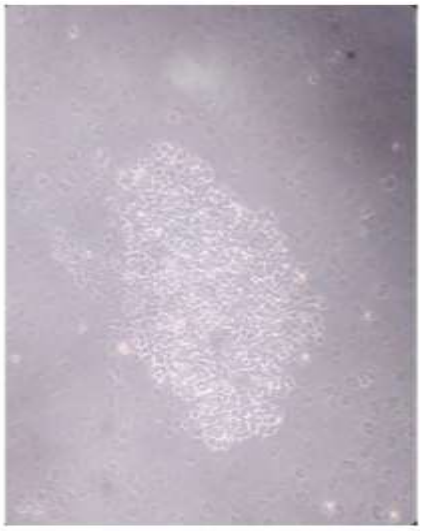


Fig 4.2d

FIG 4.2 PLAQUE FORMATION IN VERO CELL MONOLAYER INFECTED WITH BTV-23 SEROTYPE VIRUS. Fig 4.2a- Image showing two adjacent plaques surrounded with confluent healthy monolayer (4X); Fig 4.2b- Plaque at initial stage (10X); Fig 4.2c- Spreading of plaque (10X); Fig 4.2d- Spreading of plaque (40X).

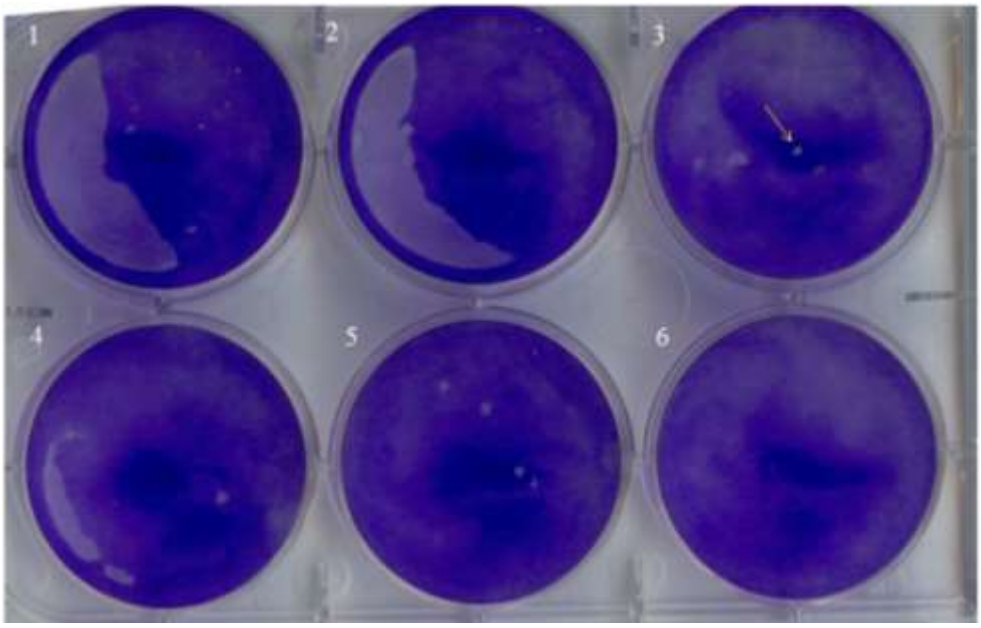


Fig 4.3a Crystal violet staining of monolayer showing plaques (bottom view)

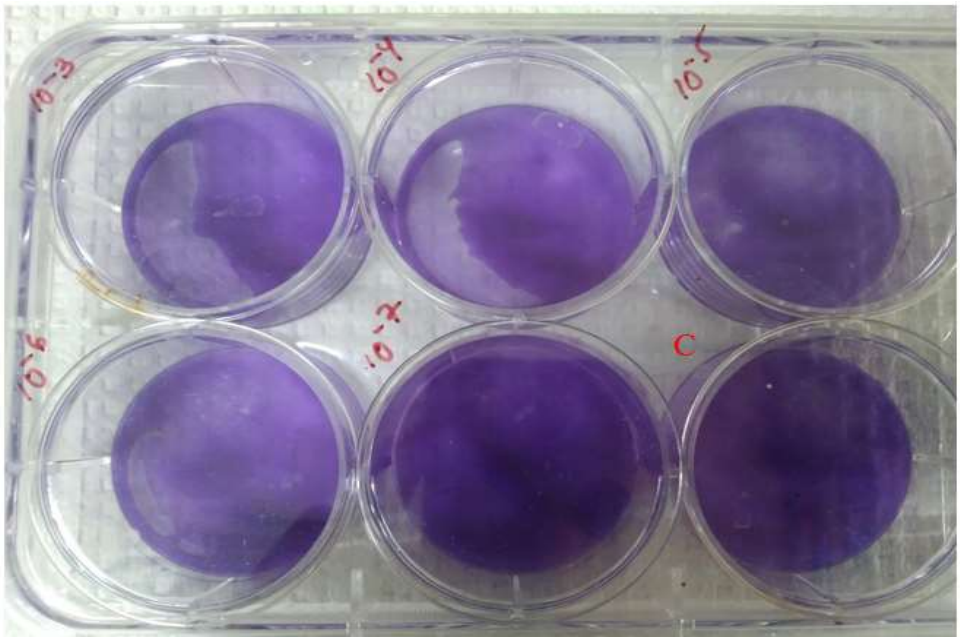


Fig 4.3b Crystal violet staining of monolayer showing plaques (upper view)

FIG 4.3 CRYSTAL VIOLET STAINING OF PLAQUES

Unstained white areas represent plaques whereas stained area represents confluent monolayer. In the above-stained plate, wells numbered 1 to 5 infected with 10^{-3} to 10^{-7} dilutions of virus respectively, and well no. 6 was kept as control i.e., added with plain MEM. Well, no.1 shows more no. of plaques due to least virus dilution (10^{-3}). Clear plaques were observed in well no.2 and 3 ($10^{-3}$and 10^{-4}).

4.4 MOLECULAR CONFIRMATION OF PLAQUE VIRUS

4.4.1 Gel Electrophoresis of RNA

Nucleic acid was extracted from BTV infected Vero cell culture fluid from third-round plaque purification by the Trizol method. The extracted RNA was subjected to 1% agarose gel electrophoresis and observed for the 10 segmented (ladder-like) pattern of BTV RNA (Fig 4.4). All isolates were showing a clear segmented pattern of the ds RNA genome (Fig 4.4).

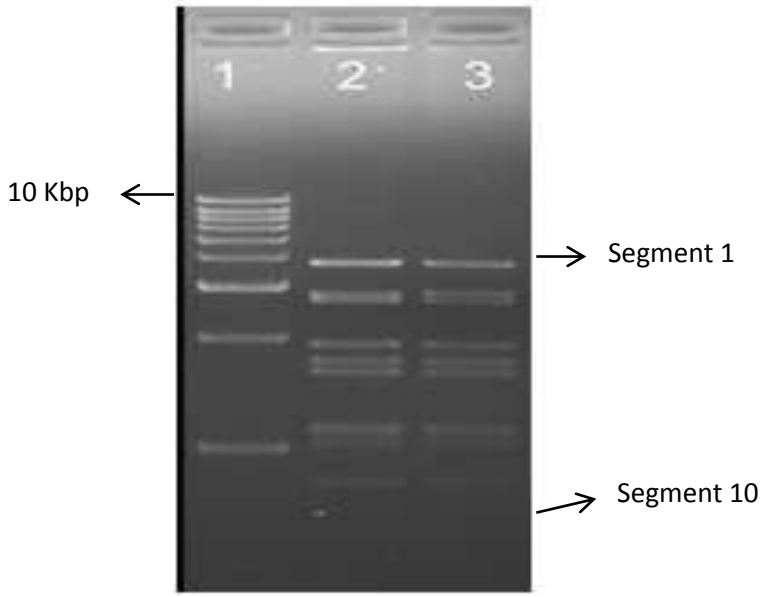


Fig 4.4 Agarose gel electrophoresis of plaque purified BTV-23 RNA
Lane 1- 1 kbp ladder; Lane 2 & 3- Segmented dsRNA of BTV 23 genome.

4.4.2 Confirmation of Plaque Purified BTV-23 by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reverse transcription - Polymerase chain reaction was done in two steps. Reverse transcription (RT) was carried out for plaque purified extracted RNA showing a segmented pattern of BTV RNA, using random hexamers to synthesize cDNA from RNA. This cDNA was used further as a template in PCR for group-specific confirmation of BTV by *NS3* gene-specific primers producing amplicon of 98 bp (Fig 4.5).

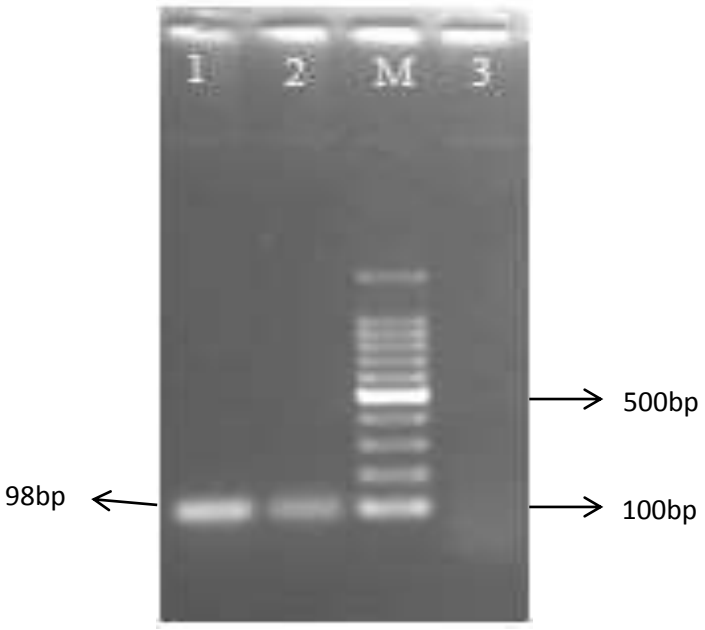


Fig 4.5 NS3 group-specific RT-PCR confirming BTV 98bp
Lane 1: positive template control; Lane 2: sample;
Lane M: 100bp DNA ladder; Lane 3: No Template control

4.4.3 Quantitative / Real-time PCR

Confirmation of plaque purified BTV-23 with other serotypes was done by Real time-PCR. The cDNA which was confirmed with group-specific BTV by *NS3* gene-specific primer was used as a template in RT-PCR against available primers to BTV serotypes (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 and 24), placed in separate PCR tubes along with no template control (NTC) for each type. The PCR product was analyzed based on the C_t value. The C_t value of the sample detected as strong positive shows very less C_t value and the negative sample shown as undetected by real-time PCR / was found to be similar to that of NTC indicating the absence of BTV serotype.

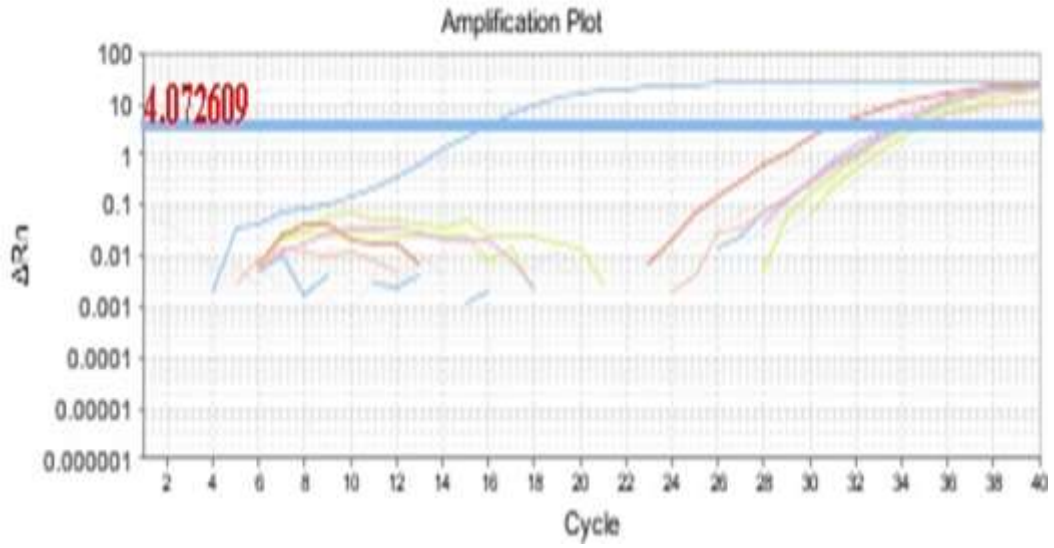


Fig 4.6 Amplification plot showing molecular typing of BTV-23 with all other available BTV serotypes by Real-time PCR (qPCR). Amplification curve showing C_T values of typing of BTV with all available primers (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 & 24 primers) placed along with no template control (NTC). Sample showed low C_T value (positive) with BTV-23 specific primer (first curve to the left) and amplification with other BTV specific primers undetermined or similar to NTC (amplification curve to the right).

4.4.4 Conventional PCR

Molecular confirmation of plaque purified BTV-23 with other serotypes was also done by conventional PCR. The cDNA which was confirmed with group-specific BTV by *NS3* gene-specific primer was used as a template in conventional polymerase chain reaction against available primers to BTV serotypes (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 and 24), placed in separate Polymerase chain reaction tube along with positive control and no template control (NTC) for each type. PCR products were analyzed by gel electrophoresis. Amplification was observed with primers specific for BTV-23 only i.e, 1370bp amplicon (Fig 4.7b). No amplification was observed with remaining serotype-specific primers. Thus the plaque purified virus was confirmed as BTV-23.

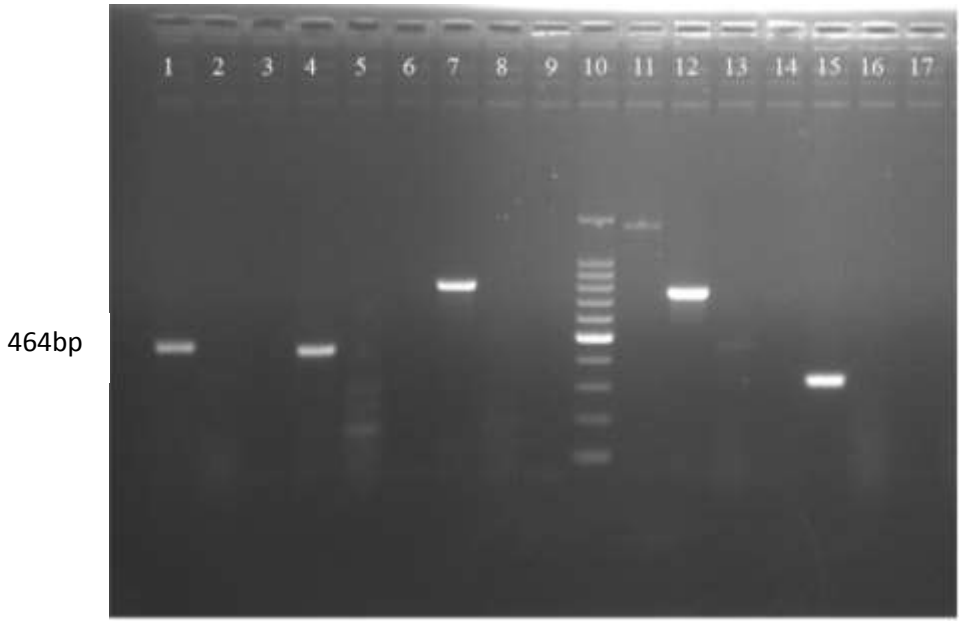


FIG 4.7 MOLECULAR TYPING OF BTV-23 BY CONVENTIONAL PCR

Fig 4.7a Gel Electrophoresis of BTV-4, 5, 10, 12 & 24

Lane -1 positive control for BTV-4:464 bp	Lane-2 Sample cDNA with BTV-4 primers	Lane-3 Negative control
Lane -4 positive control for BTV-5:444 bp	Lane-5 Sample cDNA with BTV-5 primers	Lane-6 Negative control
Lane -7 positive control for BTV-10:800 bp	Lane-8 Sample cDNA with BTV-10 primers	Lane-9 Negative control
Lane 10: 100bp ladder		
Lane -12 positive control for BTV-12: 750 bp	Lane-13 Sample cDNA with BTV-12 primers	Lane-14 Negative control
Lane -15 positive control for BTV-24: 319 bp	Lane-16 Sample cDNA with BTV-24 primers	Lane-17 Negative control

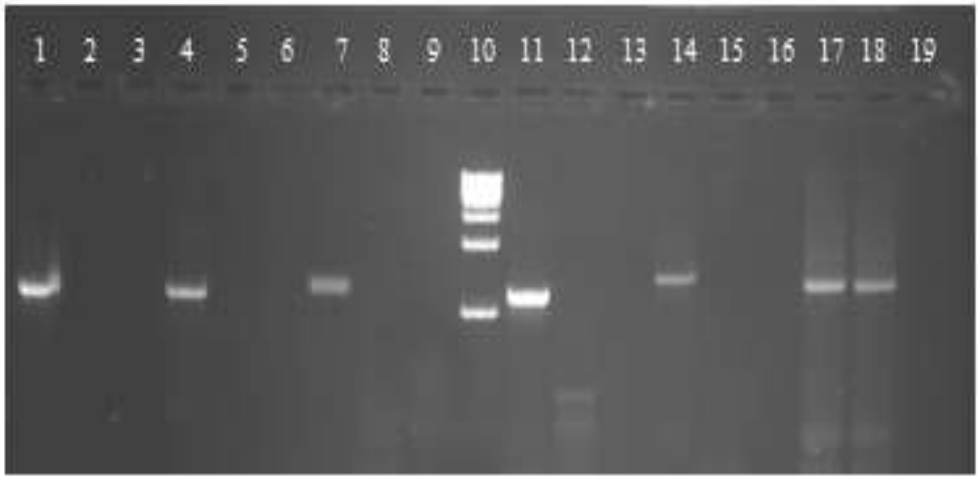


FIG 4.7 MOLECULAR TYPING OF BTV-23 BY CONVENTIONAL PCR

Fig 4.7b Gel Electrophoresis of BTV-1, 2, 9, 16, 21 & 23

Lane -1 positive control for BTV-1:1180 bp	Lane-2 Sample cDNA with BTV-1 primers	Lane-3 Negative control
Lane -4 positive control for BTV-2:1167 bp	Lane-5 Sample cDNA with BTV-2 primers	Lane-6 Negative control
Lane -7 positive control for BTV-9:1224 bp	Lane-8 Sample cDNA with BTV-9 primers	Lane-9 Negative control
Lane 10: 1kbp ladder		
Lane -11 positive control for BTV-16: 1196 bp	Lane-12 Sample cDNA with BTV-16 primers	Lane-13 Negative control
Lane -14 positive control for BTV-21: 1380 bp	Lane-15 Sample cDNA with BTV-21 primers	Lane-16 Negative control
Lane -17 positive control for BTV-23: 1370 bp	Lane-18 Sample cDNA with BTV-23 primers	Lane-19 Negative control

4.5 TITRATION OF THE VIRUS

After molecular confirmation of plaques, $TCID_{50}$ of the virus was calculated as it was passaged six times in the Vero cell line during three rounds of plaque purification.

96-well plate was observed regularly at 18-24 h intervals. Cytopathic effect was observed by the 3rd day in all replicates of lower dilutions (10^{-1} to 10^{-2}) and only started in some replicates of higher dilutions (10^{-3}). Cytopathic effect was read 5th dpi. and details are given in table 4.1 $TCID_{50}$ was calculated as per Reed & Muench (1938).

Table 4.1 $TCID_{50}$ calculation of plaque purified BTV-23 serotype.

S. No	Virus Dilution	No. of wells infected	CPE positive wells	CPE negative wells	Cumulative positive	Cumulative negative	% of negative
1	10^{-1}	8	8	0	29	0	100
2	10^{-2}	8	8	0	21	0	100
3	10^{-3}	8	8	0	13	0	100
4	10^{-4}	8	4	4	5	4	55.5
5	10^{-5}	8	1	7	1	11	8.3
6	10^{-6}	8	0	8	0	19	0
7	10^{-7}	8	0	8	0	27	0
8	10^{-8}	8	0	8	0	35	0

The virus titre was calculated as $10^{4.11}$/100 µL (or) $10^{5.11}$/mL according to Reed and Muench's (1938) method.

The other BTV serotypes (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21 and 24) available in the Department of Biotechnology. These BTV serotypes were passaged two times in T25 flask, $TCID_{50}$ was calculated and used in serum neutralization tests. Titres for BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 and 24 are given in table 4.2

Table 4.2 $TCID_{50}$ titres of available BTV types.

BTV Serotype	$TCID_{50}$ calculation
BTV-1	$10^{3.42}$/100µL
BTV-2	$10^{3.49}$/100µL
BTV-4	$10^{4.2}$/100µL
BTV-5	$10^{3.5}$/100µL
BTV-9	$10^{4.5}$/100µL
BTV-10	$10^{4.37}$/100µL
BTV-12	$10^{3.57}$/100µL
BTV-16	$10^{5.2}$/100µL
BTV-21	$10^{4.8}$/100µL
BTV-23	$10^{4.11}$/100µL
BTV-24	$10^{4.38}$/100µL

4.6 ANIMAL INOCULATION

4.6.1 Selection of Animals

Deccani sheep breed (n=36) was screened with c-ELISA for the presence of BTV-antibodies (Fig 4.8). Among them three seronegative sheep were selected for the study.

FIG 4.8 SCREENING OF DECCANI SHEEP FOR SERO-NEGATIVE ANIMALS BY C-ELISA-Screening of sheep for BTV antibodies and selection of seronegative sheep. Colored wells indicate no antibodies against BTV (seronegative), Colorless – antibodies have been detected

On day 0, two animals were infected with BTV-23 serotype ($10^{4.11}$ $TCID_{50}$ / 100µL i.e., 1ml intradermal and 1ml subcutaneous). Another animal was injected with uninfected plain MEM and was kept as control. Thereafter the booster injection was given on the 15^{th}day with the same dose. The animals were monitored for 45 days during which the clinical signs, temperatures, and serological parameters were studied.

4.6.2 Clinical Signs and Lesions

Sheep developed pyrexia in evening hours at 6 dpi (104.2^{o}F and 104.3^{o}F) which persisted for 4 days and it exhibited nasal discharges on 10 dpi (Fig 4.9a). The control animal was found to be asymptomatic (Fig 4.9b) during the entire study period (101.9^{o}F- 103.2^{o}F).

Fig 4.9a

Fig 4.9b

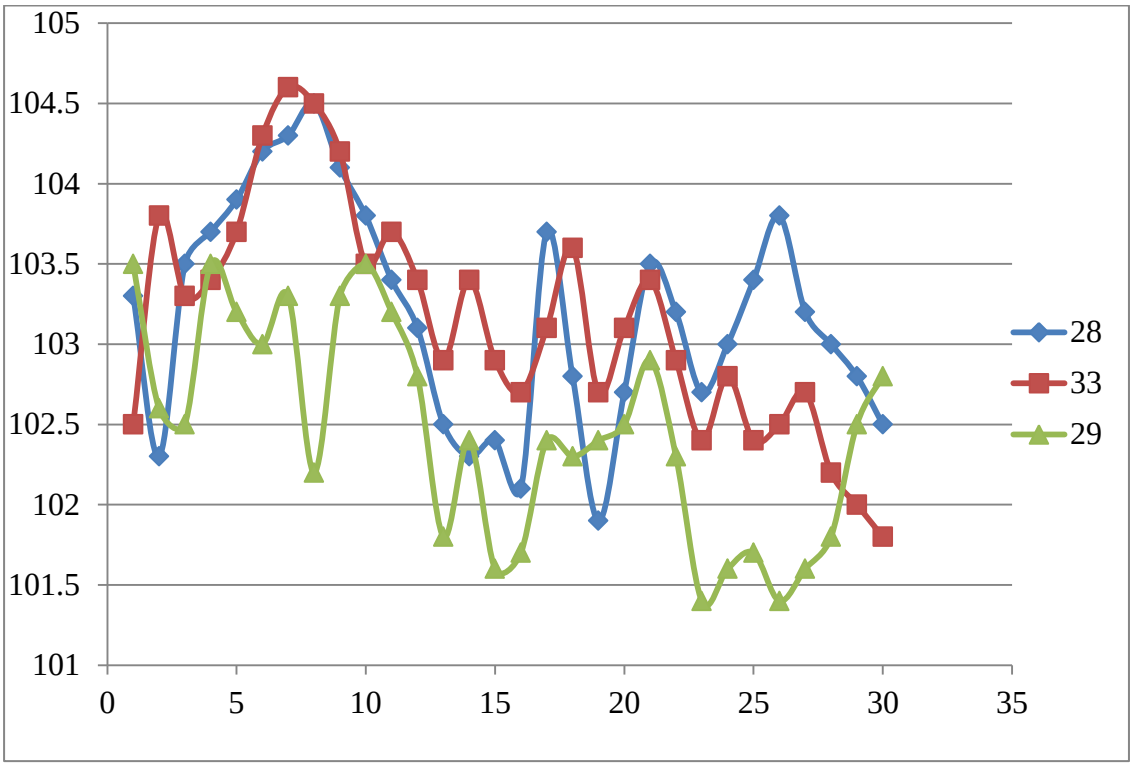
Fig 4.9 CLINICAL SYMPTOMS OF BT IN EXPERIMENTAL INOCULATED SHEEP AND CONTROL ANIMAL. Fig 4.9a Infected sheep showing nasal discharges; Fig 4.9b Control animal without nasal discharges on the same day.

4.6.3 Daily Body Temperature

The body temperatures of each animal were recorded every day for 45 days after the inoculation of the virus. All sheep showed raise in rectal temperatures 6-7 dpi in BTV-23 infection ranging 104.2^{o}F – 104.6^{o}F, whereas the control animals exhibited no pyrexia. The gradual temperature changes are shown in graphical presentation for individual animals (Fig 4.10) and peak temperatures of all infected animals are shown in Table 4.3

Table 4.3 Peak temperatures of all the animals with respective days of post-infection

Sheep tag no	BTV-23	
	Dpi	Temperature ^{o}F
28	8	104.5
33	7	104.6
29 (control)	5	103.5



Sheep tag no. 28 & 33- infected with BTV-23.
Sheep tag no. 29- control animal (plain media).

FIG 4.10 GRAPH SHOWING DAILY TEMPERATURES OF SHEEP INFECTED WITH BTV. It represents the temperature details of the infected and controlled animal. Infected animals showed pyrexia between 6-10dpi (103.9^{o}F – 104.6^{o}F). The control animal maintained normal temperature during the entire study (101.4^{o}F-103.5^{o}F).

4.7 SERUM NEUTRALIZATION TEST

Serum Neutralization test was carried out in Vero cells to study the presence of type-specific neutralizing antibodies to BTV-23. The absence of CPE in infected wells of Vero cells indicates the presence of neutralizing antibodies in the test serum.

4.7.1 Screening of Hyper-Immune Serum for BTV-23 specific Antibodies by SNT

Serum samples were collected from control and infected animal at the 7th day and 15th-day interval and SNT was done for confirmation of seroconversion. Animal tag no. 28 was found to seroconvert on 7th day of inoculation, animal tag no. 33 seroconverted between 7th-15th days of post-inoculation (dpi) while control animals remained seronegative throughout the duration of study.

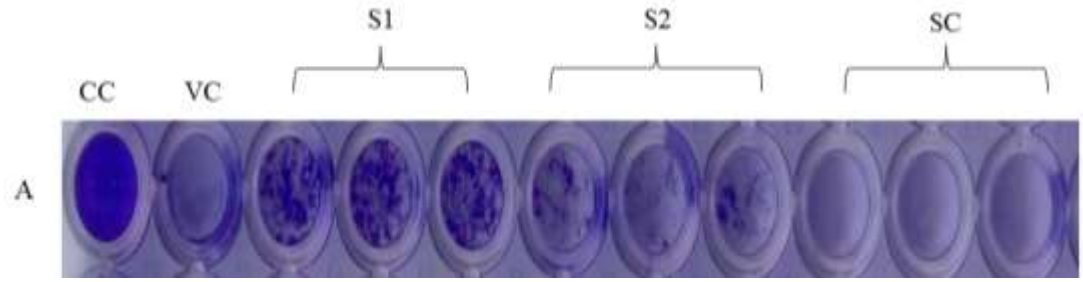


Fig 4.11 Serum Neutralization Test

Fig 4.11a Serum neutralization test (SNT) panel 'A' showing the SNT of serum collected on 7th day. Wherein CC-cell control wells; VC-virus control wells; S1& S2 serum collected from animal infected with BTV-23 i.e., animal tag no.28 & 33 respectively; SC - serum from control animal tag no. 29.

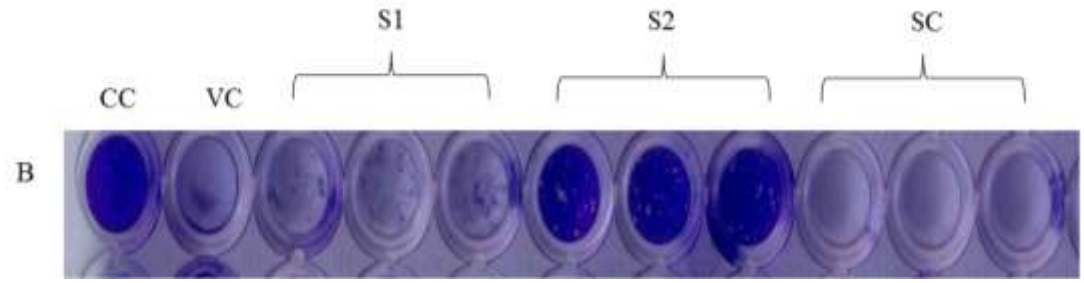


Fig 4.11 Serum Neutralization Test

Fig 4.11b Serum neutralization test (SNT) panel 'B' showing the SNT of serum collected on 15th day. Wherein CC-cell control wells; VC-virus control wells; S1& S2 serum collected from animal infected with BTV-23 i.e., animal tag no.28 & 33 respectively; SC - serum from control animal tag no. 29.

4.7.2 Detection of Antibody Titre of Hyper-Immune sera by SNT

SNT was performed as described in section 3.9 to estimate type-specific antibody titre in serum of BTV-23 inoculated sheep. The animal developed neutralizing antibodies by 7th dpi. The control animal didn't develop any antibodies. Neutralizing antibody titres of serum collected from 28th dpi was done using different dilutions of serum with 100 $TCID_{50}$ titre virus, which were estimated against homologous plaque purified BTV-23 (Fig 4.12) and are given in Table 4.4

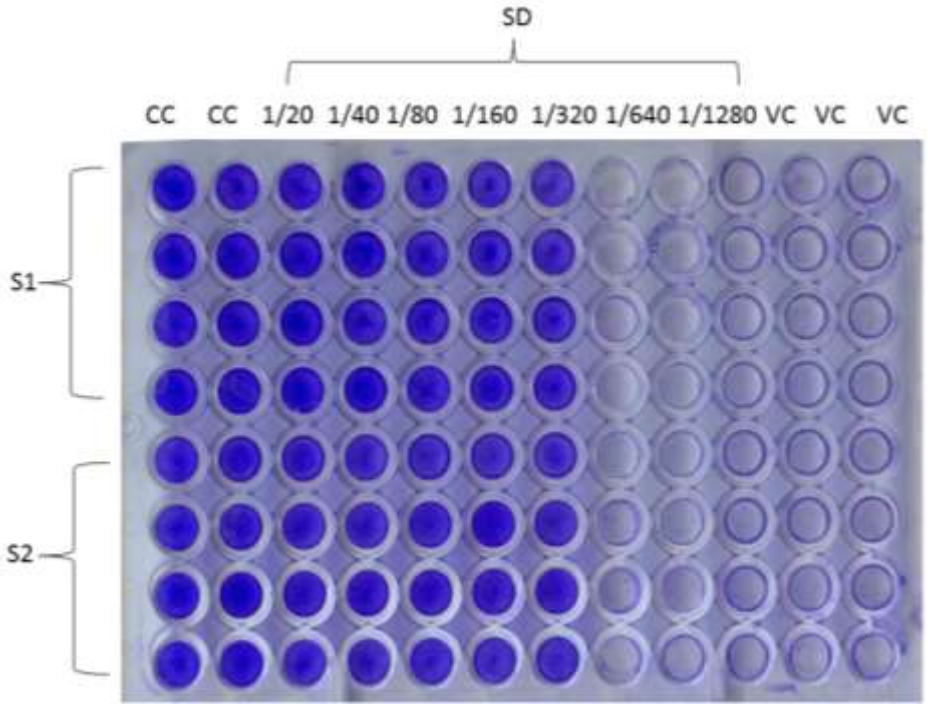


Fig 4.12 Serum neutralization test (SNT) of serum collected on 28th dpi done in quadruplicates. Wherein CC- cell control; VC-virus control; SD- serum dilutions; S1 & S2- serum collected from animal infected with BTV-23 i.e., animal tag no. 28 & 33 respectively.

Table 4.4 Serum Neutralization titres

Animal tag no.	Neutralizing antibody titer 28th dpi
28	320
33	320

4.8 CROSS-NEUTRALIZATION STUDIES

For cross-neutralization studies, serum of 28th day was tested against all available plaque purified serotypes (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 and 24). In these studies, BTV-23 Hyper Immune Serum (HIS) didn't show any cross-neutralization with other available plaque purified serotypes (Fig 4.13).

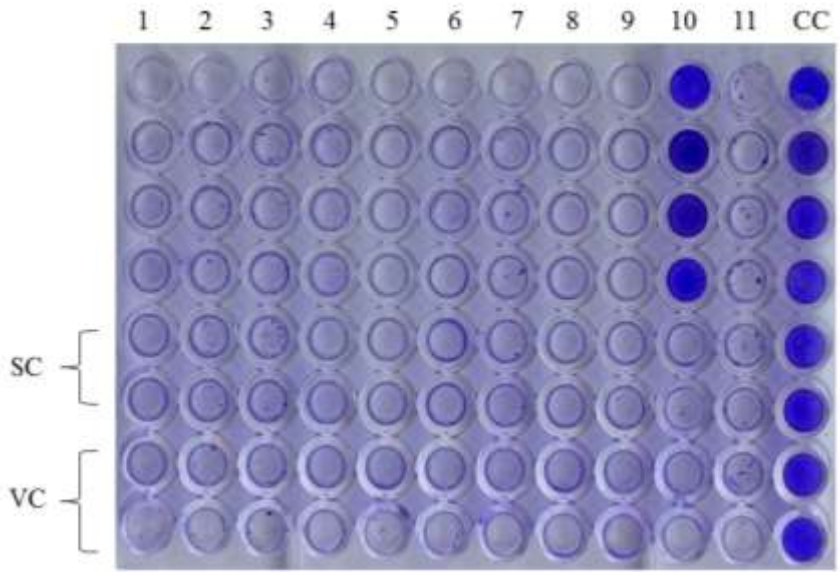


Fig 4.13 Cross Neutralization of BTV-23 HIS with other available serotypes from the serum collected on the 28th day, HIS collected from animal tag no. 28. Wherein CC- cell control; SC-serum from control animal ; VC-virus control; Wells from 1 to 11 infected with – BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 & 24 serotypes respectively.

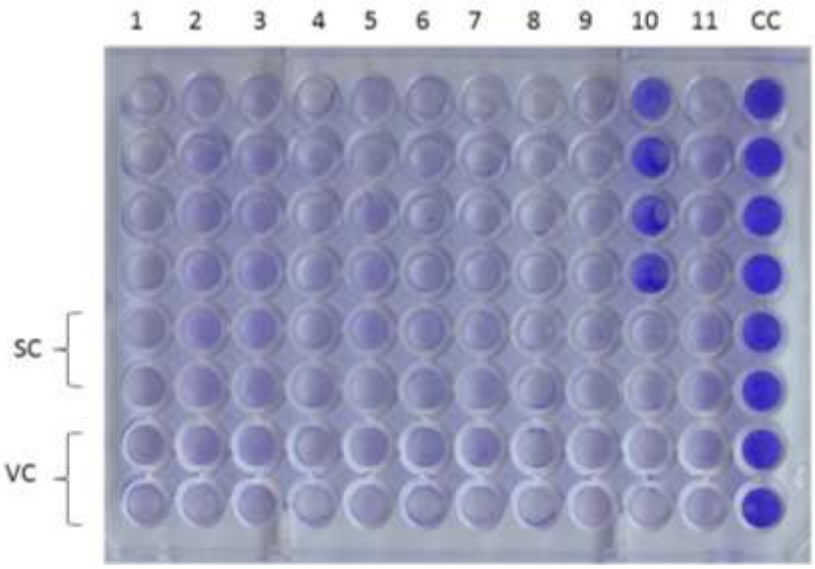


Fig 4.14 Cross Neutralization of BTV-23 HIS with other available serotypes from the serum collected on the 28th day, HIS collected from animal tag no. 33. Wherein CC- cell control; SC- serum from control animal ; VC-virus control; Wells from 1 to 11 infected with – BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 & 24 serotypes respectively.

DISCUSSION

CHAPTER V

DISCUSSION

Bluetongue (BT) is an infectious, non-contagious, haemorrhagic, arthropod transmitted viral disease of domestic and wild ruminants. Sheep are the most susceptible species where severe clinical disease is seen. The disease is caused by the bluetongue virus (BTV), which belongs to the genus *Orbivirus* of the subfamily Sedoreovirinae and family Reoviridae and it is transmitted by several species of *Culicoides* biting midges.

Bluetongue Virus infection causes severe economic losses. Bluetongue is listed under the category of multiple species disease by the Office International des Epizooties (OIE, 2021). Bluetongue is endemic in India; exotic sheep are more susceptible than indigenous and crossbred sheep (Bayliss *et al.,* 2001). However, outbreaks do occur in indigenous breeds as seen in Indian local breeds due to the prevalence of highly virulent strains.

At present 35 serotypes of BTV are recognized worldwide (Ries *et al*., 2021). In India, at least 24 serotypes have been recognized based on serology and virus isolation (Thota *et al.,* 2021). It is controlled mainly by vaccination. Currently, in India, a pentavalent inactivated vaccine having a serotypes composition of BTV-1, 2, 10, 16 and 23 is available (Reddy *et al.,* 2010). From 2017- 2019 prevalence of BTV-1, 2, 4, 5, 12, 16 and 24 was high (Naresh *et al.,* 2020 and Thota *et al.*, 2021). In recent times the prevalence of BTV-23 has rarely been detected. Therefore the current study aimed at cross-neutralization studies of BTV-23 for possible replacement in the vaccine.

The current research work was on plaque purification and cross-protection studies of bluetongue virus serotype-23 (BTV-23).

5.1 PROPAGATION OF BLUETONGUE VIRUS

In the present study, the BTV-23 isolate which was maintained in the BHK-21 cell line previously had been adapted to the Vero cell line. Initially, BTV-23 was passaged 5 times in Vero cells to obtain a high infective titre and used for plaque purification. In the first two passages, a distinct cytopathic effect (CPE) appeared 5-6 days after inoculation. In later passages, CPE was observed within 72 h of incubation which was in agreement with the observations of Krishnajyothi *et al.* (2016). Srikanth Reddy (2018) observed complete CPE at 96 h post-infection in the first two passages and then to 72 h from the third passage. The CPE observed were rounding, clumping of dead cells and finally detaching from the attached surface, these observations were in agreement with Sekar *et al*. (2009).

5.2 PLAQUE PURIFICATION

Bluetongue Virus-23 was plaque purified three times in Vero cells using the agarose overlaying method. Serial 10-fold dilutions of the stock virus were made from 10^{-3} to 10^{-7} dilutions infected on to monolayer of each well of the 6-well plate. After 3 days of incubation suspected plaque areas of minute size were noticed which became more prominent from the 4^{th} day of infection, which was found similar in agreement with Srikanth Reddy (2018), Srinivas *et al.* (2018) and Kesavulu Naidu (2019). Cooper's (1962) observation of the incubation time for the development of plaques was found to be 40 h after infection. This could be due to plaque formation described in the agar-cell suspension method, for the cells which can't form a monolayer.

The plaque morphology observed in this study was almost clear, circular with similar sizes (1-1.5 mm). On prolonged incubation, increase in plaque size was noticed. Dulbecco (1952) observed plaque size with a diameter of 2-4mm. The small

size of plaques might be due to increased agarose concentration (1.2%) in overlay as observed by Howell et al. (1967). In addition, according to Blanchard and Stott (1989) cells grown on 10% FBS in MEM produce small plaques.

Further, almost parallel results but not exact were obtained with Howell *et al.* (1967) and Dulbecco (1952) about the linear relationship between the number of plaques and virus concentration inoculated to monolayer. The plaques were observed better in the wells infected with 10^{-4} dilution stock virus. More no. of plaques developed in 10^{-3} dilution stock virus. Few plaques were observed in 10^{-5} and 10^{-6} virus dilution. Srikanth Reddy (2018) and Srinivas *et al.* (2018) performed viral dilution from 10^{-2} to 10^{-6} and observed 10-12 plaques in 10^{-4} dilution stock virus, but no plaque developed in 10^{-5} and 10^{-6} virus dilution. The plaque morphology and results were similar to Srikanth Reddy (2018), Srinivas *et al.* (2018), and Kesavulu Naidu (2019). Plaque purified BTV-23 was titrated and $TCID_{50}$ was calculated according to Reed and munch's method. The titre obtained was $10^{4.11}$/100µL.

5.3 MOLECULAR CONFIRMATION

Gel electrophoresis of extracted RNA revealed the segmented nature of the genome, which was found similar to the BTV RNA gel electrophoresis in 1% agarose by Thota *et al.* (2021). Group-specific confirmation of BT done with *NS3* gene, amplicon size of 98bp was observed in gel electrophoresis, a result obtained found similar to Sharanya (2018). Serotype confirmation by RT-PCR was conducted by Real-time PCR and conventional PCR using available primers of BTV types (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 and 24). In Real-time PCR amplification observed with BTV-23 primers, C_t value obtained 15.9, was as amplification with other primers was found to be similar to NTC. In convention PCR, PCR product was analyzed by gel

electrophoresis and amplicon size of 1370 bp was observed with BTV-23 specific primers. No amplification was observed with primers specific for other serotypes. Amplification of positive cDNA of each serotype yielded the expected size of products with respective primers. The results are in accordance with the conclusions of Reddy *et al.* (2016), Srinivas *et al.* (2018), and Kesavulu Naidu (2019) regarding VP2-based serotype-specific RT-PCR where VP2 is the BTV outer capsid protein encoded by segment-2 of BTV. VP2 is the major determinant of virus serotype. The sequences of segment-2 vary between different serotypes and the nucleotide sequence of segment-2 is available for BTV-1 to BTV-29. Therefore VP2 based molecular typing assay of BT was developed (Maan *et al*., 2012) and the same was used in this study.

5.4 ANIMAL INOCULATION

Bluetongue Virus seronegative Deccani sheep between the age group of 6m-1yr were selected based on c-ELISA. Rao *et al.* (2016b) mentioned that Deccani sheep are less severely affected, and low mortality and faster recovery we noticed compared to other breeds (Nellore). So deccani breed was selected for the study.

A 2ml dose of $10^{4.11}$ $TCID_{50}$/100µL BTV-23 was inoculated into the sheep in the shoulder region (1mL intradermal and 1mL subcutaneous). Intradermal (I/D) inoculation is similar to the natural route of transmission and it is effective in mounting good immune response. I/D inoculation results in early seroconversion and high antibody titre (Umeshappa *et al*., 2011).

After inoculation with BTV-23, both the inoculated sheep developed pyrexia on 6 dpi with 104.2^{o}F and 104.3^{o}F recorded temperature. Pyrexia was maintained for 4-6 days which was in line with previous observations of Sreenivasulu and Subbarao

(2000). However, pyrexia was not observed in either animal upon booster dose, which might be due to the development of neutralizing antibodies which was found similar in observation by Srikanth Reddy (2018), Srinivas *et al.* (2018), and Kesavulu Naidu (2019). Clinically, both animals exhibited mild signs such as pyrexia at 6 dpi and nasal discharge developed at 10 dpi. Schulz *et al*. (2018) inoculated BTV- 4 in sheep resulted in a mild clinical sign – pyrexia (39.7-40.9^{o} C) between 2dpi-10dpi and nasal discharge, mild cough developed between 6 -10 dpi. These results were in agreement with the Srinivas *et al.* (2018), and Kesavulu Naidu (2019) where mild clinical signs-pyrexia and nasal discharge noticed, whereas Reddy *et al.* (2018) observed clinical signs in inoculated sheep such as pyrexia, nasal discharge edema of buccal region and hyperemia of the buccal mucosa.

These mild clinical signs may be due to the virus attenuation attained upon being passaged in the Vero cell line several times. Similar results were made previously by Eschbaumer *et al.* (2010). Caporale *et al*. (2014) mentioned the experimental inoculation of sheep with cell culture adapted BTV results in mild clinical manifestation. MacLachlan *et al*. (2009) and Verwoerd and Erasmus (2004) mentioned the severity of BT clinical signs and disease depends on the animal species, breed and virus strain infected.

5.5 SERUM NEUTRALIZATION STUDIES

Serum Neutralization Tests were carried out as it is considered the gold standard for typing and also to study the neutralization and cross-neutralization behaviour of plaque purified BTV-23 hyperimmune serum (HIS).

After inoculation of BTV-23 to sheep, serum was collected at the different intervals and SNT was done to confirm sheep were seroconverted and to know the antibody titre. Serum was collected on 7 dpi from infected and control group animals. Serum Neutralization Test was done with the homologous virus and one of the animals (tag no.28) was found to develop antibodies/seroconverted. Other inoculated animal (tag no. 33) didn't develop antibodies by the 7 dpi. The serum is collected at 15 dpi before being given the same dose as a booster. Serum Neutralization Test was done with serum collected at 15 dpi. In the animal tag no. 28, a decrease in antibody was observed based on CPE and crystal violet staining. Animal with tag no. 33 was found to develop antibodies 15 dpi, i.e., it may have seroconverted between 7-15 dpi. These observations were in agreement with Perez de diego *et al*. (2012) who mentioned an increase in antibodies level in sheep vaccinated with BTV-1 till 14 dpi and a later decrease in antibody level was noticed at 20 dpi, following a booster an increase in antibodies level was again observed. After booster vaccination, serum was collected on 28 dpi and SNT was done, it is found that both the animals had developed antibodies and neutralizing antibodies levels increased based on CPE and crystal violet staining, titre was calculated as 1/320 for both the animals. The control animal remained seronegative throughout the entire study. Oura *et al*. (2009) observed sheep infected with BTV showed an undetectable level of neutralizing antibodies after the first vaccination and had high antibody titres after the second vaccination. Srikanth Reddy (2018), Srinivas (2018) and Kesavulu Naidu (2019) observed BTV inoculated sheep seroconverted by 7^{th} dpi and neutralizing antibodies titre were increased and animals remained seropositive during their entire study.

5.6 CROSS-NEUTRALIZATION STUDIES

In cross-neutralization studies, BTV-23 hyperimmune serum didn't cross neutralize the available BTV-1, 2, 4, 5, 9, 10, 12, 16, 21 and 24 serotypes. Erasmus (1990) described the serological relationship of 24 BTV serotypes, based on cross-protection tests in sheep and observed BTV-23 cross-protection towards BTV-8 and 18. Fay *et al.* (2021) observed two-way cross-protection between BTV-23 and BTV-8, and weak cross-protection was seen between BTV-23 and BTV-18. The BTV serotypes 8 & 18 isolates were not taken in the current study and BTV-23 hyperimmune serum didn't cross neutralize with available serotypes.

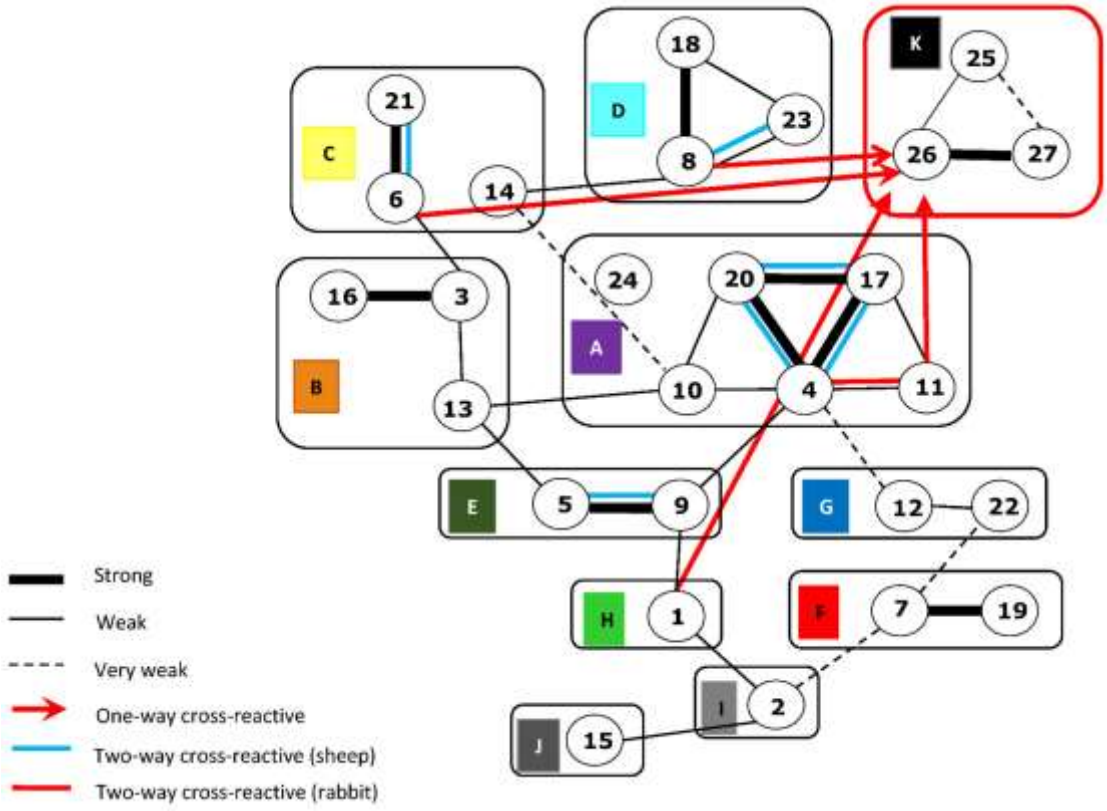


Fig 5.1 Antigenic relationship between BTV serotypes (Fay *et al.*, 2021).

Phylogenetic analysis of seg-2 of 24 BTV serotypes grouped the BTV-23, BTV-18 and BTV-8 into nucleotype-D (Maan *et al*., 2007). Umeshappa *et al*. (2011) immunized sheep with the BTV-1 vaccine, challenged with BTV-23 and observed heterologous cross-protection toward BTV-23. The phylogenetic analysis of VP2 and VP5 protein of BTV-1 is close related to BTV-23. Therefore BTV-1 showed one-way cross-protection to BTV-23. However, since BTV also circulates as different topotypes based on the geographic region, that might have played a role in absence of any cross reactivity of BTV-23 with other tested BTV serotypes, in the current study

The prevalence of BTV serotypes during the year 2002-2011 were BTV-1, 2, 9, 10, 12, 16 and 21 in the state of Andhra Pradesh (Reddy *et al*., 2016). From 2017-2019 BTV prevalence was BTV-1, 2, 4, 5, 12, 16 and 24 (Naresh *et al.,* 2020 and Thota *et al.,* 2021). The prevalence of BTV-8 and BTV-18 were not recorded during recent times and therefore not included in the study.

Conclusion

Bluetongue Virus-23 was successfully plaque purified in Vero cells and on infection into seronegative Deccani sheep elicited mild clinical signs. Further antibody response was noticed from 7 dpi. Bluetongue Virus-23 hyperimmune serum didn’t cross neutralize with available BTV serotypes. Plaque purified BTV serotype was confirmed as BTV-23 by molecular confirmation, and the hyperimmune serum raised will serve as a major tool in future neutralization assays for BTV serotype confirmation.

SUMMARY

CHAPTER VI

SUMMARY

Bluetongue (BT) is an economically important infectious, non-contagious, arthropod transmitted viral disease of domestic and wild ruminants. It is caused by the BTV and is mainly transmitted by some species of *Culicoides* biting midges. Bluetongue virus is a segmented ds-RNA virus belonging to the genus *Orbivirus* under the family Reoviridae. Sheep and white-tailed deer are the most susceptible host. It is placed in the Office International des Epizooties listed multispecies disease (OIE, 2021). In the present study, the BTV-23 serotype was taken for plaque purification and the ability of BTV-23 antiserum to cross-neutralize with other available serotypes (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21 and 24) was studied.

First, BHK-21 adapted BTV-23 serotype was grown in Vero cell line supplemented with 1% MEM to get a high titre and for plaque purification. Vero adapted BTV-23 plaque purification was done and the titre of BTV-23 was calculated as $10^{4.11}$/100 µL. Thus, plaque purified BTV-23 was then used for molecular and serological confirmation.

For molecular typing, extraction of RNA was done from plaque purified BTV-23 cell culture supernatant and subjected to 1% agarose gel electrophoresis, which revealed 10 segmented nature of ds-RNA BTV genome. Then, the BTV-23 was checked against type-specific primers of BTV types (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 and 24) by RT-PCR. Amplification was seen with BTV-23 primers and the size of

the amplicon was 1370 bp, no amplification was observed with the remaining BTV primers. Bluetongue virus-23 plaque purified cell culture fluid was inoculated into two BTV seronegative sheep and control animals inoculated with plain MEM media.

Bluetongue Virus inoculated sheep developed mild clinical signs such as pyrexia (104.2 and 104.3^{o}F) on 6 dpi and nasal discharges on 10 dpi. Sera of blood collected from BTV inoculated and a control animal on 0, 7, 15 and 28 dpi was subjected to SNT. It was revealed that BTV inoculated sheep seroconverted from 7 dpi and remained seropositive throughout the experimental study and control remained seronegative throughout the entire study.

In serum neutralization studies using BTV-23 hyperimmune serum against plaque purified BTV-23 virus, antibody titre was estimated to be 1/320 by 28 dpi. In cross-neutralization studies, BTV-23 hyperimmune serum didn't cross neutralize with any available BTV serotypes (1, 2, 4, 5, 9, 10, 12, 16, 21 and 24). Plaque purified BTV serotype was confirmed as BTV-23 by molecular confirmation and the hyperimmune serum raised will serve as a major tool in future neutralization assays for BTV serotype confirmation and cross neutralization studies.

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