

CHARACTERIZATION OF BLUETONGUE VIRUS -12 (BTV-12)

By

J. SRINIVAS

B.V.Sc. & A.H

RVM/15-61

**MASTER OF VETERINARY SCIENCE
(VETERINARY BIOTECHNOLOGY)**



**DEPARTMENT OF VETERINARY BIOTECHNOLOGY
COLLEGE OF VETERINARY SCIENCE, RAJENDRANAGAR
P.V. NARSIMHARAO TELANGANA VETERINARY UNIVERSITY
HYDERABAD.**

FEBRUARY, 2018

CHARACTERIZATION OF BLUETONGUE VIRUS-12 (BTV-12)

By

J. SRINIVAS

B.V.Sc. & A.H

RVM/15-61

**THESIS SUBMITTED TO
P.V. NARSIMHARAO TELANGANA VETERINARY UNIVERSITY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF
MASTER OF VETERINARY SCIENCE
(VETERINARY BIOTECHNOLOGY)**



**DEPARTMENT OF VETERINARY BIOTECHNOLOGY
COLLEGE OF VETERINARY SCIENCE, RAJENDRANAGAR
P.V. NARSIMHARAO TELANGANA VETERINARY UNIVERSITY
HYDERABAD.
FEBRUARY, 2018**

CERTIFICATE

I certify that **Mr. JAKKULA SRINIVAS** has satisfactorily prosecuted the course of research and that the thesis entitled "**CHARACTERIZATION OF BLUETONGUE VIRUS -12 (BTV-12)**" submitted is the result of original research work 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 him for a degree of any University.

Date:

3/2/18

Place: Hyderabad

Handwritten signature of Dr. P. Kalyani in blue ink, with the date 3/2/18 written below it.

Dr. P. KALYANI

(Major Advisor)

CERTIFICATE

This is to certify that the thesis entitled "**CHARACTERIZATION OF BLUETONGUE VIRUS-12 (BTV-12)**" submitted in partial fulfilment of the requirements for the degree of **MASTER OF VETERINARY SCIENCE** of the **P.V. NARSIMHA RAO TELANGANA VETERINARY UNIVERSITY, HYDERBAD**, is a record of the bonafide research work carried out by **JAKKULA SRINIVAS** under my 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 for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of the investigations have been duly acknowledged by the author of the thesis.



Dr. P. KALYANI

Chairman of the Advisory Committee

Thesis approved by the Student's Advisory Committee.

Chairman: Dr. P. KALYANI

Department of Veterinary Biotechnology
College of Veterinary Science
Rajendranagar, Hyderabad-500030



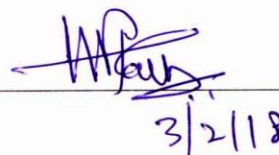
Member: Dr. Y. NARASIMHA REDDY

Professor and Head
Department of Veterinary Microbiology
College of Veterinary Science
Rajendranagar, Hyderabad-500030.



Member: Dr. M. RAMAKOTI REDDY

Principal Scientist
Directorate of Poultry Research
Rajendranagar, Hyderabad-500030.



LIST OF CONTENTS

CHAPTER NO.	TITLE	PAGE NO.
I	INTRODUCTION	1-3
II	REVIEW OF LITERATURE	4-23
	2.1 Bluetongue	4
	2.2 Epidemiology of bluetongue	4
	2.2.1 History	4
	2.2.2 Distribution	4
	2.2.3 Host	5
	2.2.4 Transmission	5
	2.2.5 Clinical signs	5-6
	2.2.6 Bluetongue virus	6-7
	2.2.6.1 Outer capsid proteins	7
	2.2.6.2 Inner capsid (core) proteins	8
	2.2.6.2.1 Major core proteins	8
	2.2.6.2.2 Minor proteins	8-9
	2.2.6.3 Non-structural proteins	9
	2.3 Propagation of BTV in cell cultures	10-11
	2.4 Plaque purification	11-13
	2.5 Typing of BTV isolates	13
	2.5.1 Molecular typing	14-15
	2.5.2 Serological typing	16-17
	2.5.2.1 Cross Neutralization Studies	17-18
	2.6 Animal experimental studies	18-20
	2.7 VP2 gene sequencing analysis	20-21

	2.8 Growth characters of bluetongue virus in cell cultures	22-23
III	MATERIALS AND METHODS	24-43
	3.1 Cell culture material	24
	3.1.1 Glassware and plastic ware	24
	3.2 Cleaning and sterilization	24
	3.2.1 Glassware	24
	3.2.2 Plastic ware	24-25
	3.2.3 Liquid material	25
	3.3 Preparation of essentials	25
	3.3.1 Media	25
	3.3.2 Phosphate buffer saline	25
	3.3.3 Trypsin (0.1%)	26
	3.3.4 10% formalin	26
	3.3.5 1% crystal violet solution	26
	3.3.6 Trypan blue	26
	3.3.7 Sea plaque agarose	26
	3.4 Cell lines	26
	3.4.1 Maintenance of cell lines	27
	3.4.2 Cell count	27
	3.4.3 Cell seeding	27
	3.5 Bluetongue virus serotype-12	27
	3.5.1 Adaptation of BTV-12 to Vero cell line	27-28
	3.6 Titration of the virus	28
	3.7 Plaque purification of BTV	28-29
	3.7.1 Confirmation and selection of the plaques	29

3.7.2 Molecular confirmation of plaque purified virus	30
3.8 Freeze thawing of cell culture fluid	30
3.8.1 RNA isolation	30-31
3.8.2 Reverse transcription polymerase chain reaction (RT-PCR)	31
3.8.2.1 Complementary DNA synthesis (c DNA)	31
3.8.2.2 PCR set up	32-34
3.9 Agarose gel electrophoresis	35
3.10 Titration Of Virus	36
3.10.1 Protocol for crystal violet staining	36-37
3.11. Selection Of Animals	37-38
3.11.1 Housing of experimental animals	39
3.11.2 Dose and route of inoculum	39
3.11.3 cELISA for serum samples	39
3.12. Serum neutralization	39-40
3.13 VP2 gene sequencing	40
3.13.1 Primers	40
3.13.2 Polymerase chain reaction	40
3.13.3 Agarose gel electrophoresis	41
3.13.4 Purification pcr product	41
3.13.5 Estimation of DNA concentration	41
3.13.6 DNA sequencing	42
3.13.7 Phylogenetic analysis	42
3.14 Growth characters of BTV-12 in cell lines	42
3.14.1 Fixation	42
3.14.1.1 Cleaning of cover slips	42

	3.14.1.2 Sterilization of cover slips	43
	3.14.1.3 Gelatin –coating protocol	43
	3.14.1.4 Seeding	43
	3.14.2 Fixing and staining of cells	43
IV	RESULTS	44-75
	4.1 Maintenance of cell lines	44
	4.2 Vero cell line infection	44
	4.3 Titration of virus	44
	4.4 Plaque purification of BTV	46-48
	4.5 Molecular confirmation of plaque virus	48
	4.5.1 Gel electrophoresis of RNA	48
	4.5.2 Confirmation Of Plaque Purified BTV -12 Reverse transcription polymerase chain reaction (RT-PCR)	48
	4.6 Titration of virus	53
	4.7 Animal inoculation	54
	4.7.1 Signs and lesions	54
	4.7.2 Antibody response to virus inoculation	55
	4.8 Neutralization studies	55
	4.9 VP2 gene sequencing and phylogenetic analysis of BTV-12 VP2	59
	4.9.1 Sequencing	59
	4.9.2 Sequence analysis	66
	4.9.2.1 Multiple sequence alignment	66
	4.9.2.2 Phylogenetic analysis	71
	4.10 BTV-12 in Vero cells	73
V	DISCUSSION	76-81
	5.1 Propagation of bluetongue virus	76

	5.2 Plaque purification	76-77
	5.3 Molecular confirmation	77
	5.4 Animal inoculation	77-78
	5.5 Serum neutralization studies	78-80
	5.6 VP2 gene sequencing and phylogenetic analysis of BTV12 VP2	80-81
	5.7 Growth characters of BTV-12 in Vero cells	81
VI	SUMMARY	82-83
	LITERATURE CITED	84-97

LIST OF TABLES

Table No.	Particulars	Page No
1	Segment 2 specific Primers sequence (5'-3') used for PCR	33-34
2	Infection of 96-well plate for TCID50 calculation	36
3	Titration of BTV-12 in Vero cells	46
4	TCID50 calculation of Plaque purified BTV-12 serotype	53
5	TCID50 titres of available BTV types	54
6	Immune response in experimental animals(by cELISA)	55
7	Serum Neutralization titres	56
8	Cross neutralization studies	56

LIST OF ILLUSTRATIONS

Figure No	Title	Page No
1	Vero Cell Monolayers	45
1.A	Healthy monolayer (100X)	45
1.B	Cells infected with BTV12 (100X)	45
2	Plaque Formation In Vero Cell Monolayer Infected With BTV-12	47
2.A	Single individual plaque surrounded with confluent healthy monolayer (40X)	47
2.B	Spreading plaque (40X)	47
2.C	Plaque at initial stage (100X)	47
2.D	Spreading plaque (100X)	47
3	Crystal violet staining for plaques	49
3.A	Crystal violet staining of monolayer showing plaques (upper view)	49
3.B	Crystal violet staining of monolayer showing plaques (bottom view)	49
4	Agarose gel electrophoresis of plaque purified BTV-12 RNA	50
5	MOLECULAR TYPING OF BTV -12	51-52
5.A	Gel electrophoresis of BTV-4, 10, 12 and 24	51
5.B	Gel electrophoresis of BTV-1, 2, 9, 16 21 and 23	52
6	Clinical symptoms of BT in experimental inoculated sheep	57
6.A	Infected sheep was showing nasal discharges on 13dpi.	57

6.B	Control animal without nasal discharges	57
7	Temperatures record of experimental animals	58
8.A	Chromatogram of forward sequence	60-62
8.B	Chromatogram of reverse sequence	63-65
9	Clustal W Multiple nucleotide sequence alignment of BTV 12 (isolated from the current study) with partial sequences of other bluetongue viruses (obtained from Gene Bank)	67-70
10	Phylogenetic analysis of BTV-12 India/ 2017	72
11	Cytopathic effect of BTV 12 in Vero cell line(H & E staining)	74-75
11.A	Monolayer after 24hrs.PI (100X)	74
11.B	Monolayer after 24hrs. PI (40X)	74
11.C	Rounding and clumping of infected monolayer after 48 hrs. PI (100X)	74
11.D	Rounding and clumping of infected monolayer after 48 hrs .PI (40X)	74
11.E	Complete detachment of infected monolayer after 72 hrs. PI (100X).	75
11.F	Complete detachment of infected monolayer after 72 hrs. PI (40X).	75
12	Phylogenetic analysis of BTV12 VP2	79

ACKNOWLEDGEMENTS

*Fervently and modestly, I extol the genuine co-operation and affection offered to me by my venerable philosopher and guide **Dr.P.KALYANI**, Assistant Professor, Department of Veterinary Biotechnology, College of Veterinary Science, Rajendranagar, Hyderabad, PVNR TVU, from the initiation of the work to the ship-shaping of the manuscript. The present work bears at every stage the impression of her work counsel, sustained interest, careful and seasonal criticism and meticulous attention to details, for without her guidance and valuable suggestions, this work could not have seen the light of the day. It was indeed a rare privilege for me to work under her unending inspiration and indomitable spirit.*

*I deem it my privilege in expressing my heartfelt gratitude to the members of the Advisory Committee **Dr .Y. Narasimha Reddy**, Professor and University Head, Department of Veterinary Microbiology, College of Veterinary Science, Rajendranagar, for his suggestions, generous help, affectionate guidance, constant encouragement and deliberate counsel during the course of investigation and execution of the thesis.*

*I am extremely grateful to have been guided by **Dr.M. Ramakoti Reddy**, Principal Scientist, Directorate of poultry research , Rajendranagar, Hyderabad, the member of my advisory committee for supporting me in every aspect of my research.*

*I am extremely thankful to **Dr.K.Dhanalakshmi**, Associate Professor, Department of Microbiology, College of Veterinary Science, Rajendranagar, Hyderabad, for her valuable suggestions during my research work.*

*I would like to thank **Dr.M.Srinivas, and Dr.K.Kavitha**, Assistant Professors, Department of Microbiology, College of Veterinary Science, Rajendranagar, for their valuable suggestions and constant words of encouragement during this research work.*

*I owe my humble and heartfelt thanks to **Sunil Ragunath Patil SRF** at Department of Veterinary Biotechnology who helped me during my entire research work by giving ideas to solve the problems.*

*I would like to thank **B.Sushmitha R.A** at Department of Microbiology, for her concern, moral support and timely help during the investigation.*

*I am very much thankful to **Dr.M.Srikanth Reddy**, CTF, Department of Veterinary Microbiology, Hyderabad, for his response towards my doubts at any time with patience.*

*I am highly thankful to **Dr.N.Ramesh**, Ph.D scholar, Department of Veterinary A.H &Extension, IVRI, for his help, care, encouragement and valuable advices during this work.*

*I humbly express my thanks to **Dr.J.Raju**, Ph.D scholar, Department of Animal Nutrition, Hyderabad for their cooperation and help.*

*I would like to thank my seniors **Dr.Shiva Jyothi,Dr.B.Rajashekar, Dr.Santhosh, Dr.Yashitha,Dr.Shirisha, Dr.Muzzer, Dr.Jahangeer, Dr.Samatha, Dr.Jyothsna** and juniors **Dr.Keshav Naidu, Dr.Sharnaya, Dr.Sairam Sandeep, Dr.Purushotham, and Dr.Abilash** for their suggestions and cooperation.*

*I am happy to remember my colleagues **Dr.Raju,Dr.Himaja, Dr.Swathi, Dr.Sravan and Dr.Swathi** for their cooperation and moral support during execution of work.*

*I acknowledge the help and assistance of **Sudarshan, Mohan Krishna ,Savitri, Mahender** and **Ananthaiah**, Non-teaching staff of Department of Veterinary Microbiology and Veterinary Biotechnology.*

*It gives me immense pleasure to express my profound sense of gratitude and sincere thanks to **Dr.L. Atchamamba**, Assistant Professor,(Library & Information Science) and staff of library **Rajesh** and **Kishore** of College Library for their help in providing the necessary facilities to carryout research work.*

*A special thanks to **Dr. Venkatesh**, P.G.Scholar, Department of Veterinary pathology, Hyderabad for his cooperation and help.*

*A very special thanks to my juniors **Dr. G.Naresh Yadhav**, **Dr.Anesh, karunakar,Dr.Sravan, Akhil and Saiprasad** for their cooperation and moral support especially during animal experiment without whom it is difficult to me.*

*I am very glad to acknowledge the encouragement, support and help offered by my friends **Dr.Anil**, **Dr.Pothanna**, **Dr.Shiva Rama Krishna**, **Dr.Venkatesh**, **Dr.Manohar** **Dr.Narendhar,Dr.Ashok,Dr.Madhu,Dr.Venumadhav,Dr.sampath** and **Dr.Visheshwar**.*

*It is pleasure to express my adorance to my beloved parents **Mr.J.Rajamouli** and **Smt. J.Mallikamba** for making me capable of reaching this peak of success and upbringing me with precious value. It is a cherish to express my deep sense of love to my Brother **Nagaraju** and my lovely sister **Gouthami**for providing me strength to pursue my study.*

(J.SRINIVAS)

LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
μ	Micron
μg	Microgram
μl	Microliter
°C	Degree Celsius
°F	Fahrenheit
AGID	Agar gel immunodiffusion
BHI	Brain Heart Infusion
BHK	Baby Hamster Kidney
BT	Bluetongue
BTV	Bluetongue virus
Bp	base pair
cDNA	Complementary deoxy ribo nucleic acid
cELISA	Competitive ELISA
CO ₂	Carbon dioxide
CPE	Cytopathic Effect
DMEM	Dulbecco's modified Eagle's medium
dNTP	Deoxynucleotide triphosphate
ds RNA	Double stranded Ribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic acid
ELISA	Enzyme linked immune sorbant assay
F	Forward

FBS	Foetal Bovine Serum
Fig.	Figure
FMDV	foot-and-mouth disease virus
G	Gram
hrs.	Hours
I.U	International Unit
Kbp	Kilo base pair
KCl	Potassium chloride
KH ₂ PO ₄	Potassium dihydrogen phosphate
MEM	Minimum essential medium
Mg	Milligram
MgCl ₂	Magnesium chloride
ml	Milliliter
mm	Millimeter
mM	milli molar
Min.	Minutes
MOI	Multiplicity of infection
Mol.wt	Molecular weight
MTSN	Microtitre serum neutralization
MuMLV	Moloney murine leukemia virus
NS	Non-structural proteins
NaCl	Sodium chloride
NaHCO ₃	Sodium bi carbonate
Na ₂ HPO ₄	Disodium hydrogen phosphate
NDV	New castle disease virus

OD	Optical Density
OIE	Office International des Epizooties
OPD	o-Phenylenediamine dihydrochloride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
pH	Potential of Hydrogen
PPRV	peste des petits ruminants virus
PI	Percentage inhibition
Rpm	Revolutions per minute
R	Reverse
RBC	Red blood cells
RNA	Ribonucleic Acid
RT	Reverse transcription/ Reverse transcriptase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
SDA	Sabouraud's Dextrose Agar
SNT	Serum neutralization test
TAE	Tris acetate EDTA
TCID50	Tissue culture infective dose50
T25	25cm2 tissue culture flask
VNT	Virus neutralization Test
VP	Viral protein
WEE	Western equine encephalomyelitis

w/v	Weight/Volume
-----	---------------

NAME OF THE AUTHOR : J.SRINIVAS

TITLE OF THE THESIS : CHARACTERIZATION OF
BLUETONGUE VIRUS -12(BTV-12)

DEGREE TO WHICH IT IS SUBMITTED : MASTER OF VETERINARY SCIENCE

FACULTY : FACULTY OF VETERINARY SCIENCE

DISCIPLINE : VETERINARY BIOTECHNOLOGY

MAJOR ADVISOR : Dr .P. KALYANI

UNIVERSITY : P.V. NARSIMHA RAO TELANGANA
VETERINARY UNIVERSITY

YEAR OF SUBMISSION : 2018

ABSTRACT

Bluetongue (BT) is an infectious, non-contagious disease of ruminants and camelids transmitted by culicoides biting midges. It is caused by bluetongue virus (BTV), a segmented double-stranded RNA virus of genus Orbivirus under family Reoviridae; and placed in the Office International des Epizooties (OIE) of diseases (Sperlova and Zendulkova, 2011). It has been recognized to exist as 29 different serotypes until now (Maan et al., 2016).

The present study was taken up with the objective of plaque purification of BTV-12 isolated from Kurnool in 2016 and its characterization. Animal experimentation was carried out in sheep by inoculating Vero cell adapted BTV-12 to study its pathogenicity,

immune response to it and also to find out any cross reactions between BTV-12 with other serotypes available in the department (BTV-1, 2, 4, 9, 10, 16, 21, 23 and 24). Sequence analysis of partial VP2 segment of BTV-12 and growth characters of BTV-12 in Vero cell line were also included in this study.

BTV-12 isolate adapted to Vero cell line had a titre of $10^{6.116}$ /ml. The plaque purified virus revealed 10 segmented genome on agarose gel electrophoresis. Plaque purification was confirmed since RT-PCR amplified only a product of 750bp with BTV-12 specific primers. The purified virus was not amplified by other primers specific for BTV-1, 2, 4, 9, 10, 16, 21, 23 and 24.

Plaque purified virus was inoculated into one BTVseronegative sheep with a dose of 1ml of $10^{6.116}$ /ml TCID₅₀. The animal exhibited mild clinical symptoms including pyrexia (103.8°F-105.1°F) and nasal discharges on 13dpi.

Further, this animal developed BTV antibodies as measured by cELISA from 7 days of post inoculation and remained seropositive throughout the experimental period of 28 days. Serum was also tested for type specific BTV-12 antibodies by SNT. It was found that titres ranged from 1/40 on 7 dpi. to 1/320 by 28 dpi. However BTV-12 hyper immune serum from infected animals was also found to cross neutralize BTV-1. This needs further studies.

Further, partial VP2 gene of BTV 12 was sequenced and analysed. Phylogenetic analyses revealed that BTV-12 of the current study had closest sequence similarities with the Indian and South Africa isolates of BTV-12 available in Gene Bank.

Infection of BTV12 in Vero cell line revealed that CPE started to appear by 48hrs post infection with complete detachment of the monolayer taking place by 72hrs post infection.

CHAPTER-I

INTRODUCTION

Agriculture is the primary source of income in India where nearly 70% of the Indian population depends on it. Agriculture makes the highest contribution of 15.1% to Indian GDP. Livestock sector alone with its 4.1 % contribution to the agriculture GDP, became the backbone of Indian agro economy (Islam *et al.*, 2016).

In this context, sheep plays an important role in the livelihood of a large percentage of small and marginal farmers, landless labourers engaged in sheep rearing. A number of rural based industries use wool and sheep skins as raw material. In India sheep are mostly maintained on natural vegetation on common grazing lands, wastelands and uncultivable lands, stubble of cultivated crops and top feeds (tree lopping). Sheep are mostly reared for wool, meat and milk. Sheep skin and manure constitute important source of earning, the latter particularly in southern India.

A number of viral diseases affects sheep; importantly sheep pox, goat pox, Peste des Petitis Ruminants (PPR), foot and mouth disease (FMD) and bluetongue (BT), among which BT is one of the most economically important diseases of sheep.

Bluetongue (BT) is an infectious, non-contagious arthropod borne viral disease of wild and domestic ruminants caused by bluetongue virus (BTV) the type species of the genus Orbivirus and belongs to the family Reoviridae (Mertens *et al.*, 2004). BTV infects most of the domestic and wild ruminant and causes BT disease primarily in sheep characterised by severe clinical signs such as fever, lameness (coronitis), swelling

and cyanosis of lips and tongue. It is listed as 'notifiable by office international des Epizooties (OIE, 2006).

BTV's genome consists of ten segments of double-stranded RNA (dsRNA) encoding 7 structural proteins (VP1 to VP7) organised into two concrete shells i.e., the inner and outer capsid and 4 non-structural proteins (NS1, NS2, NS3 and NS3a) (Roy 2008).

Currently, a total of 29 serotypes of BTV are recognized worldwide (Maan *et al.*, 2016). In India, 23 serotypes have been recognized based on serology and/ or virus isolation (Sairaju *et al.*, 2013). Recently, Krishnajyothi *et al.* (2016) isolated BTV-24 from an outbreak during 2010 in Telangana State and Hemadri *et al.* (2016) isolated BTV-5 from Karnataka state.

In endemic areas such as the southern parts of India, where mixed infections with more than one serotype have been commonly observed, it is mandatory to plaque purify the samples and typing of plaque purification virus by Virus neutralization test (VNT) (Reddy *et al.*, 2015).

For typing of BTV molecular techniques such as RT-PCR are available but the major limitation of RT-PCR is variation in sequence between and within a toptotype (Maan *et al.*, 2012a and Susmitha *et al.*, 2012). Due to genetic diversity observed within and between serotypes and toptotypes of BTV, primers designed based on some strains may fail to detect other strains. For example, BTV-2 primers designed based on the eastern toptotype virus of Andhra Pradesh could not detect a BTV-2 virus isolated from the neighbouring state of Tamil Nadu in 2003, for which a separate primer set based on western toptotype of BTV had to be designed (BTV-2W). Therefore, a combination of RT-PCR with other tests such as VNT may be required to resolve the issues arising due to RT-PCR based typing (Reddy *et al.*, 2015).

For serological typing of BTV, type-specific sera raised against reference viruses are used in VNT but some of the Asian viruses (BTV-3 and BTV-9) differ substantially from the reference viruses (Maan et al., 2012b;). For example, VP2 gene of Indian BTV-9 has 70% similarity with both BTV-9 and BTV-5 reference viruses. Further, the isolate could not be neutralized by sera to reference BTV-9. Therefore, it is essential to characterize individual isolates and raise type-specific sera against different serotypes isolated locally (Rao et al., 2014).

Keeping these factors in view, the present study was taken up with the following objectives:

1. Propagation of BTV-12 (Isolated from Kurnool (7/KNL/2016) in Vero cell line.
2. Plaque purification of BTV-12 and confirmation by molecular and serological methods.
3. Sequencing of segment 2 of BTV-12.
4. Comparison of VP2 sequence of BTV-12 with available sequences in Gene Bank.
5. Growth characters of BTV-12 in cell lines.

CHAPTER-II

REVIEW OF LITERATURE

2.1 BLUETONGUE

Bluetongue (BT) is an infectious, non-contagious disease of ruminants and camelids transmitted by *Culicoides* biting midges. It is caused by the bluetongue virus (BTV) and is placed in the Office International des Epizooties (OIE) of diseases (Sperlova and Zendulkova, 2011).

2.2 EPIDEMIOLOGY OF BLUETONGUE

2.2.1 HISTORY

BT was initially reported at the end of the 18th century in South Africa after an import of fine-wool sheep from Europe (Spreull 1905) and was described as “Malarial Catarrhal Fever” or “Epizootic Catarrh of Sheep” (Hutcheon 1902 and Spreull 1905). The name bluetongue” originated from the African term “bloutong”, which was used to describe the distinctive cyanotic tongue of severely affected sheep (MacLachlan et al., 2009).

2.2.2 DISTRIBUTION

Bluetongue virus occurs throughout temperate and tropical areas of the world, between latitudes 40°S and 53°N where *Culicoides* midges are present (Schwartz-Cornil et al., 2008). The outbreak of BT depends on the diverse climatic conditions, population and breeds of sheep. In India, BT outbreaks were observed in exotic and cross breed sheep but recent outbreaks were observed in native sheep breeds (Chand et al., 2015). In

India, first outbreak of BT in sheep and goats from Maharashtra state was reported by Sapre (1964).

2.2.3 HOST

The vertebrate hosts for BTV include both domestic and wild ruminants, such as sheep, goat, cattle, buffalo, deer, most species of African antelope and other Artiodactyla such as camels. Although antibodies to BTV and virus antigen or nucleic acid or live virus have been demonstrated in some carnivores, felids, black and white rhinoceroses and elephants. The role of non-ruminant species in BTV epidemiology is considered minimal. However a higher incidence of clinical disease has been observed in cattle infected with BTV 8 in Europe. Some breeds of sheep are more susceptible to disease than others. With the result, in some countries BTV infections of livestock can occur unobserved and be detected only by active surveillance (Daniels *et al.*, 2004).

2.2.4 TRANSMISSION

The vector for BTV was first reported as *Culicoides* biting midges after it was first isolated from *Culicoides* *simicola* in South Africa (Du Toit 1944). This finding was later confirmed by Walker and Davis (1971), Braverman *et al.* (1981) and Mellor (1990). Though there are 1300-1400 species of *Culicoides* (Mellor *et al.*, 2000), BT is naturally transmitted by only 30 *Culicoides* species (Meiswinkel *et al.*, 2008).

Mellor *et al.* (2000) reported that these midges are most active from one hour before sunset to one hour after sunrise. The virus in vectors can replicate at a temperature above 15°C. The virus shows the highest activity in the temperature range from 13°C to 35°C (Tweedle and Mellor, 2002).

2.2.5 CLINICAL SIGNS

Bluetongue outbreaks were recorded as acute or sub-acute form among ruminants and is characterized by congestion, edema and hemorrhages (Sellers 1981). The incubation period of BT is 6 - 9 days (Gibbs and Greiner 1988). The first sign of the disease is rise in body temperature (104-108 °F), which continues for 5 -7 days. During the early febrile reaction there is rise in respiration rate, hyperaemia and swelling of buccal, nasal and ocular mucosa (Sapre 1964 and Harbola et al., 1982). There is catarrhal nasal discharge and frothy salivation from mouth. The nasal discharge may be serous, muco-purulent or bloody. Swelling of lips, licking of lips and nostrils is followed by cracking of epidermis at the commissures of lips along with oedema of face, sub-maxillary space and occasionally ears and eyelids. The mucosa of gums, cheeks and tongue may ulcerate. Tongue becomes swollen, markedly congested and may protrude from the mouth. Because of the cyanotic appearance of the tongue (Spreull 1905; Theiler 1906 and Bekkeret *al.*, 1934), the name bluetongue has been assigned to the disease. But this sign is transitory and may be often missed. The other related clinical signs in acute cases are arching of the back muscle stiffness, lethargy and anorexia. Later the affected animals develop marked lameness due to coronitis with petechial haemorrhage around the coronary band and periople (Gambles 1949 and Moulton 1961).

Cattle were naturally infected with BTV but generally showed no clinical signs (Luedkeet *al.*, 1970). But viremic bulls might shed BTV in the semen (Bowne et al., 1985). Recent reports in cattle showed symptoms of reproductive disorders and fetal abnormalities as per reports. (Dal Pozzoet *al.*, 2009 and Santman-Berendset *al.*, 2010).

2.2.6 BLUETONGUE VIRUS

Bluetongue virus, a segmented double-stranded RNA virus of genus Orbivirus under family Reoviridae, has been recognized to exist as 29 different serotypes until now (Maan *et al.*, 2016).

The virus is a non-enveloped virus with a genome of approximately 19,200 base pairs composed of ten linear segments of double-stranded RNA (dsRNA). These 10 dsRNA segments are packaged within a triple-layered icosahedral protein capsid (approximately 90 nm in diameter) (Huismans *et al.*, 1981).

The viral RNA is enclosed by a double capsid protein shell, the outer shell consists of two polypeptides (VP2, VP5), the inner core contains two major (VP3, VP7) and three minor (VP1, VP4, VP6) polypeptides (Verwoerd *et al.*, 1972) and Non-structural proteins (NS1, NS2, NS3 and NS3A) probably participate in the control of BTV replication, maturation and export from the infected cell (Eschbaumer *et al.*, 2009).

2.2.6.1 OUTER CAPSID PROTEINS

VP2

It is the outermost BTV protein forming a large triskelion on the virus particle surface (Hewat *et al.*, 1992) which is coded by the genomic segment-2 (Mertens *et al.*, 1984) and is responsible for receptor binding, hemagglutination and eliciting serotype-specific neutralizing antibodies (Hassan *et al.*, 1999).

VP5

It is the smallest of the outer capsid proteins and the second most variable of the BTV genome segments (Maan *et al.*, 2008). It is coded by genomic segment 6 (Verwoerd *et al.*, 1972). It also influences the virus serotype (Demaula *et al.*, 2000) and also possess membrane fusion activity (Hassan *et al.*, 2001). VP2 and VP5 can

influence the specificity of reactions between the virusparticle and the neutralizingantibodies (Cowley and Gorman, 1989; Mertens *et al.*, 1989).

2.2.6.2 INNER CAPSID (CORE) PROTEINS

2.2.6.2.1Major core proteins

VP3

It is coded by L3 RNA segment and was found to be highly conserved among BTV serotypes (Ghiasi *et al.*, 1985). It plays important role in the structural integrity of the virus core (Hassan *et al.*, 2001).

VP7

VP7 is coded by small RNA segment7 (S7). This is the major protein which comprises almost 1/3rd of the total protein of BTV. It is the main component of the capsomeres on the surface of the BTV core particle (Huismans *et al.*, 1987b)and contains group- specific antigenic determinants (Huismans& Erasmus 1981).

2.2.6.2.2 Minor core proteins

VP1

It is encoded by L1 genome segment and has been suggested to act as viral RNA polymerase involved in elongation of RNA (Roy *et al.*, 1988 and Urakawa *et al.*, 1989).

VP4

It is the second minor protein of inner core virions and is coded by M4 segment which is known to have guanylyltransferase, transmethylese1 and transmethylese 2 activities, that catalyses the capping of BTV mRNA (Mertens *et al.*, 1992).

VP6

Nucleic acid binding protein coded by the segment 9 of BTV genome, has a molecular mass of 35 KDa (Roy *et al.*, 1990). It acts by ATP binding activity and displays RNA-dependent ATPase and helicase activities (Stäuber *et al.*, 1997).

2.2.6.3 NON-STRUCTURAL PROTEINS

NS1

Is the major non-structural protein encoded by highly conserved genome segment M6. Polymerization of NS1 produces large numbers of high molecular weight tubular structures in the infected cells (Huismans and Els 1979). The presence of these tubules is characteristic feature of BTV replication in infected cells (Urakawa and Roy 1988).

Hwang *et al.* (1993) reported that NS1 protein could be used as a group specific diagnostic reagent. NS1 found to be an essential protein involved in virus replication and morphogenesis (Owens *et al.*, 2004).

NS2

Is the only viral encoded protein that is phosphorylated in BTV infected cells (Grubman *et al.*, 1983 and Huismans *et al.*, 1987a). It is an important component of the matrix of viral inclusion bodies (VIB). These are the sites of viral replication and

assembly (Eaton *et al.*, 1988). It has high affinity for ss RNA and plays an active role in replication and encapsidation (Huismans *et al.*, 1987c).

NS3 and NS3A

These two non-structural proteins are indeed related to each other and that both are encoded by the S10 gene (French *et al.*, 1989) and help in release of BTV virion from the insect cells (Hyatt *et al.*, 1993).

2.3 PROPAGATION OF BTV IN CELL CULTURES

McPhee *et al.* (1982) observed replication of Australian BTV serotype 1 and 20 in BHK-21, Vero, foetal ovine lung, mouse fibroblast, *Aedes albopictus* cells, and 11 day old chicken embryos. At high multiplicity of infection (MOI) all vertebrate cell lines showed a clear cytopathic effect (CPE) whereas at low MOI, CPE was almost absent in all except BHK-21 cells. The invertebrate cell line (*Aedes albopictus*) showed no signs of CPE at higher or lower MOI. BHK-21 cells produced much higher yields of virus (1x10⁸ PFU/0.2 ml) than other cell lines.

Ghalib *et al.* (1985) reported that BHK-21 cells grown in Eagle's MEM supplemented with 10% FCS and 10% tryptose phosphate broth and antibiotics. When washed twice with medium supplemented with antibiotics and then virus was infected to monolayer, adsorbed at room temperature for 30 min at MOI of 10 PFU/cells, after 48 h. infected cells aggregated together forming small and large round clumps.

BTV serotype-1 was adapted by Bhat *et al.* (1996) to C6/36, an insect (*Aedes albopictus*) cell line which grow as round cells in a loosely adherent monolayer. After 24 hrs of incubation, thinning of infected monolayer was seen. After 48 hrs of incubation, infected cells aggregated together forming small and large clumps and after 72 hrs detachment of cell sheet and cell lysis were seen.

Babu et al. (2005) propagated BTV-2 and MBN isolate in BHK-21 cell line. The cytopathetic effect of the virus isolate was characterized by swollen spindle shaped cells, granulation of cytoplasm and finally detachment of monolayer by 96-120 hr post infection.

Sekaret *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 and infected with 165 BTV infected blood samples, collected during high febrile period. RBC was separated and lysed with distilled water then the supernatant after centrifugation was used to infect 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. Despite the same time taken for CPE, high virus titre values were obtained in Vero cell line than in BHK-21. Virus titre at 12th passage was estimated as $10^{10.3}$ - $10^{10.8}$ TCID₅₀/ml in Vero cells and $10^{8.1}$ - $10^{8.8}$ TCID₅₀/ml in BHK₂₁ cells. From the above observations they concluded that Vero cell line exhibited maximum infectivity titre than BHK-21 cell line.

Gollapalliet *al.* (2012) reported that BHK-21 cells grown in Dulbecco's modified Eagle's medium (DMEM) with 5% foetal bovine serum (FBS) were infected with BTV at a multiplicity of 0.1 TCID₅₀ and after 48-72 hrs 80% cytopathic effect was observed, the infected cell lysate was harvested and used as the source of virus.

Reddy *et al.* (2015) reported that collected heparinized blood from BT suspected sheep was washed with phosphate-buffered saline (PBS) (pH 7.2) and lysed in sterile distilled water. The blood cell lysate was pelleted and resuspended in PBS and inoculated to 10-day-old embryonated chicken eggs by intravenous route and incubated in a humidified chamber at 32°C. The embryos were observed for 5 days, for lesions which

showndiffuse haemorrhages. This is homogenizedand used for inoculation of BHK-21 cells. The cellswere incubated at 37°C with intermittent change in mediaand observed for 15-20 days for BTV-like cytopathology.

2.4 PLAQUE PURIFICATION

Dulbecco (1952) observed plaque formation for the first time by animal viruses on tissue culture monolayer using agarose overlaying method. In this study, western equine encephalomyelitis (WEE) was infected to chicken embryo monolayer and plaques were observed with a diameter of 2-4mm as bright areas against a dark background after 3 days of incubation. Then the same protocol was carried with New castle disease virus (NDV) to observe plaques. Plaques formed by these two viruses were distinguished under the microscope. From these results author concluded that possibility of distinguishing the plaques produced by different viruses will be valuable to study problems where two different viruses were involved. Along with these observations, concept of nearly one-to-one ratio between the no.of plaques and the no.of infective virus particles were established.

Howell *et al.* (1967) followed agarose overlaying method to study plaque formation by 16 tissue culture adapted and 14 egg adapted strains of bluetongue virus in mouse fibroblast cells. During this entire study, cells were maintained in modified eagle's medium with 10% FBS. Plaques were formed by each strain of tissue culture and egg adapted strains but they differ morphologically in size, shape and perimeter. These variations were observed between different immunological types and also between tissue culture and egg adapted strains of the same type. Egg adapted strains formed plaques earlier than tissue culture adapted ones. Plaques were observed from 3rd day onwards. They also observed that increase in agarose concentration and depth will result in decrease in size and number of plaques. Linear relationship between the plaque

count and the relative virus concentration was demonstrated which supported that each plaque was produced by one virus particle.

Bouianger and Frank (1975) reported plaque reduction neutralization test used for different BTV isolates. All the isolates studied caused plaque formation in L-929 cells. The plaques appeared in 48 hrs and stabilized by day four post inoculation and used it further in the study.

The genomic diversity of an Indian isolates of BTV was studied by Prasad *et al.* (1998) who purified the virus to prevent mixed infections in BHK21cells and used it further in the study.

Kumaret *al.* (2016) reported that cocultured Vero/BHK-21cells were used for isolation of pestedespetitsruminantsvirus(PPRV)andfoot-and-mouth disease virus(FMDV) respectively. They observed cytopathetic effect of both viruses at 6 blind passages (BP6) level and performed plaque assay in Vero cells. Eleven plaques werepickedandresuspendedin500µlMEM and tested them for PPRV andFMDV-specific gene segment byPCR where 10plaques belonged to PPRV. NoFMDVspecific amplificationcould beobserved inany plaque and one plaque was negative for both PPRV and FMDV genomes.

Reddy (2016) reported that BTV-4 was plaque purified for three times in Vero cells using agaroseoverlaying method. Serial 10-fold dilutions of stock virus were made and 10^{-2} to 10^{-6} dilutions were infected on to monolayer of each well of 6-well plate. After 3 days of incubation suspected plaque areas of minute size were noticed which became more prominent from 4th day onwards.They also observed that increase in agarose concentration and depth will result in decrease in size and number of plaques.

2.5 TYPING OF BTV ISOLATES

For effective control of bluetongue disease, an early diagnosis and effective prophylactic measures like vaccination, are required. The main obstacle for this is the existing number of serotypes (29 serotypes). Hence an 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 (Blacksellet *al.*, 1993) and molecular methods using PCR technique (Mertenset *al.*, 2007).

2.5.1 MOLECULAR TYPING

Dangler *et al.* (1990) developed PCR using NS1 gene specific primers for detection of BTV in bovine blood samples. The PCR test was found to be very sensitive and could detect two femtograms of BTV RNA in the samples.

Akita *et al.* (1992) developed a RT-PCR based on highly conserved genome segment 10 by amplifying a 251 bp product. This test could detect a wide spectrum of BTV isolates of USA and Israel. Later this protocol was standardized for detection of BTV in different clinical samples viz, infected whole blood, semen and spleen.

Bandyopadhyayet *al.* (1998) developed a RT-PCR method for detecting BTV in infected cell culture fluid and clinically suspected samples by amplifying a portion of M6 gene which codes for group specific NS1 protein. Similarly Prasad et al. (1998) also standardized the protocol by amplifying 274 bp of NS1.

BTV-type specific RT-PCR assay was also developed by Maanet *al.*, (2004) based on amplification of genome segment S2 for identification of the BTV serotypes - 1, 2, 4, 9 and 16 by designing specific primer pair of each bluetongue virus type. Further the PCR assay was also used for identification and differentiation of field and vaccine strains.

Dahiya *et al.* (2005) designed new set of primer pair of VP2 gene of BTV1 and BTV23 which successfully amplified the cell culture grown six BTV1 and BTV23 Indian isolates respectively, but not vice-versa and other serotypes. They suggested that the serotype primer have potential for detection of BTV1 and BTV23 by using reliable and specific RT-PCR assay.

Mertens *et al.* (2007) using PCR technique full length genome sequence can be utilized to design primers for amplifying desired length of different genome segments to identify serogroup as well as serotype of BTV isolates.

Biswas *et al.* (2010) isolated and confirmed BTV-1 serotype from goats by RT-PCR method by targeting segment-2 and compared phylogenetically with other BTV-1 isolates in India and Australian sequences.

Prasad *et al.* (2013) used group specific RT-PCR based on NS1 gene and it showed a single 274bp amplicon of expected size confirming as positive for BTV. The sample was subjected to typing PCR to determine the serotype it possessed using 24 serotype specific primer pairs based on VP2 gene. The expected amplicon size (648bp) was observed without any nonspecific implication in 1% agarose electrophoresis and they confirmed the serotype as BTV-10.

Reddy *et al.* (2015) developed RT-PCR based on Indian isolates of BTV (serotypes 1, 2, 9, 10, 12, 16, 21 and 23) and this was applied to type 52 isolates. It was found that multiple serotypes are circulating, with involvement of more than one serotype infecting individual animals and herds over a period in a given area.

Krishna Jayathi *et al.* (2016) isolated BTV-24 from sheep of 2010 outbreak in Medak district, Telangana state, India. After group specific RT-PCR confirmation as BTV, serotype specific RT-PCR targeting seg-2 was carried out with primers of common circulating serotypes in India, followed by primers of 26 worldwide serotypes

of BTV. In all these RT-PCRs isolate was found to amplify with BTV-24 primers only. From these results and along with further sequencing analysis, the investigators confirmed the presence of BTV-24.

2.5.2 SEROLOGICAL TYPING

Della -portal *et al.* (1983) reported the presence of antibodies against bluetongue group virus in Australian and Papua New Guinea cattle and Australian sheep. They tested 16,213 sheep serum samples by group test and 155 serum samples by Serum neutralization test. They found 45 % samples were positive for BTV.

Squire (1989) tested serum samples from 103 sheep and 24 cattle experimentally infected with one of three serotypes of BTV isolated in Australia for antibody to BTV using serum neutralization and AGID tests. The AGID test failed to detect antibody in 28% (29/103) of sheep which had seroconverted in the SNT.

Sreenivasulu and SubbaRao (2004) infected sheep with BTV-2 and observed that neutralizing and precipitating antibodies appeared on 8 and 9 dpi and maintained till the end of the experiment.

Dubayet *al.* (2006) used SNT for determining the prevalence of bluetongue disease and EHDV in mule deer. The sera samples were screened by AGID and positive samples were tested for both mono specific Abs and cross neutralizing Abs by SNT. Based on the results the prevalence of serotypes BTV-11 and BTV-10 were determined in USA.

Sairajuet *al.* (2013) performed SNT for serum samples collected from combined Andhra Pradesh. Collected serum samples were confirmed as BTV positive by group specific cELISA and then type specific serotyping was carried out using available

serotypes (BTV-1, 2, 9, 10, 21, and 23). Among the 52 samples, BTV-1 was neutralized in 50 % sera followed by BTV-2 in 44.23%, BTV-10 in 26.92 %, BTV-9 in 21.15 % and BTV-23 in 15.38 % sera samples. 32.69% of 52 samples didn't neutralize any available serotype suggesting that existence of serotypes other than those tested.

De Campos Nogueira *et al.* (2016) studied seroprevalence against BTV serotype 4 in cows older than 1 year age in Brazil. Virus neutralization test (VNT) performed in Vero cell lines maintained in MEM. A total of 1716 serum samples were used in 1:10 to 1:640 serial dilutions in order to detect antibodies against BTV-4. After 4-5 days incubation, it was concluded that among 1716 animals, 1,483 (86%) were found positive for BTV-4.

2.5.2.1 CROSS NEUTRALIZATION STUDIES

Balam *et al.* (2011) used reciprocal cross neutralization test (modified micro neutralization test) to identify neutralizing antibodies to BTV serotypes and concluded that SNT can find a weak antigenic relationship present between the BTV serotypes.

Zulu and Venter (2014) studied possible cross-protection of different BTV serotypes in sheep (in vivo) based on previous cross-neutralization reports of specific BTV serotypes in vitro studies. Investigators selected BTV-4 as the vaccine for this study and cross-neutralization studies were carried out against BTV-1, 9, 10, 11. During this study BTV seronegative sheep were infected with BTV-4 at a titre of 4.2×10^6 log₁₀ TCID₅₀/ml. After 28 dpi serum samples were collected from vaccinated animals and then challenged with BTV-1, 9, 10, 11 each serotype to each animal and monitored for BTV specific clinical signs. Cross-protection was estimated by observing clinical signs and by SNT assay against challenged types as BTV-9 = BTV-11 > BTV-10 >> BTV-1. Upon these observations, investigators concluded that BTV-4 serum was involved in cross-neutralization with BTV-9, 10 serotypes.

Reddy (2016) reported that in their study 1 ml contain $10^{6.46}$ TCID₅₀ of plaque purified BTV-4 virus was inoculated to sheep via intra dermal route and subcutaneous routes on 0 day and 15th 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.

2.6 ANIMAL EXPERIMENTAL STUDIES

Ghalib et al. (1985) used 10 sheep to study clinical, virological and serological response caused by BTV serotypes 10, 11, 13 and 17. 2 ml of pure BTV containing 10^7 pfu/ml was injected to 3-6 months old sheep via intravenously, intradermal and subcutaneous routes. All the infected animals developed viraemia (10^4 F- 10^6 F) as early as Day 3 P.I. and the maximum viraemia was reached by Day 7 P.I. along with excessive salivation and dyspnoea, but no mouth lesions were observed. All the infected animals developed antibodies by 10th day of post inoculation, respectively, which was observed to maintain till the end of the experiment (28dpi).

Jeggoet *al.* (1984) raised hyper immune serum to BTV serotype 3 in sheep to assess the role of neutralizing antibodies in passive immunity to BTV infection. In this study BTV-3 was adapted and plaque purified in BHK-21 cell line. After plaque purification 1 ml containing 10^6 TCID₅₀/ml of BTV-3 was inoculated to sheep via intra dermal route. Animal developed neutralizing antibodies on 10th day of inoculation with a titre of <1:10 and peak titre i.e., 1:180 was developed by 18th day of inoculation. These antibodies were shown to be type specific *in vivo*, by inoculating this hyper

immune serum into another animal and when challenged with BTV-3 animal developed neither pyrexia nor viraemia.

Monaco et al. (2004) reported that 30 pregnant cattle were vaccinated with the bivalent BTV-2/BTV-9 vaccine. The vaccine contained $10^{4.37}$ TCID₅₀/ml BTV-2 and $10^{4.24}$ TCID₅₀/ml BTV-9. BTV-2 titres were detected in the circulating blood of 22 (73.3%) vaccinated animals between day 8 and day 28 P.V and viraemia peaked on day 11 P.V. BTV-9 caused detectable viraemia in 25 (83.3%) cattle commencing on day 4 P.V and lasting 24 days and the maximum viral titre was detected on day 9 P.V. None of the negative controls developed detectable viraemia. Antibodies against BTV-2 were detected in 29 animals commencing on day 16 p.v with the highest titre on day 42 P.V. All animals except one developed an immune response to BTV-9; VN antibody was detected on day 18 P.V, two days later than BTV-2, and BTV-9 antibody titre peaked on Vaccines day 35 P.V and none of the control animals developed antibody against BTV-2 or BTV-9.

Sreenivasulu and Subba Rao (2004) used sheep and goat to study clinical, virological and serological response caused by BTV serotype 2 (BTV-2) which was isolated previously during natural outbreak of sheep in Andhra Pradesh. 10 ml of BTV-2 with a titer of $10^{5.5}$ TCID₅₀/ml at 10th passage was injected to 2 years old sheep and goat via intra dermal and subcutaneous routes. Sheep developed pyrexia of 39.5°C on 6 dpi. 40.5°C on 7 dpi. along with mild congestion of mucocutaneous borders of lips between 10-11 dpi. Goats didn't show either pyrexia or clinical signs. But humoral immune response was developed by sheep as well as goat on 8th and 9th day of inoculation, respectively, which was observed to maintain till the end of the experiment (15dpi).

Reddy (2016) raised hyper immune serum against to BTV serotype4 in sheep. In this study BTV-4 was adapted and plaque purified in Vero cell line. After plaque purification 1 ml of $10^{6.46}$ TCID₅₀ of BTV-4 virus was inoculated to sheep via intra dermal route and subcutaneous routes on 0 day and 15th day. Sheep had developed mild pyrexia of 103.6° F on 6dpi, which persisted for 4 days along with nasal discharges, edema of buccal area and lips between 10-11 dpi. Both animals developed humoral immune response on 7th day of post inoculation, which was observed to maintain till the end of the experiment (28dpi).

2.7 VP2 GENE SEQUENCING ANALYSIS

Yamaguchi *et al.* (1988) sequenced the L2 gene of BTV2 of USA and compared with L2 gene sequence of other USA BTV serotypes BTV10, 11, 13, and 17. The L2 gene of BTV2 was found to have 42.5% homology with BTV 13 where homology of 40-41% was noticed with BTV10, 11 and 17.

A comparative sequence analysis of VP2 sequences from a number BTV serotypes was done by Gould and Pritchard (1990) which led to the grouping of individuals serotypes in to nucleotypes. Containing serotypes whose VP2 nucleotide and amino acid sequence showed a high degree of homology (~70%). VP2 comparisons between serotypes of different nucleotypes had a lower level of homology (~50-55%).

Yamakawa *et al.* (1994) sequenced the L2 gene segment of BTV 23 isolated from Australia and compared with L2 gene segments of USA BTV2, 10, 11, 13 and 17 and Australian serotype BTV1. The comparison revealed that Australian serotype BTV1 and BTV23 were closely related to BTV of USA than other four serotypes. A homology of 22% was noticed among the seven serotypes studied.

Pritchard and Gould (1995) compared the partial sequence of L2 gene of BTV1 isolated from India and Australia. The viruses were found to have 87.6% and 91.2% nucleotide and amino acid homology respectively.

Breard *et al.* (2003) determined molecular relationship between isolates of the BTV2 Corsican wild type viruses isolated during 2000 and 2001, and the attenuated BTV2 vaccine by comparing corresponding sequence of genome segments 2, 7 and 10 with each other and already published sequences available in database. The results indicated complete genetic stability between the isolates of the Corsican BTV2 and 17.9% divergence between the nucleotide sequences of S10 from the BTV2 wild type and vaccine viruses. Based on these differences, primers were selected that could be used to differentiate between the wild type and vaccine viruses.

Maanet *et al.* (2007) sequenced and analysed full length L2 Segments of all 24 BTV reference serotypes. Each L2 segment was found to have a single open reading frame. All BTV serotypes were found to have terminal hexanucleotides (5' GUUAAA....ACUUAC 3') with the exception of BTV24 which was found to have G instead of A at sixth position from 3' end of the position strand. Comparison of L2 gene nucleotide sequence revealed a variation of 29 to 59% between reference strains of BTV serotypes. Variation of 22.7% to 72.9% was noticed on comparison of amino acid sequence of VP2 protein. Based on L2 gene sequence and phylogenetic analysis, the 24 serotypes were grouped into ten distinct nucleotypes (A-J).

Prasad *et al.* (2013) sequenced and analysed VP2 segment of BTV10 to determine the identity of novel isolate with rest of the global isolates in the Gene Bank. The data analysed showed that novel BTV10 isolates share 100% nucleotide sequence identity with four USA isolates and one of the Indian isolate and it showed more than

95% nucleotide identity with six other USA isolates. The novel BTV10 isolate showed only 88.7% and 89.5% nucleotide identity with South Africa and France BTV 10 isolate.

Krishnajyothi *et al.* (2016) designed sets of primers based on the segment 2 of Western BTV24 and used them for sequencing. Sequence analysis indicated that it is related to Western viruses and the data analysed showed that BTV24 isolate shared 94% sequence identity with the reference strain of BTV24, 92% with an Israeli isolate and 89% with Central and South American isolates. Phylogenetic analysis of all these sequences indicated that BTV24 is more related to South African and Israeli isolates than to the Central and South American isolates.

2.8 GROWTH CHARACTERS OF BLUETONGUE VIRUS IN CELL CULTURES

Goutham Roy and Mehrotra (1999) adapted BTV isolates into BHK21 cells and observed cellular destruction after 24 hours of infection. The H & E staining of the infected BHK21 monolayers, 48 hours post-infection, revealed several intracytoplasmic, eosinophilic, perinuclear inclusion bodies along with vacuolation in cytoplasm and giant cell formation. Similar observations were also made by Meenambigai *et al.* (2006) when propagated an isolate of BTV in BHK21 cell line and Vero cell line.

Three serotypes (2, 9, and 15) of BTV were propagated successfully in Vero cell line by Krishnajyothi (2007) and Sandhya (2007). Distinct cytopathic effect appeared 4-5 days after inoculation. The earliest sign observed, as early as 72-78 hrs. post inoculation was scattered focal rounding of cells. Within next 24-36 hrs, cell destruction extended rapidly and was characterized by degeneration, sloughing of cells from the surface, loss of cell architecture followed by cell detachment by 100-120 hrs. post infection.

Balam(2011) infected the BHK21 cell lines with BTV-2 at 36th passage level and noticed CPE as early as 24 hours PI, with initial rounding and detachment of cells, characteristic ballooning and clumping of cells, swollen spindle-shaped cells and long cytoplasmic bridges appeared by 48 hours PI, complete detachment of monolayer by 72 hours PI.

Subhadra (2014)infected the BHK21 cell lines with BTV-2 at 6th passage level and noticed CPE as early as 24 h PI, with initial rounding and detachment of cells. Characteristic CPE of BTV with rounding, clumping of cells and granulation in cytoplasm wasobserved. Swollen spindle shaped cells and long cytoplasmic bridges appeared by 48 h. Finally complete detachment of the cells from the surface occurred and the detached cells appeared as clumps.

CHAPTER - III

MATERIALS AND METHODS

3.1 CELL CULTURE MATERIAL

3.1.1 GLASSWARE AND PLASTIC WARE

Glassware manufactured by Borosil and Schott Duran were used in this study. Plastic ware of different suppliers i.e., tissue culture flasks by TPP, multiwell plates of COStar, centrifuge tubes and micropipette tips of Axygen and GeNei brand were used during this study.

3.2 CLEANING AND STERILIZATION

3.2.1 GLASSWARE

Glassware were soaked in 5% neutral detergent (Labolene) for 30mins. Then scrubbed and rinsed thoroughly under running tap water till all traces of soap were removed. This was followed by washing three times in deionized water. After that, glassware was inverted and allowed for drying. Dried glassware was covered initially with aluminium foil, butter paper and tied with thread. The packed glassware was sterilized in hot air oven at 160°C for 2hrs.

3.2.2 PLASTIC WARE

Plastic ware (like filter assembly, screw caps) was soaked in lukewarm water with added detergent for 30 minutes and rinsed thoroughly in tap water followed by three times in deionized water. Washed material was inverted for air dry on blotting paper.

Screw caps were wrapped in aluminium foil for sterilization. Filter assemblies were loosely tightened by placing 0.22 μ membrane filter and packed in brown paper. Thus packed plasticware was sterilized by autoclaving at 121°C and 15lb/ 20minutes.

3.2.3 LIQUID MATERIAL

Liquid material 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. 41500-034) was dissolved in 1000ml of autoclaved millipore water in a conical flask gradually with constant mixing. After all the constituents were dissolved completely, 2.2gm of NaHCO₃ was added (as per manufacturer) and sterilized by filtration using 0.22 μ membrane filter. Then after 5ml of antibiotic anti mycotic solution (100X concentration, HIMEDIA LOT: 0000224846 contained 10,000U Penicillin, 10mg streptomycin, 25mg Amphotericin B per ml in 0.9% normal saline) was added. 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 incubator at 37°C for one week. After sterility check, the prepared media was properly sealed and stored at 4°C.

3.3.2. PHOSPHATE BUFFERD SALINE (PBS)

8g of NaCl, 0.2g of KCl, 2.44g of Na₂HPO₄, 0.24g of KH₂PO₄ were added in a glass bottle to make volume upto 1 Lt. The pH of the solution was adjusted to 7.2, finally this solution was sterilized by autoclave.

3.3.3. TRYPsin (0.1%)

0.1 gm of trypsin (Sigma, T-4799 E.C No 232-650-8), 0.2 gm of EDTA, 0.05 gm of glucose were added in a sterile glass bottle and the volume was made up to 100 ml with autoclaved 1X PBS. It was kept for over-night stirring for proper mixing, then next day the prepared solution was filtered through 0.22 µ membrane filter using filter assembly and stored at 4°C.

3.3.4. 10% FORMALIN

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

3.3.5.1% CRYSTAL VIOLET SOLUTION

1gm. of crystal violet powder was dissolved in 100ml of 70% methanol to infected cell culture plates.

3.3.6. TRYPAN BLUE (0.4%)

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

3.3.7. SEA PLAQUE AGAROSE

1.5gm of Sea plaque agarose powder (Cambrex Bio Science, Cat.No.50100) was dissolved in 50ml of millipore water and sterilized by autoclaving at 121°C, 15lb/20 minutes and then stored at room temperature.

3.4. CELL LINES

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

3.4.1. MAINTENANCE OF CELL LINES

Vero cells were maintained in tissue culture flask with 5% growth medium. After formation of healthy 80-90% confluent monolayer the growth medium was decanted completely and washed with 1X PBS to remove any dead cells followed by incubation of the monolayer with 1X Trypsin at 37°C for 5 min until the monolayer detached. 2ml of growth medium with 5% FBS was added and then flushed for 5-6 times for uniform distribution of the cells. The detached cells were flushed in growth medium and cells were incubated in a carbon dioxide incubator at 37°C with 5% CO₂.

3.4.2. CELL COUNT

0.4% of trypan blue solution (w/v) was added to equal amount of cell suspension in an eppendorf tube and kept aside for 5 minutes. Cell count was carried out using haemocytometer and cover slip in position under the inverted microscopic.

3.4.3. CELL SEEDING

Cells were seeded at the concentration of 2×10^4 cells per ml into tissue culture flasks (T25) and incubated at 37°C in 5% CO₂ incubator until complete monolayer was formed.

3.5. BLUETONGUE VIRUS SEROTYPE-12

BHK-21 cell culture adapted bluetongue virus serotype-12 confirmed by type specific PCR is available in the Department of Biotechnology, C.V.Sc, Rajendranagar.

3.5.1. ADAPTATION OF BTV-12 TO VERO CELL LINE

BHK-21 cell line adapted BTV-12 serotype was passaged 3 times in Verocells to show cytopathic effect (CPE) within ideal time. At 80-90% confluence of monolayer in tissue culture flask growth medium was discarded and washed with 1XPBS. Then, 2ml of 1% MEM with FBS was added along with 50µl of stock virus and incubated at 37°C in 5% CO₂ incubator. After 1hr adsorption, 4ml of 1% MEM with FBS was added and kept in CO₂ incubator along with control flask in which only maintenance medium was added without virus. Thus infected flasks were subjected to microscopic examination for every 18-24hrs. Infected flask was compared with control flask for CPE in Vero cells which is characterized by vacuole formation, rounding and clumping of cells and eventually complete thinning of monolayer.

3.6. TITRATION OF THE VIRUS

Vero cells were seeded at the rate of $1-2 \times 10^4$ per well in MEM in 96 well plate with 10% FBS and incubated in CO₂ incubator at 37°C. At 80-90% confluence, cells were infected with 100µl of 10^{-1} to 10^{-8} serially diluted Virus in plain MEM medium, each dilution in a separate row in replicates of eight along with two cell control rows incubated at 37°C in 5% CO₂ incubator. After 1hr adsorption, 100µl of 1% MEM was added to every infected and control wells. Plate was observed at 18-24 hrs. interval for

characteristic CPE under inverted microscope and final readings were taken on 5th day of infection. Virus titre was calculated according to Reed and Munch method (1938).

3.7. PLAQUE PURIFICATION OF BTV

6-well tissue culture plates were used for plaque purification in which each well was seeded with 1×10^6 Vero cells in 10% growth medium and incubated at 5% CO₂ level at 37°C. BTV-12 was serially diluted 10^{-1} from to 10^{-6} in plain medium, in six wells, monolayer of five wells were infected with 1ml of different virus dilution starting from 10^{-2} to 10^{-6} and 6th well was kept as cell control. This plate was kept in incubator with 5% CO₂ at 37°C for 1hr. involving swaying for every 10-15 minutes to ensure virus adsorption.

During this incubation time, already prepared 3% sea plaque agarose was liquefied in microwave and maintained at 42°C in water bath.

After incubation, inoculum was drained off completely with the help of 1ml micropipette to remove unadsorbed virus without disturbing the monolayer and immediately overlaid with 10ml of 2X MEM with 2% FBS; 3% sea plaque agarose mixture in 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, overlaid plates were allowed to solidify and then transferred to incubator. The plates were observed daily for plaque formation. Plaques which are more distinct and isolated from others were collected with the help of micropipette in 200 µl of 10% MEM. Ten plaques per plate were collected and infected to Vero cells in 12-well plate for virus propagation.

3.7.1. CONFIRMATION AND SELECTION OF THE PLAQUES

Vero cells were seeded in 12 well tissue culture plates at concentration of 2×10^4 cells per ml; likewise 2ml of this cell suspension per well. Monolayer in each well was infected with 200µl 10% MEM in which plaque was dissolved. To each well 1.5ml of 1% growth medium was added and incubated at 37°C in CO₂ incubator at 5% CO₂. Plates were observed under inverted microscope at 18-24hr interval and resultant cell culture fluid showing CPE was collected and used in next round of plaque purification.

The same procedure was carried out for another two times. For each successive purification, previous round plaque which was more independent from others was selected.

3.7.2. MOLECULAR CONFIRMATION OF PLAQUEPURIFIED VIRUS

After plaque purifying the putative virus for 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 TCID₅₀ titre to inoculate selected animal to raise hyper immune serum (HIS) against this serotype.

3.8. FREEZE THAWING OF CELL CULTURE FLUID

At 90% CPE in a T25 flask, cell culture fluid was freeze thawed for three times. Freeze thaw involved rapid freezing of cell culture fluid at -70°C for 20 min.

3.8.1RNA ISOLATION

Bluetongue virus genome i.e., double strand RNA (ds RNA) was extracted from cell culture fluid by Acid Phenol method using TRIZOL reagent.

1) 6ml of infected Vero cell culture supernatant was centrifuged at 2600 rpm for 10min to obtain pellet.

- 2) To that pellet, 750µl of TRIZOL® was added.
- 3) The mixture was vortexed for 1min for proper mixing and incubated at room temperature for 5 min.
- 4) 250µl of chloroform was added, mixed and incubated at 4°C for 10 min.
- 5) The mixture was centrifuged at 13200 rpm for 20min at 4°C.
- 6) Aqueous phase was collected carefully and transfer to another RNase free 1.5ml Eppendorftube 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°C for overnight.
- 9) The following day this mixture was centrifuged at 13200 rpm for 20 min at 4°C.
- 10) Supernatant was discarded leaving the small pellet.
- 11) 1ml of 70% alcohol was added to pellet and centrifuged at 13200 rpm for 10 min at 4°C.
- 12) Supernatant was discarded and the pellet was air dried.
- 13) The pellet was dissolved in 30µl of nuclease free water and stored at -20°C.

3.8.2. REVERSE TRANSCRIPTASEPOLYMERASE CHAIN REACTION (RT-PCR)

3.8.2.1. COMPLEMENTARY DNA SYNTHESIS (c DNA)

For cDNA synthesis a total of 25 µl reaction mixture was prepared consisting

RNA mix and RT mix.

RNA : 10 μ l

Nuclease free water :5 μ l

15 μ l

This RNA mix was denatured at 95°C for 5 minutes followed by snap cooling for 5 minutes. Meanwhile RT mix was prepared and stored at 4°C until RNA denaturation.

RT mix:

Buffer (1X) : 5 μ l

DTT (0.01M) : 2 μ l

Random hexamer (2.5ng/ μ l) :1 μ l

dNTPs : 1 μ l

RT enzyme (MuMLV) (100U/ μ l) :0.5 μ l

RNase out : 0.5 μ l

10 μ l

After snap cooling, RT mix was added to denatured RNA mix and subjected to following conditions in PCR thermo cycler.

Annealing : 25°C/10 minutes

RT enzyme activation : 42°C/1 hour

RT enzyme inactivation : 72°C/10 minutes

Hold at 4°C

3.8.2.2. PCR SET UP:

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, 9, 10, 12, 16, 21, 23, 24) and their 5'-3' sequences were mentioned in table .

Each PCR reaction was prepared for 20 µl as given below.

cDNA	: 1.5 µl		
Forward primer (1µM)	: 0.5 µl		
Reverse primer (1µM)	: 0.5 µl		
Buffer (1x)	: 2 µl		
dNTPs (0.2-0.6mM)	: 0.5 µl		
Taq polymerase enzyme	: 0.5 µl		20µl
Nuclease free water	: 14.5 µl		

Table 1: Segment 2 specific Primers sequence (5'-3') used for PCR

BTV-1EF	5' TGT CGA GCC GAT TGA AGA TCC GTC 3'.
BTV 1ER	5' ATC GTC ATT CCG TCG TTG TGC G 3'
BTV 2EF	5' TAC GCA CCT CGT GAG AGA GA 3'.
BTV 2ER	5' GTT GGA GGA ACC AAC TTC CA 3'.
BTV 4P2F	5' GTT GGA TCT GAG AAA TGG GT 3'

BTv 4P2R	5' AAG ACA CGG ATA AGG ATT CG 3'
BTv 9EF	5' GAT GGA ACG GCT AAA CCA AA 3'
BTv 9ER	5' TGG ATA TTT GAC ACG AGC GA 3'.
BTv 10F	5' TGT ATC GTT AAG GCG AGG TCA GCA 3'
BTv 10R	5' TGT CTT CTA ACG GCC TCT CAC G 3'
BTv 12F	5' TTT AGG TGA CCA TGT GGA GAC G 3'.
BTv 12R	5' CAA CGC ACT TTC GCA AAA CC 3'
BTv 16EF	5' TCG AGG AAA GCG GAT ACC ACG T 3'.
BTv 16ER	5' CGT TGC GCT AAC TCG ACT TCG C 3'
BTv 21F	5' GCA GAT TCG TAC AAC CAA CGG CC 3'
BTv 21R	5' TTG GGA TTT GCG AGG CGC GA 3'
BTv 23F	5' GCG TTG CGA TGG ATG AGT TAG CA 3'.
BTv 23R	5' GGT GGT CAT CTC TTC ATC TTC GGG G 3'
BTv 24P3F	5' GTT TCA TGA ATT TGA AGG ACG 3'
BTv 24P3R	5' ACC TTG TGA AAT CTT AGT YTT TG 3'

(Reddy *et al.*, 2015)

PCR conditions followed in thermo cycler:

For BTv-1, 2,4,9,10,12,21,23,24

94°C for 3 minutes

94°C for 30 second

For BTv-16:

94°C for 3 minutes

94°C for 30 seconds

55°C for 30 seconds

50°C for 30 seconds

72°C for 1 min 30 sec

72°C for 1 min 30 sec minutes

72°C for 10 minutes

72°C for 10 minutes

PCR thermo cycler was set to hold at 4°C after completion of reaction.

3.9. AGAROSE GEL ELECTROPHORESIS:

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 (GeNei, Cat No. 105659).

Materials:

1. PCR products
2. Agarose (HI media)
3. 1X TAE buffer
4. Ethidium Bromide (10 mg/ml, Sigma)
5. Gel loading dye 6X (0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 25% Sucrose in distilled water stored at 4°C).
6. DNA ladder 100bp, 1 kb (GeNei)

Method:

1. Agarose powder (1.5%) was dissolved in 1X TAE buffer and heated in a microwave oven till it melted completely.

2. After letting it cool to 40°C, Ethidium Bromide was added to a final concentration of

1 μ l / 10 ml. The content was swirled gently to mix after adding.

3. The molten agarose was poured into a casting tray with positioned comb was allowed to solidify for at least 20 min.

4. Comb was removed gently from the gel tray. Electrophoresis tank was positioned with gel tray and loaded with 1X TAE buffer so that the gel was just submerged within the buffer.

5. Sample and DNA ladder were loaded with 6X loading dye and electrophoresis was run at 75 Volts (5 Volts/cm) for 1 hr.

6. The gel was transferred to gel doc having UV trans-illuminator and observed under medium wavelength for desired band.

3.10. TITRATION OF VIRUS

After confirmation by PCR, virus titre was calculated once again to know whether there was any increase in TCID₅₀ after plaque purification. Different dilutions were taken as shown below.

Table2.Infection of 96-well plate for TCID50 calculation

[illegible]

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 Controls

3.10.1. PROTOCOL FOR CRYSTAL VIOLET STAINING

After titration of virus in 96 well tissue culture plates, wells were stained with Crystal violet stain as per the method described by Mendes (1992).

- 1) The medium was discarded from the plate and the cells were washed with 1X PBS (200 μ /well).
- 2) The cells were fixed in 10% Formaldehyde (200 μ l/well) and incubated for one hour at room temperature.
- 3) Formaldehyde was discarded and the plate was washed twice with 1X PBS.
- 4) 1% Crystal violet solution (200 μ l/well) was added and kept on shaker for 1 hour.
- 5) Crystal violet solution was discarded and the cells were washed thrice with 1X PBS. The plates were dried at room temperature.

3.11. SELECTION OF ANIMALS

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

cELISA was done using the bluetongue antibody test kit supplied by the Veterinary Diagnostic Technology, Inc., USA, according to the kit protocol (Afsharet 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 1:5 in dilutionbuffer.
- 3) Precoated plates were washed with wash buffer twice.
- 4) 50µl of test and negative, weak positive, strong positive controls were added to wells.
- 5) After adding test and control sera, immediately 50µl 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 1X diluting buffer.
- 8) At the end of incubation period, the reactants were discarded from the wells and washed with wash buffer thrice.
- 9) Diluted conjugate (100µl) was added to each well and incubated at room temperature for one hour.
- 10) Buffer substrate solution was prepared by dissolving the contents of buffer substrate capsule in 100 ml of deionized water.
- 11) 2mg of OPD(1mg/2.5ml) tablet is dissolved in 5 ml buffer substrate solution. After preparing the OPD buffer substrate solution, the wells were washed 5 times with washing buffer.

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

13) After 10 minutes, 50µl 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 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.

3.11.1. HOUSING OF EXPERIMENTAL ANIMALS

Two BTV seronegative Deccani sheep (6-8 months) were selected for use in present study and were shifted to insect proof experimental animal house at College of Veterinary Science, Rajendranagar, Hyderabad.

3.11.2. DOSE AND ROUTE OF INOCULUM

After deworming and acclimatization, 1ml of $10^{6.116}$ /ml TCID₅₀ virus was inoculated to sheep by two routes i.e., 0.5ml via intradermal; 0.5ml via subcutaneous routes at shoulder region. For control animal, 1ml of plain MEM was given to compare results. Before inoculation animals were examined for their general health status i.e., temperature and physical appearance. Selected site for infection was shaved and sterilized with 70% ethanol. On 15th day of infection one more injection of same dose was given to both animals.

From the day of inoculation animals were examined regularly for typical clinical signs. Temperatures were recorded twice daily and serum samples were collected in sterile serum vacutainers (BD Vacutainer® Ref.365078) on 0, 7, 14, 21 and 28 days post inoculation (dpi.).

3.11.3. cELISA FOR SERUM SAMPLES

cELISA was performed as described in 3.11 for serum samples of each animal to assess the antibodies level or seroconversion.

3.12. SERUM NEUTRALIZATION (SNT)

Serum neutralization study was carried out with BTV-12 using HIS at different dilutions (1/20, 1/40, 1/80, 1/160) and cross neutralization studies with available plaque purified virus types (BTV-1, 2, 4, 9, 10, 16, 21, 23, 24). Briefly, the method was as follows. 1:20 serum dilution was prepared in maintenance medium in sterile deep well plate with the volume of 250 µl (12.5 µl serum + 237.5 µl maintenance medium). 250µl of medium containing 100 TCID₅₀ virus per 50µl was added to each well. The serum virus mixtures were incubated for 1hr at 37°C with 5% CO₂. 100 µl of incubated mixture was added to well with Vero cell monolayer in 96 well microtiter plate in quadruplicates. Four wells were kept as virus controls and four wells as cell controls in the plate. The plates were incubated for 5 days at 37°C with 5% CO₂. Plates were observed under inverted microscope at 18hr interval for CPE and the final titre was taken on the 5th day.

3.13. VP2 GENE SEQUENCING

3.13.1. PRIMERS

BTV 12 primers: Primers weredesigned for bluetongue virus serotype 12 using partial VP2 gene sequence (750bp) of BTV-12 IND2016/118 (GenBank: MF615238.1)

VP2 FTTT AGG TGA CCA TGT GGA GAC G

VP2 R CAA CGC ACT TTC GCA AAA CC

3.13.2. POLYMERASE CHAIN REACTION (PCR)

PCR conditions followed in thermo cycler:

94°C for 3 minutes

94°C for 30 seconds	}	35cycles
55°C for 30 seconds		
72°C for 1min 30 sec		

72°C for 10 minutes

PCR thermo cycler was set to hold at 4°C after completion of reaction.

3.13.3. AGAROSE GEL ELECTROPHORESIS

Gel electrophoresis was carried out as described in 3.9 to confirm the specificity of the PCR product by comparing the migration distances with 100 bp DNA ladder (GeNei, Cat No. 105659).

3.13.4. PURIFICATION OF PCR PRODUCT

The PCR amplified DNA was purified using QIAGEN MinElute PCR Purification Kit as per the manufacturer's instructions with suitable modifications. All centrifugations were done at 13,000rpm. Briefly the procedure was followed as:

1. Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and mix
2. Place a MinElute column in a provided 2 ml collection tube in a suitable rack.
3. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 min

4. After discarding the flow-through, the MinElute column was placed back into the same tube.

5. To wash, add 750 µl Buffer PE to the MinElute column and centrifuge for 1 min. Discard flow-through and place the MinElute column back in the same tube. Centrifuge the column for an additional 1 min at maximum speed.

6. Place the MinElute column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 10 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the centre of the membrane, let the column stand for 1 min, and then centrifuge for 1 min.

3.13.5. ESTIMATION OF DNA CONCENTRATION

Purity and concentration of the DNA and was estimated by finding the ratio of OD at 260/280nm. preparations with OD 260/280 value of 1.8 and 2 respectively were considered to be pure for DNA and RNA, respectively.

3.13.6. DNA SEQUENCING

The DNA sequencing was done from Xcelris Labs Ltd, Gujarat using VP2 gene specific primers as mentioned in 3.13.1. (for purified PCR products).

3.13.7 PHYLOGENETIC ANALYSIS

The sequences of partial segment 2 of BTV 12 isolate obtained after sequencing were compared with other available sequences in GENEBANK using NCBI BLAST (www.ncbi.nlm.nih.gov/blast) and phylogenetic tree was reconstructed with MEGA7 software to study the evolutionary relationship between the BTV-12 and other bluetongue serotypes.

3.14. GROWTH CHARACTERS OF BTV -12 IN CELL LINES

Growth characters of BTV12 in cell lines was observed by H&E staining of infected Vero cell lines at time points post infection (12, 24, 36, 48 and 72hrs). Briefly the procedure was followed as:

3.14.1. FIXATION:

3.14.1.1. Cleaning of cover slips:

Washing Protocol:

To wash the cover slips use a cleaning solution of 57% ethanol, 10% sodium hydroxide. Pour cleaning solution over cover slips and shake (gently!) for 2 hours at room temperature. Then rinse cover slips well with distilled water, allow for air dry and then autoclaved.

3.14.1.2. Sterilization of cover slips.

To sterilize the cover slips before use, they are kept under the UV light for 20-30 minutes.

3.14.1.3 Gelatin-coating protocol:

Immerse the cover slips in 0.1% gelatin in deionized H₂O solution and let stand for 10 minutes. Then aspirate off and let the slips air dry in the hood for 15 minutes.

3.14.1.4. SEEDING

Place the sterile and coated cover slips into a new sterile 6-well culture plate, in which each well was seeded with 1×10^6 Vero cells in 10% growth medium and

incubated at 5% CO₂ level at 37°C. After observing 80-90% confluence, they were infected with BTV 12 virus.

3.14.2.FIXING AND STAINING OF CELLS

Infected monolayers on coverslips were taken out carefully from 6wells plate. The coverslips were washed thrice with PBS. Then the coverslips were fixed in methanol for five minutes. Later haematoxylin was added to the coverslips and incubated for 10 min. then the coverslips were immersed in water for 25 min. Later, eosin was added to the coverslips and incubated for 30 sec. Then the coverslips were washed with absolute alcohol for 10 sec. After complete drying the coverslips were examined under the inverted microscope.

CHAPTER IV

RESULTS

4.1 MAINTENANCE OF CELL LINES

On seeding with Vero cells, the cell culture flasks exhibited 90% confluency within 24-48 hrs. under optimum conditions (5% CO₂ at 37⁰C). These cell culture flasks were used for the infection purpose.

4.2 VERO CELL LINE INFECTION

BHK-21 adapted BTV-12 was adapted to Vero cells by passaging 3 times. Infection was done on 90% confluent monolayer of Vero cells.

In the first passage, cytopathic effect was seen by 4th day of infection and complete CPE was observed by 5-6 days. In the next two passages, CPE was observed between 36-48 hrs. as rounding and clumping of dead cells. Complete peeling off of monolayer was observed within 72 hrs. of infection (Fig 1.B). Control monolayer didn't show any CPE (Fig 1.A).

4.3 TITRATION OF THE VIRUS

Titration was done in a 96-well plate and observed regularly at 18-24 hr. intervals. CPE was observed by 3rd day in all replicates of lower dilutions (10^{-1} to 10^{-3}) and had only started in some replicates of higher dilutions (10^{-4}). CPE was read on 5th dpi. and details are given in Table 3. TCID₅₀ was calculated as per Reed & Muench (1938).

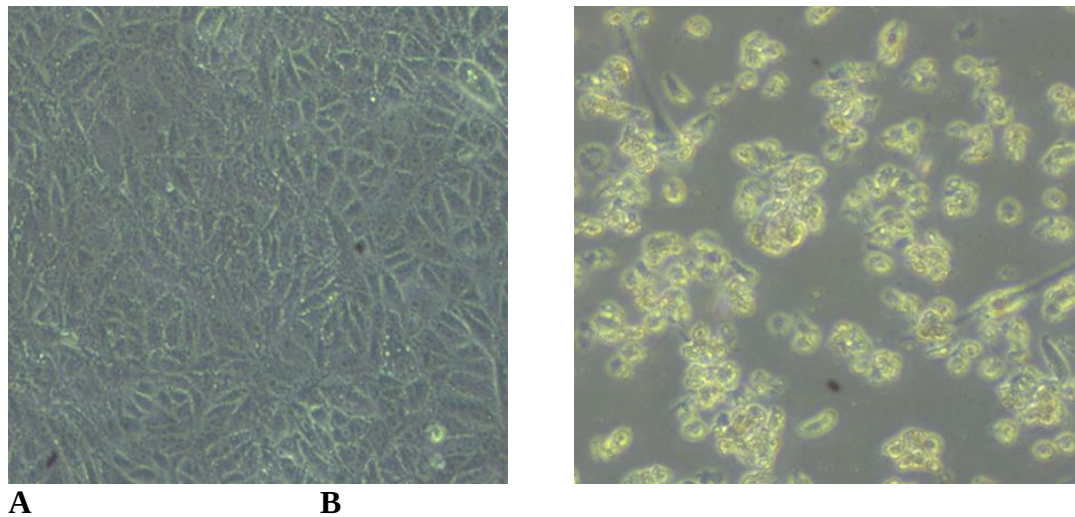


FIG. 1: VERO CELL MONOLAYER

Fig A - Healthy monolayer (100X)

Fig B – Cells infected with BTV12. It shows complete detachment of monolayer after 72hrs of PI (100X)

Table3: Titration of BTV-12 in Vero cells

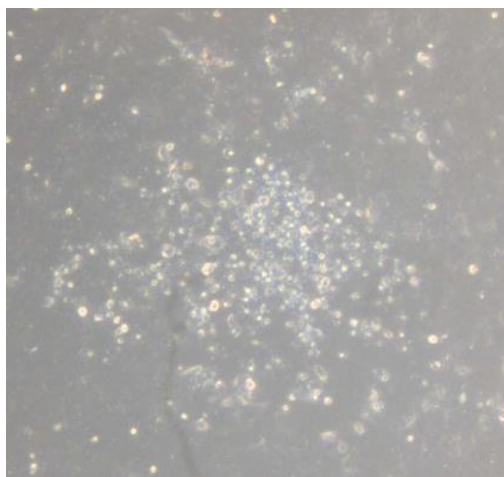
S.No.	Virus dilution	No.of wells infected	CPE positive wells	CPE negative wells	Cumulative positive	Cumulative negative	% of CPE
1	10^{-1}	6	6	0	24	0	100
2	10^{-2}	6	6	0	18	0	100
3	10^{-3}	6	6	0	12	0	100
4	10^{-4}	6	6	0	6	0	100
5	10^{-5}	6	0	6	0	6	0
6	10^{-6}	6	0	6	0	12	0
7	10^{-7}	6	0	6	0	18	0
8	10^{-8}	6	0	6	0	24	0

Titre was determined as $10^{4.5}$ /100 μ l (or) $10^{5.5}$ /ml.

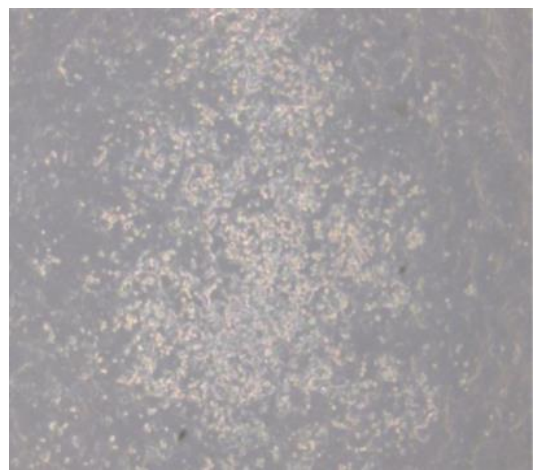
4.4 PLAQUE PURIFICATION OF BTV

Sea plaque agarose overlayed method was done as described in chapter 3.7. Plate was observed for plaque formation every 18-24hours which revealed plaques from 3rd day onwards. Plaques were best identified in low power magnification (Fig 2.A) compared to high power magnification (Fig 2.C).Plaques in different sizes; and different

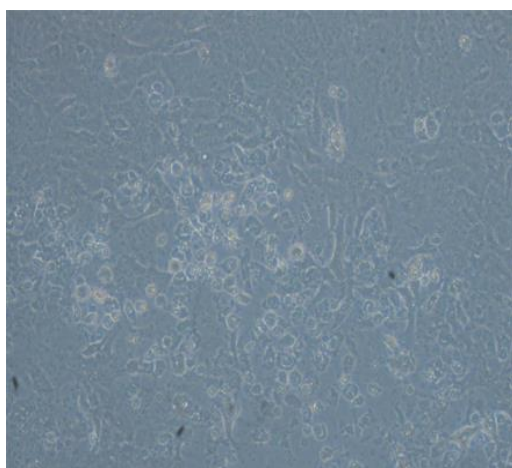
magnifications are shown from Fig 2.A to 2.D. Plaques of approximately not more than 1mm in diameter were visible to naked eye against light source as pale areas in monolayer. Among wells infected with 10^{-2} to 10^{-6} virus dilutions, only 10^{-2} to 10^{-4} dilutions produced plaques where as monolayer infected with 10^{-5} and 10^{-6} dilutions revealed no plaques. The infected plate was also stained with 1% crystal violet solution to observe plaques (Fig .3.A & B). Plaques remained as white spots in the stained monolayer.



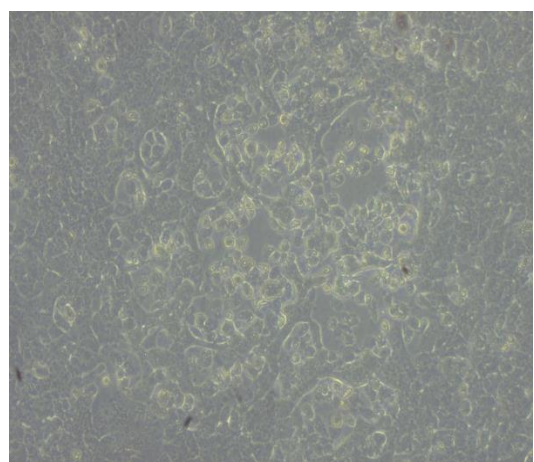
A



B



C



D

FIG.2 : PLAQUE FORMATION IN VERO CELL MONOLAYER INFECTED

WITH BTV-12

Fig A-Single individual plaque surrounded with confluent healthy monolayer (40X).

Fig B- Spreading plaque (40X); Fig C- Plaque at initial stage (100X).

Fig D- Spreading plaque (100X).

Plaques collected from 6-well plate infected the Vero cells in 12-well plate, also showed CPE within 3 days of infection, therefore confirming that collected plaque contained infectious virus. Among these, CPE showing cell culture fluid of respective plaque, particularly which is more isolated from others in monolayer of 6-well plate was used for successive rounds of plaque purification. Finally, selected plaque from third purification was grown in T25 flask and the resultant cell culture fluid was used for molecular confirmation and characterization.

4.5 MOLECULAR CONFIRMATION OF PLAQUE PURIFIED VIRUS

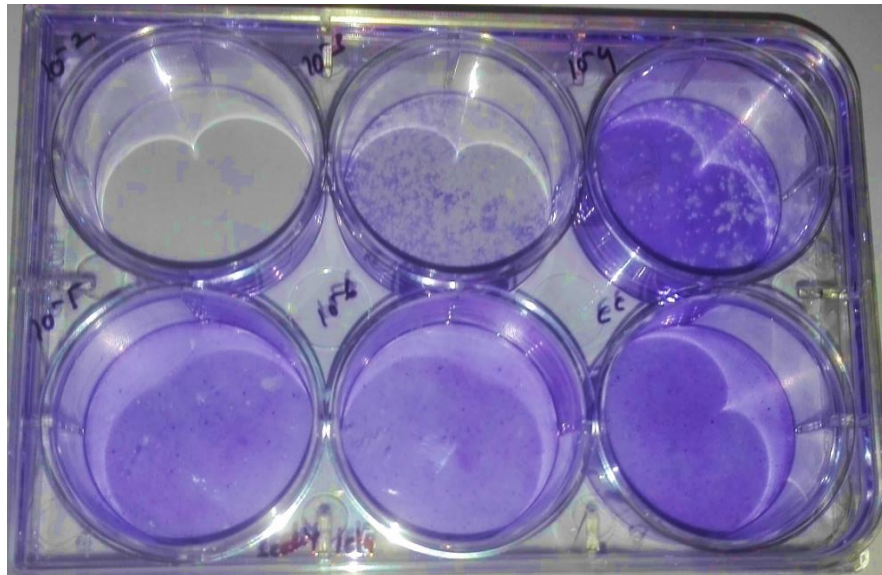
4.5.1 GEL ELECTROPHORESIS OF RNA

RNA extracted from BTV infected Vero cell culture fluid from third round plaque purification was analysed in 1.5% agarose gel. All isolates were showing clear segmented pattern of ds RNA genome with clearly resolved 8 segments and two unresolved segments (Fig 4).

4.5.2. CONFIRMATION OF PLAQUE PURIFIED BTV-12 BY REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

First, cDNA was synthesized from extracted RNA and stored at -20°C . The cDNA was used as template in polymerase chain reaction against available primers to BTV serotypes (BTV-1, 2, 4, 9, 10, 12, 16, 21, 23 and 24), in separate PCR tubes along with positive and negative controls for each type. PCR products were analysed by gel electrophoresis. Only BTV-12 serotype specific primers showed specific PCR amplicon of 750bp with sample cDNA (Fig.5 A), and did not give any amplification with remaining serotype specific primers (Fig.5 B). Thus the isolate was confirmed as BTV-12.

A



B

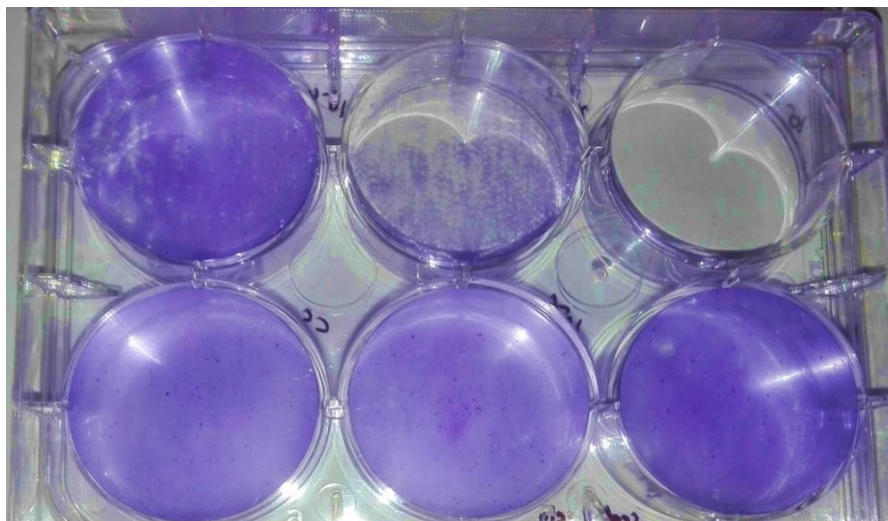


FIG3:CRYSTAL VIOLET STAINING OF PLAQUES

Fig.3 A: Crystal violet staining of monolayer showing plaques (upper view).

Fig.3.B: Crystal violet staining of monolayer showing plaques (bottom view).

Unstained white areas represent plaques where as stained area represent confluent monolayer. In the above stained plate, wells numbered 1 to 5 infected with 10^{-2} to 10^{-6} dilutions of virus respectively and well no. 6 kept as control i.e., infected with plain MEM. Wells no.1 showing complete CPE due to least virus dilution (10^{-2}). Clear plaques observed in well no.2 and 3(10^{-3} and 10^{-4}).

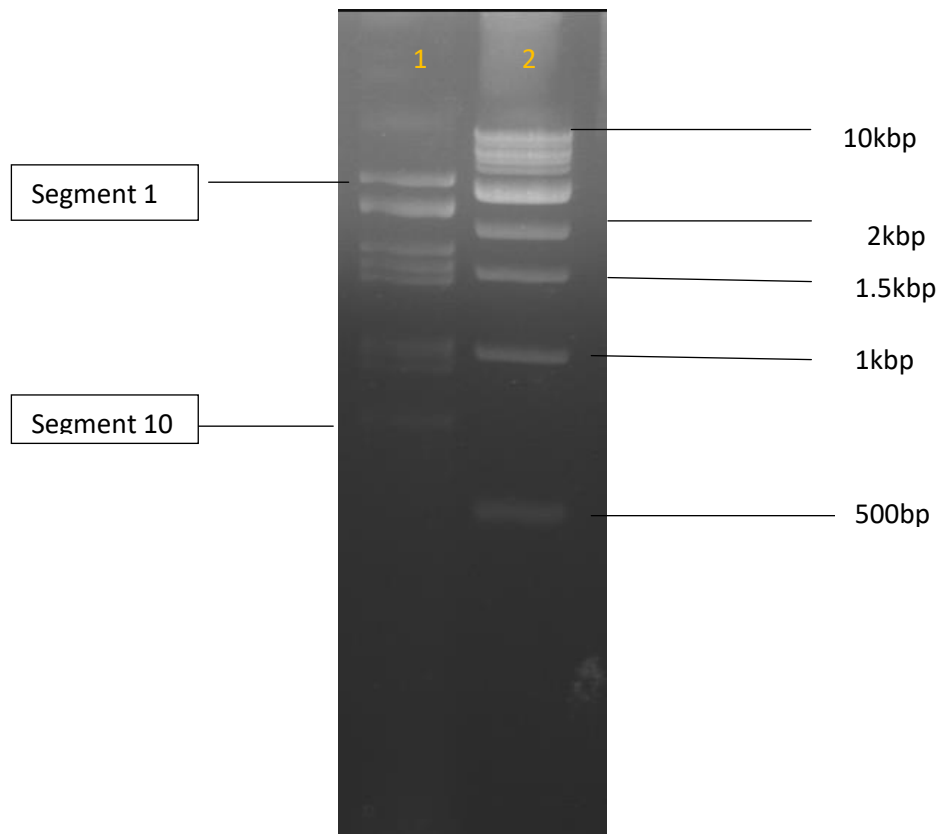
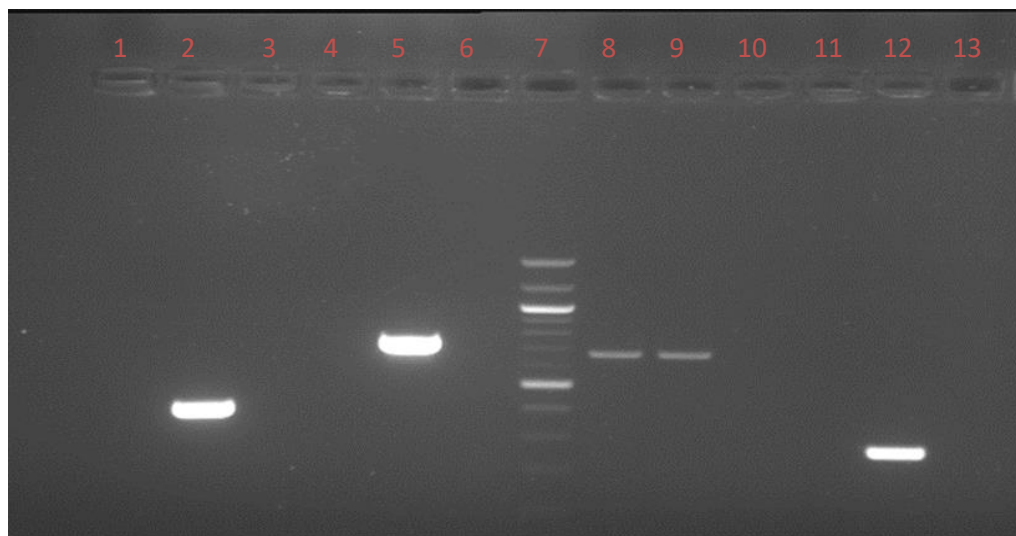


Fig 4:Agarose gel electrophoresis of plaque purified BTV-12RNA

Lane 1- Segments of BTV 12genome

Lane 2- 1 kbp ladder



A

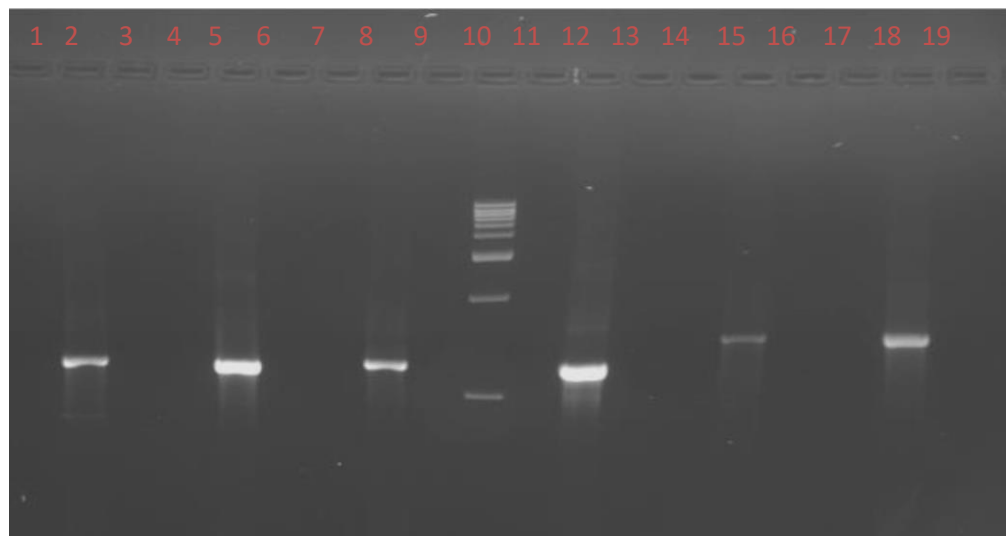
FIG. 5:MOLECULAR TYPING OF BTV -12

Fig A: Gel electrophoresis of BTV-4, 10, 12 and 24.

After third round of plaque purification, the virus study was confirmed as BTV-12 by RT-PCR using type specific primers. 750 bp amplicon was observed with primers specific for BTV- 12 only (Fig. 5.A :well no 8). No amplification was observed with other primers for BTV – 4, 10 and 24 which are commonly prevalent in India. Hence the plaque purified virus was confirmed as BTV-12.

Lane 1: Sample cDNA with BTV-4 primers	Lane 2: Positive control for BTV-4: 464 bp	Lane 3: Negative control
---	---	---------------------------------

Lane 4: Sample cDNA with BTV-10 primers	Lane 5: Positive control for BTV-10: 800 bp	Lane 6: Negative control
Lane 7: 100 bp ladder		
Lane 8: Sample cDNA with BTV-12 primers	Lane 9: Positive control for BTV-12: 750 bp	Lane 10: Negative control
Lane 11: Sample cDNA with BTV-24 primers	Lane 12: Positive control for BTV-24: 319 bp	Lane 13: Negative control



B

FIG .5: MOLECULAR TYPING OF BTV -12

Fig. B:Gel electrophoresis of BTV-1, 2, 9, 16 21 and 23.

No amplification was observed with primers specific for BTV-1, 2, 9, 16,21and 23 which are commonly prevalent in India .Hence plaque purified virus was confirmed as BTV-12.

Lane 1: Sample cDNA with BTV-1 primers	Lane 2: Positive control for BTV-1: 1180bp	Lane 3: Negative control
---	---	---------------------------------

Lane 4: Sample cDNA with BTV-2 primers	Lane 5: Positive control for BTV-2: 1167bp	Lane 6: Negative control
Lane 7: Sample cDNA with BTV-9 primers	Lane 8: Positive control for BTV-9: 1200 bp	Lane 9: Negative control

Lane 10: 1 kbp ladder

Lane 11: Sample cDNA with BTV-16 primers	Lane 12: Positive control for BTV-16: 1200 bp	Lane 13: Negative control
Lane 14: Sample cDNA with BTV-21 primers	Lane 15: Positive control for BTV-21: 1388 bp	Lane 16: Negative control
Lane 17: Sample cDNA with BTV-23 primers	Lane 18: Positive control for BTV-23: 1370 bp	Lane 19: Negative control

4.6 TITRATION OF THE VIRUS

After confirmation of plaques, TCID₅₀ of the virus was once again calculated as it was passaged for 4 times in Vero cell line during three rounds of plaque purification. The 96-well plate readings are given in Table 4.

The virus titre was calculated as $10^{5.116}/100 \mu\text{l}$ (or) $10^{6.116}/\text{ml}$ according to Reed and Munch (1938) method. Increase in virus titre was observed as compared to the titre before plaque purification.

TCID₅₀ was calculated for available types (BTV-1, 2, 4, 9, 10, 16, 21, 23, and 24) which were used in serum neutralization tests. Titres for BTV-1, 2, 4, 9, 10, 16, 21, 23, and 24 are given in Table 5.

Table 4: TCID₅₀ calculation of plaque purified BTV-12serotype

S .No.	Virus dilution	No.of wells infected	CPE positive wells	CPE negative wells	Cumulative positive	Cumulative negative	% of CPE
1	10^{-1}	8	8	0	37	0	100

2	10^{-2}	8	8	0	29	0	100
3	10^{-3}	8	8	0	21	0	100
4	10^{-4}	8	8	0	13	0	100
5	10^{-5}	8	4	4	5	4	55.5
6	10^{-6}	8	1	7	1	11	8.3
7	10^{-7}	8	0	8	0	19	0
8	10^{-8}	8	0	8	0	27	0

Table 5: TCID₅₀ titres of available BTV serotypes

BTV serotype	TCID ₅₀ Calculation /ml
BTV-1	$10^{4.74}$
BTV-2	$10^{5.36}$
BTV-4	$10^{4.39}$
BTV-9	$10^{5.74}$
BTV-10	$10^{4.31}$
BTV-12	$10^{6.116}$
BTV-16	$10^{6.1}$
BTV-21	$10^{5.5}$
BTV-23	$10^{4.6}$
BTV-24	$10^{3.5}$

4.7 ANIMAL INOCULATION

BTV seronegative 8 months old sheep was inoculated with 1ml containing $10^{6.116}$ TCID₅₀ of plaque purified BTV-12 virus on 0 day and 15th day .Clinical and humoral immune response was studied in the animal.

4.7.1 SIGNS AND LESIONS:

Sheep developed pyrexia on evening hours on 6 dpi (104.3° F) which persisted for 4 days (Fig 7) and it exhibited nasal discharges on 13 dpi (Fig 6.A). Control animal was found to be normal (Fig 6.B) during the entire study period (102° F- 103.4° F) (Fig 7).

4.7.2 ANTIBODY RESPONSE TO VIRUS INOCULATION

Serum samples were collected from control and infected animal weekly once for 30 days and group-specific serologic test, cELISA for confirmation of seroconversion was done. Inoculated sheep found to seroconvert by 7th day while control remained seronegative. The animal was seropositive till the end of the experiment i.e, 28 dpi (Table 6).

**Table 6: IMMUNE RESPONSE IN EXPERIMENTAL ANIMALS
(BY cELISA)**

Days postinoculation	% Inhibition (Result) in	
	Infected sheep	Control animal
0	22.74 (Negative)	5.58 (Negative)
7	100.12 (Positive)	10.64 (Negative)
14	109.6 (Positive)	14.67 (Negative)
21	112.74 (Positive)	29.67 (Negative)
28	113.54 (Positive)	22.58 (Negative)

(PI values above 50 are considered to be for antibody positive)

4.8 NEUTRALIZATION STUDIES

SNT was performed as described in 3.12.to estimate type specific antibody titre in serum of BTV-12 inoculated sheep. Animal developed neutralizing antibodies by 7 dpi. Control animal didn't develop any antibodies. Neutralizing antibody titres of weekly collected serum samples of animals, using different dilutions of serum with 100 TCID₅₀ titre virus, were estimated against homologous plaque purified BTV-12. Weekly SN titres against plaque purified BTV-12 are given in Table 7.

Table 7. Serum Neutralization titres

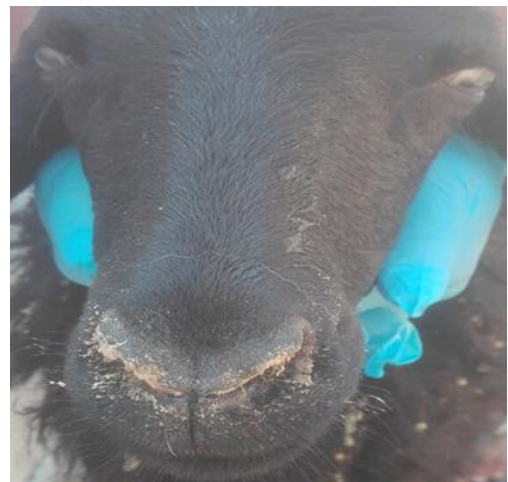
Animal	Neutralizing antibody titre				
s.no	0 dpi.	7 dpi	14 dpi	21 dpi.	28 dpi
1	0	1/40	1/160	1/320	1/320

For cross-neutralization studies, serum of 28th day was tested against all available plaque purified serotypes (BTV-1, 2, 4, 9, 10, 16, 21, 23, and 24). In these studies, BTV-1 was found be neutralized with BTV-12 serum, but didn't show any cross neutralization with other available plaque purified serotypes (Table 8).

Table 8: CROSS NEUTRALIZATION STUDIES

Plaque purified BTV serotypes	SNT result with 28 th day hyperimmune serum of BTV- 12
BTV-1	CPE –ve
BTV-2	CPE +ve

BTv-4	CPE +ve
BTv-9	CPE +ve
BTv-10	CPE +ve
BTv-16	CPE +ve
BTv-21	CPE +ve
BTv-23	CPE +ve
BTv-24	CPE +ve



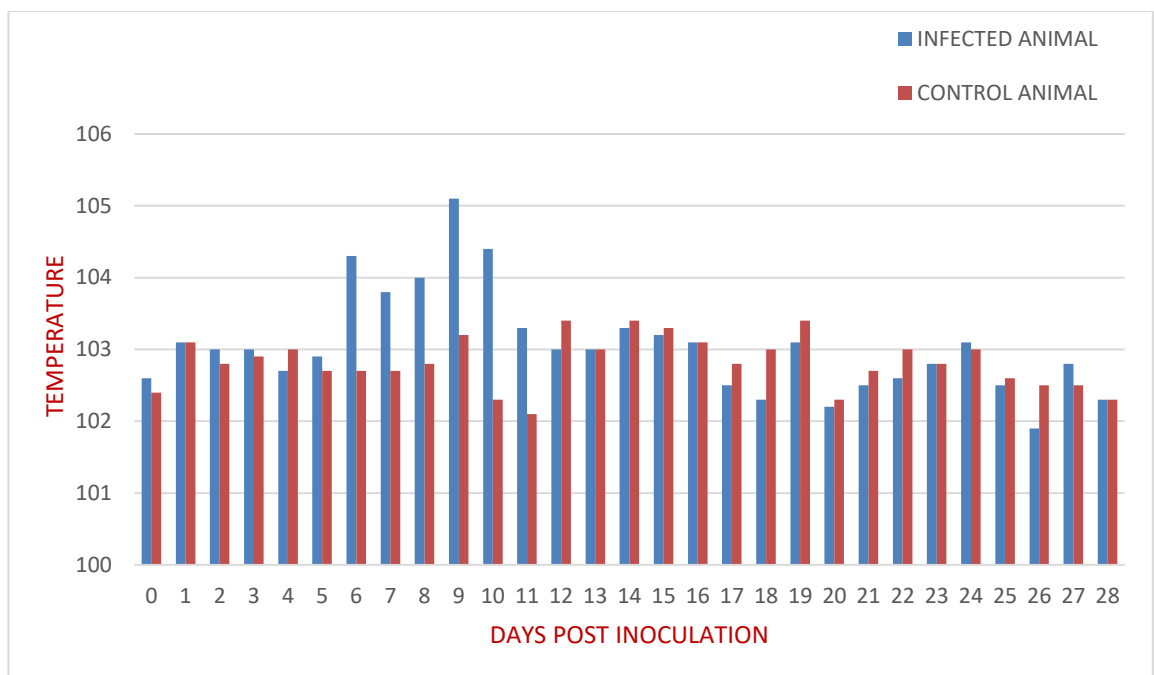
A B

**FIG 6:CLINICAL SYMPTOMS OF BT IN EXPERIMENTAL
INOCULATED SHEEP**

Fig.A: Infected sheep was showing nasal discharges on 13dpi.

Fig.B: Control animal without nasal discharges.

Fig 7: TEMPERATURES RECORD OF EXPERIMENTAL ANIMALS



It represents temperature details of infected and control animal. Infected animal showed pyrexia between 6-10dpi (103.8⁰F-105.1⁰F). Control animal maintained normal temperature during the entire study(101.9⁰ F-103.4 ⁰F).

4.9 VP2 GENE SEQUENCING AND PHYLOGENETIC ANALYSIS OF BTV12 VP2

4.9.1 Sequencing

The product with 750 bp size was sequenced at Xcelris Labs Ltd, Gujarat using VP2 gene specific forward and reverse primers. The generated forward and reverse sequence chromatograms (Fig 8.A &B) were initially edited with Chromas V 2.0 software and low quality sequences were trimmed from raw sequence data file. Further editing was carried out by BioEdit v7.0.5.3.software and necessary additional changes were made to rawsequence data file. Upon editing, the forward and reverse sequences were aligned and a consensus sequence was obtained.

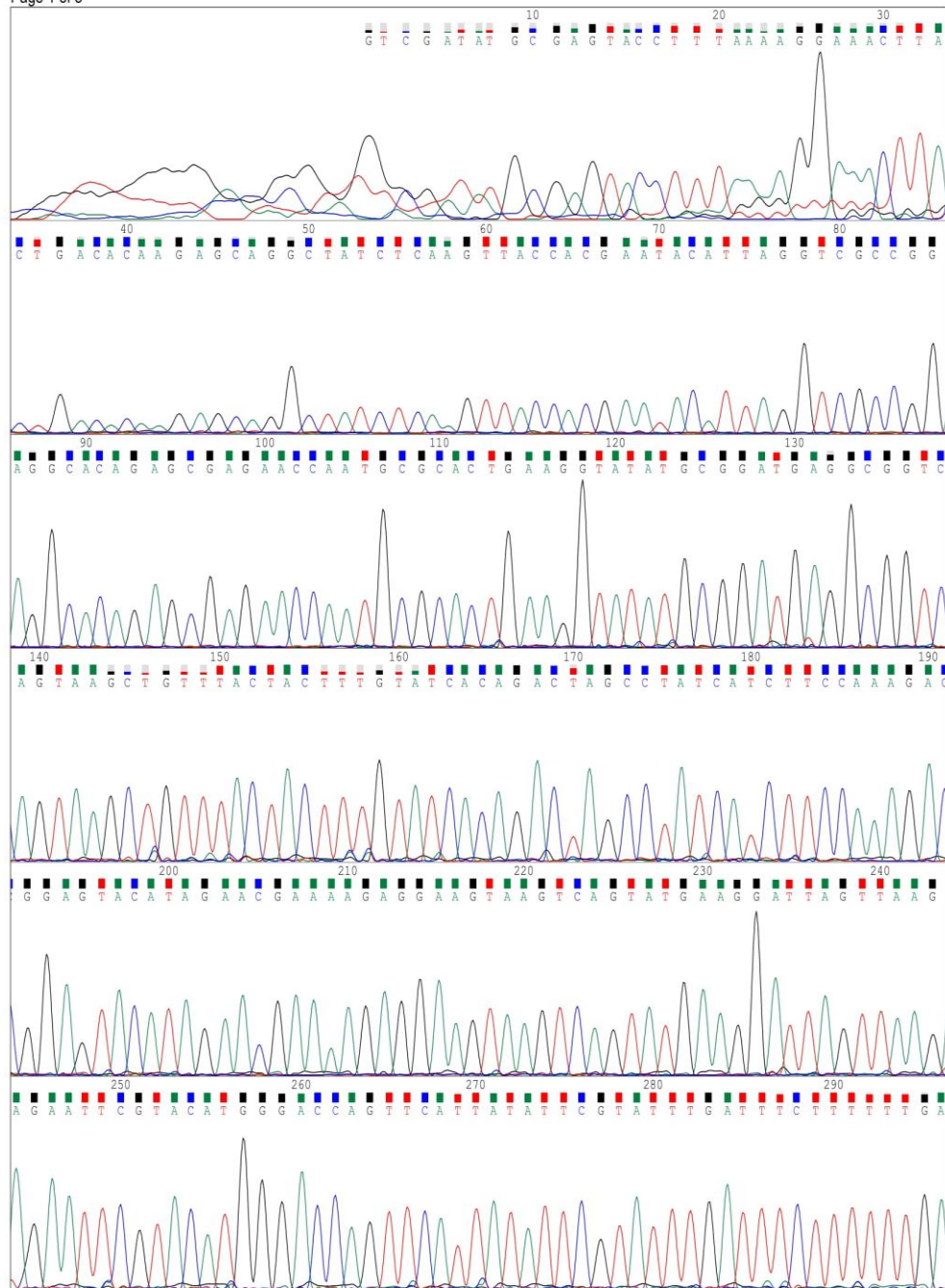


Fig8.A:Chromatogram of forward sequence(contd.)

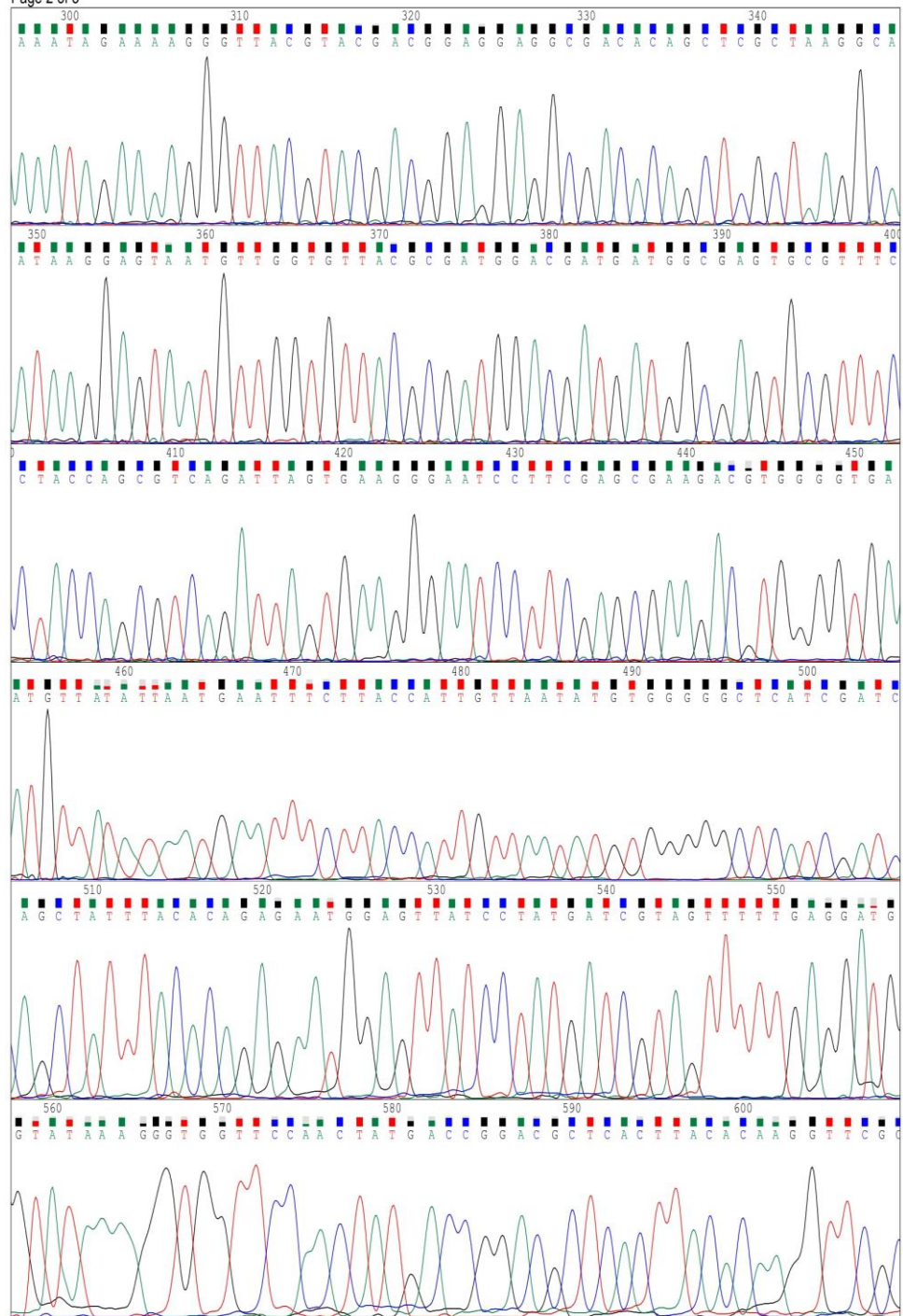


Fig 8.A:Chromatogram of forward sequence(contd,)

File: VP2-OF-BT-V12F-1_BT-12-VP2F_S017763_C08_060.ab1 Run Ended: Nov 2, 2017, 14:17:18 Signal G:4414 A:9299 T:9201 C:7029
Sample: VP2-OF-BT-V12F-1_BT-12-VP2F_S017763 Lane: 60 Base spacing: 14.97 691 bases in 8789 scans
Page 3 of 3

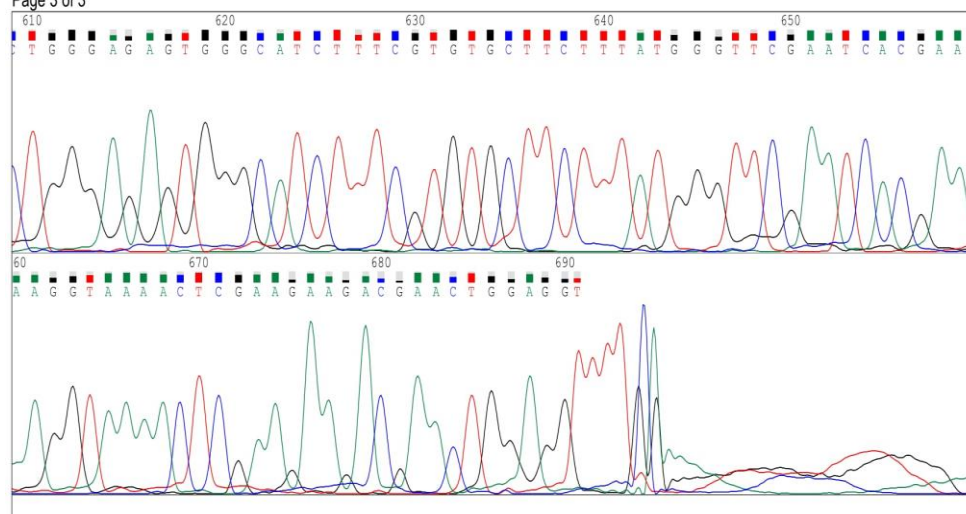


Fig 8.A:Chromatogram of forward sequence

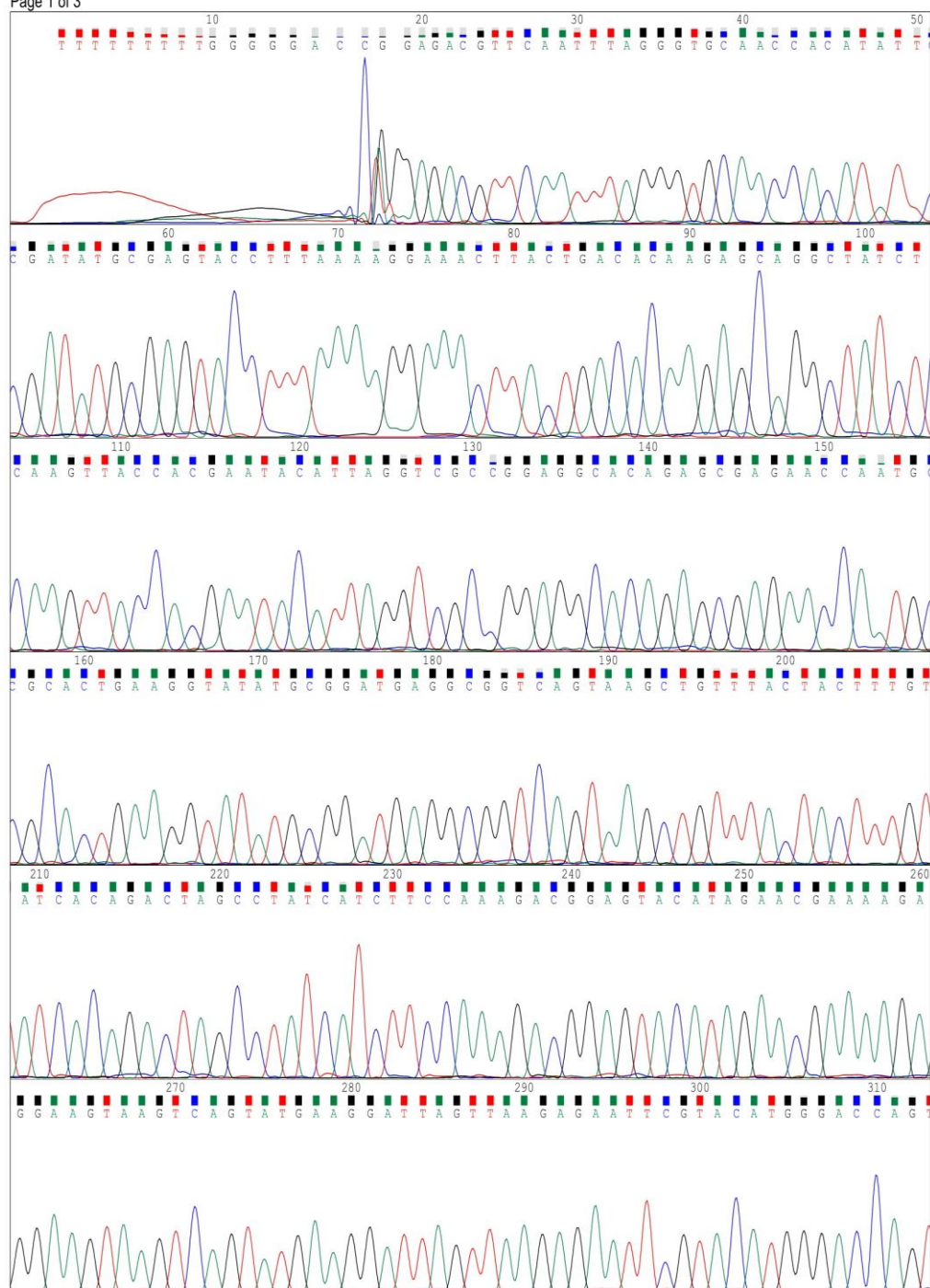


Fig 8.B:Chromatogram of reverse sequence(contd.,)

File: VP2-OF-BT-V12F-2_BT-12-VP2R_S017763_D08_058.ab1 Run Ended: Nov 2, 2017, 14:17:18
Signal C:5036 T:8795 A:12562 G:12629 Sample: VP2-OF-BT-V12F-2_BT-12-VP2R_S017763
Lane: 58 Base spacing: 14.86 708 bases in 8742 scans
Page 2 of 3

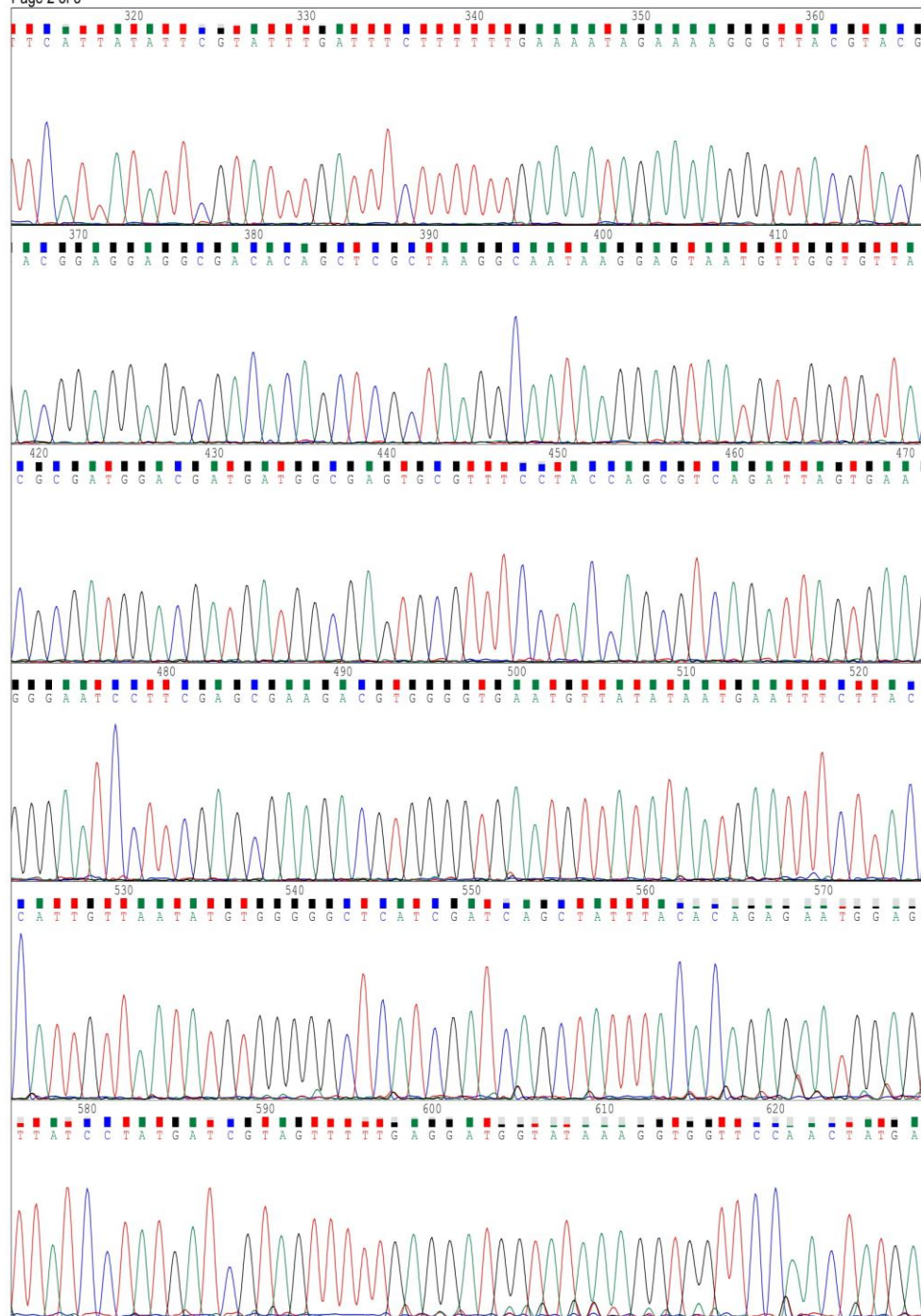


Fig 8.B:Chromatogram of reverse sequence(contd.,)

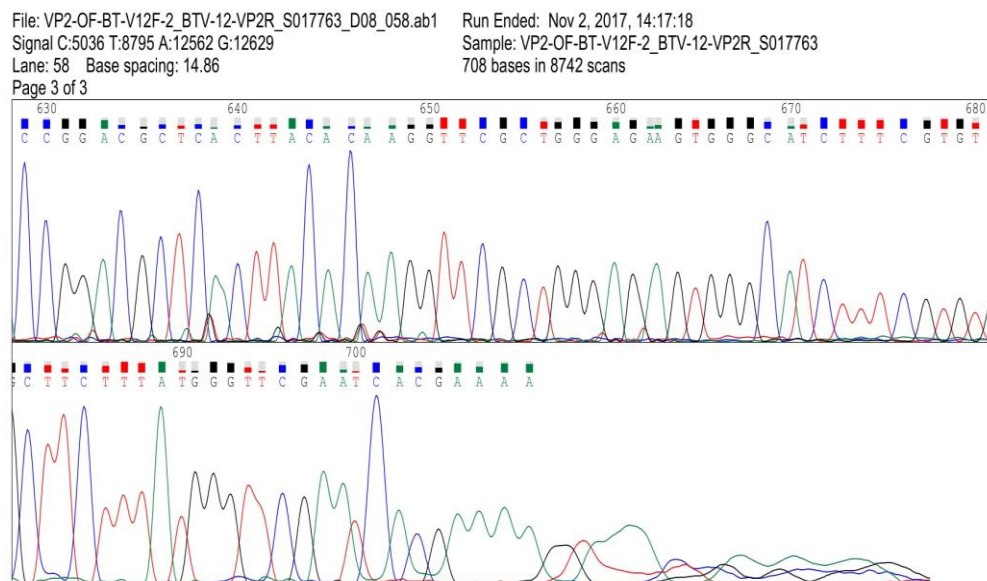


Fig 8.B: Chromatogram of reverse sequence

Consensus sequence after aligning of forward and reverse sequences:

GTCGATATGCGAGTACCTTTAAAAGGAACTTACTGACACAAGAGCAGGCT
ATCTCAAGTTACCACGAATACATTAGGTCGCCGGAGGCACAGAGCGAGAAC
CAATGCGCACTGAAGGTATATGCGGATGAGGCGGTCAGTAAGCTGTTTACT
ACTTTGTATCACAGACTAGCCTATCATCTTCCAAAGACGGAGTACATAGAAC
GAAAAGAGGAAGTAAGTCAGTATGAAGGATTAGTTAAGAGAATTCGTACAT
GGGACCAGTTCATTATATTCGTATTTGATTTCTTTTTTGAAAATAGAAAAGG
GTTACGTACGACGGAGGAGGCGACACAGCTCGCTAAGGCAATAAGGAGTA
ATGTTGGTGTTACGCGATGGACGATGATGGCGAGTGCGTTTCCTACCAGCGT
CAGATTAGTGAAGGGAATCCTTCGAGCGAAGACGTGGGGTGAATGTTATAT
AATGAATTTCTTACCATTGTTAATATGTGGGGGCTCATCGATCAGCTATTTA
CACAGAGAATGGAGTTATCCTATGATCGTAGTTTTTGAGGATGGTATAAAG
GTGGTTCCAACATATGACCGGACGCTCACTTACACAAGGTTTCGCTGGGAGAG
TGGGCATCTTTCGTGTGCTTCTTTATGGGTTCAATCACGAAAAGGTAAAAC
TCGAAGAAGACGAACTGGAGGT.

4.9.2 SEQUENCE ANALYSIS

The sequences of partial segment 2 of BTV 12 isolate obtained after sequencing were compared with other available BTV 12 VP2 sequences in GeneBank using NCBI BLAST (www.ncbi.nlm.nih.gov/blast).

4.9.2.1 MULTIPLE SEQUENCE ALIGNMENT

Using ClustalW, multiple sequence alignment was generated using MEGA 7.0 and sequence alignment generated by comparing the nucleotide sequence of BTV 12 with other sequences of bluetongue virus downloaded from NCBI-Gene Bank is presented in fig 9.

	410	420	430	440	450
BTV 12 INDIA/2017	TACCAGGTCAGATTAGTCAAGGGAATCCTTCGAGCCAACACGTGGGGTG				
INDIA 1	TACCAGGTCAGATTAGTCAAGGGAATCCTTCGAGCCAACACGTGGGGTG				
INDIA 2	TACCAGGTCAGATTAGTCAAGGGAATCCTTCGAGCCAACACGTGGGGTG				
INDIA 3	TACCAGGTCAGATTAGTCAAGGGAATCCTTCGAGCCAACACGTGGGGTG				
INDIA 4	TACCAGGTCAGATTAGTCAAGGGAATCCTTCGAGCCAACACGTGGGGTG				
SOUTH AFRICA 1	TACCAGGTCAGATTAGTCAAGGGAATCCTTCGAGCCAACACGTGGGGTG				
SOUTH AFRICA 2	TACCAGGTCAGATTAGTCAAGGGAATCCTTCGAGCCAACACGTGGGGTG				
ISRAEL 1	CACCAGTGTGAGATTAGTCAAGGGAATCCTTCGAGCCAACACGTGGGGCG				
ISRAEL 2	CACCAGTGTGAGATTAGTCAAGGGAATCCTTCGAGCCAACACGTGGGGCG				
ISRAEL 3	CACCAGTGTGAGATTAGTCAAGGGAATCCTTCGAGCCAACACGTGGGGCG				
USA 1	TACCAGTGTGAGATTAGTCAAGGGAATCCTTCGAGCCAACACGTGGGGTG				
USA 2	AACCAGTGTGAGATTAGTCAAGGGAATCCTTCGAGCCAACACGTGGGGTG				
CHINA 1					
CHINA 2					

	460	470	480	490	500
BTV 12 INDIA/2017	AATGTTATATAATGAATTTCTTACCATTGTTTATATGTTGGGGGCTCATCG				
INDIA 1	AATGTTATATAATGAATTTCTTACCATTGTTTATATGTTGGGGGCTCATCG				
INDIA 2	AATGTTATATAATGAATTTCTTACCATTGTTTATATGTTGGGGGCTCATCG				
INDIA 3	AATGTTATATAATGAATTTCTTACCATTGTTTATATGTTGGGGGCTCATCG				
INDIA 4	AATGTTATATAATGAATTTCTTACCATTGTTTATATGTTGGGGGCTCATCG				
SOUTH AFRICA 1	AATGTTATATAATGAATTTCTTACCATTGTTTATATGTTGGGGGCTCATCG				
SOUTH AFRICA 2	AATGTTATATAATGAATTTCTTACCATTGTTTATATGTTGGGGGCTCATCG				
ISRAEL 1	AATGTTATATAATGAATTTCTTACCATTGTTTATATGTTGGGGGCTCATCG				
ISRAEL 2	AATGTTATATAATGAATTTCTTACCATTGTTTATATGTTGGGGGCTCATCG				
ISRAEL 3	AATGTTATATAATGAATTTCTTACCATTGTTTATATGTTGGGGGCTCATCG				
USA 1	AGTGTATATAATGAATTTCTTACCATTGTTTATATGTTGGGGGCTCATCG				
USA 2	AGTGTATATAATGAATTTCTTACCATTGTTTATATGTTGGGGGCTCATCG				
CHINA 1					
CHINA 2					

	510	520	530	540	550
BTV 12 INDIA/2017	ATCAGCTATTTACACACACAATGGAGTTATCCTATGATCGTAGTCTTTTGA				
INDIA 1	ATCAGCTATTTACACACACAATGGAGTTATCCTATGATCGTAGTCTTTTGA				
INDIA 2	ATCAGCTATTTACACACACAATGGAGTTATCCTATGATCGTAGTCTTTTGA				
INDIA 3	ATCAGCTATTTACACACACAATGGAGTTATCCTATGATCGTAGTCTTTTGA				
INDIA 4	ATCAGCTATTTACACACACAATGGAGTTATCCTATGATCGTAGTCTTTTGA				
SOUTH AFRICA 1	ATCAGCTATTTACACACACAATGGAGTTATCCTATGATCGTAGTCTTTTGA				
SOUTH AFRICA 2	ATCAGCTATTTACACACACAATGGAGTTATCCTATGATCGTAGTCTTTTGA				
ISRAEL 1	ATCAGCTATTTACACACACAATGGAGTTATCCTATGATCGTAGTCTTTTGA				
ISRAEL 2	ATCAGCTATTTACACACACAATGGAGTTATCCTATGATCGTAGTCTTTTGA				
ISRAEL 3	ATCAGCTATTTACACACACAATGGAGTTATCCTATGATCGTAGTCTTTTGA				
USA 1	ATCAGCTATTTACACACACAATGGAGTTATCCTATGATCGTAGTCTTTTGA				
USA 2	ATCAGCTATTTACACACACAATGGAGTTATCCTATGATCGTAGTCTTTTGA				
CHINA 1					
CHINA 2					

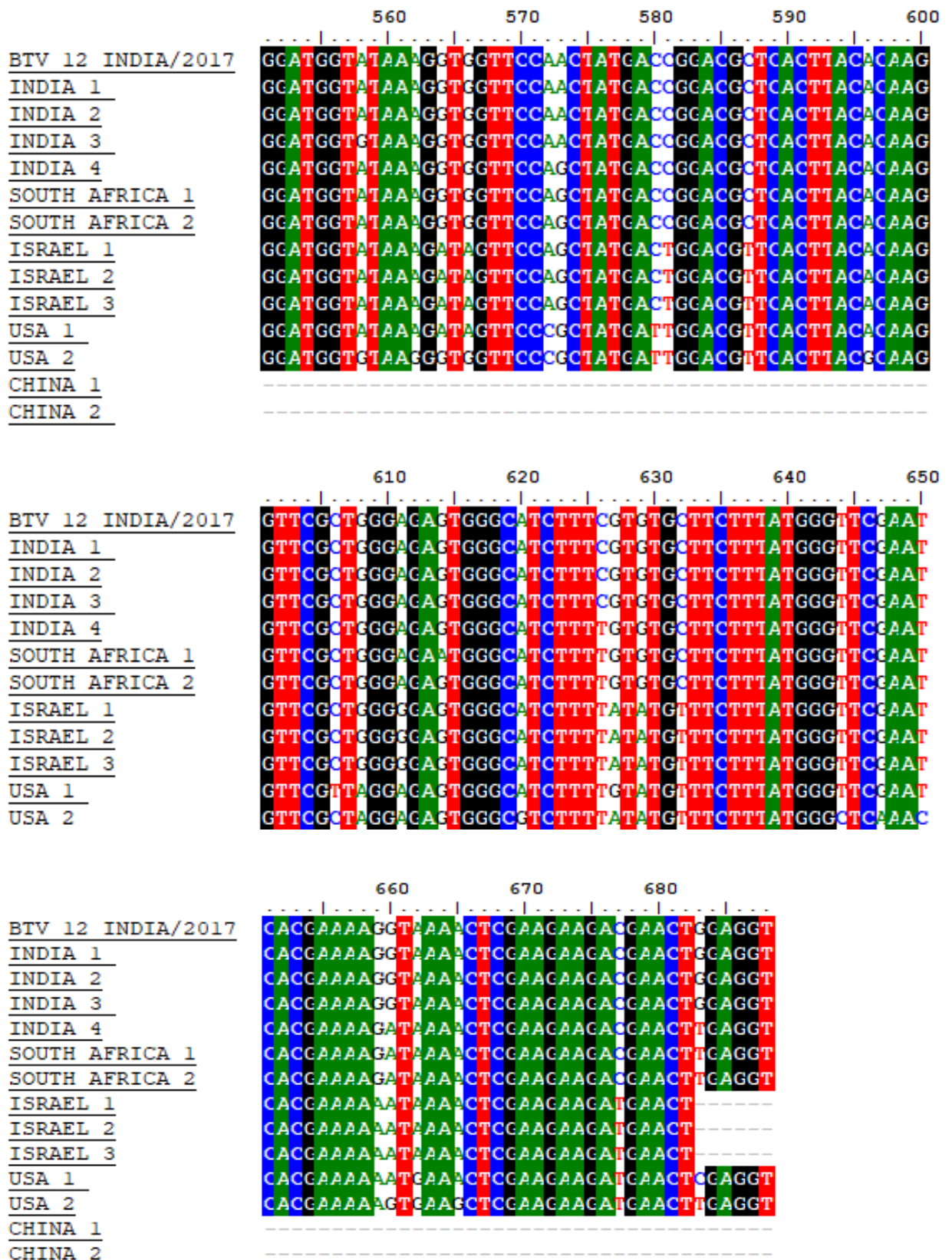


Fig 9: Clustal W Multiple nucleotide sequence alignment of BTV 12 (used in the current study) with partial sequences of other bluetongue viruses (obtained from Gene Bank) showing complete and partially conserved regions among bluetongue viruses. Isolate used in current study is labelled as BTV 12 INDIA/ 2017

4.9.2.2PHYLOGENETIC ANALYSES

Phylogenetic tree reconstruction by neighbour joining method (NJ) using MEGA7 software was done to study the evolutionary relationship between BTV12 of the current study and other bluetongue viruses (Fig.10). It revealed that BTV12 (BTV12 INDIA/2017) was very closely related to Indian BTV 12 isolates (99%). India 1 (GENE BANK ACC. NO. MF615238.1), India 2 (GENE BANK ACC.NO. KX905151.1) and India 3 (GENE BANK ACC. NO. KC662613.1). Its sequences were 97% similar to BTV12 isolates; India 4 (GENE BANK ACC. NO. JX272500.1), China1 (GENE BANK ACC. NO. KP195138.1), China 2 (GENE BANK ACC. NO. KP 221199.1) and 96% similarity was seen with South Africa 1 (GENE BANK ACC.NO.KP821100.1) and SouthAfrica 2 (GENE BANKACC. NO. AJ585133.1) isolates. It has 90% similarity with Israel 1 (GENE BANK ACC. NO. KT946761.1), Israel2 (GENE BANK ACC. NO. KT946760.1), Israel3 isolates (GENE BANK ACC. NO. KT946759.1) and 87-88% similarity with USA 1 (GENE BANK ACC. NO.KX164090.1) and USA 2 (GENE BANK ACC. NO KX164080.1) isolates were observed.

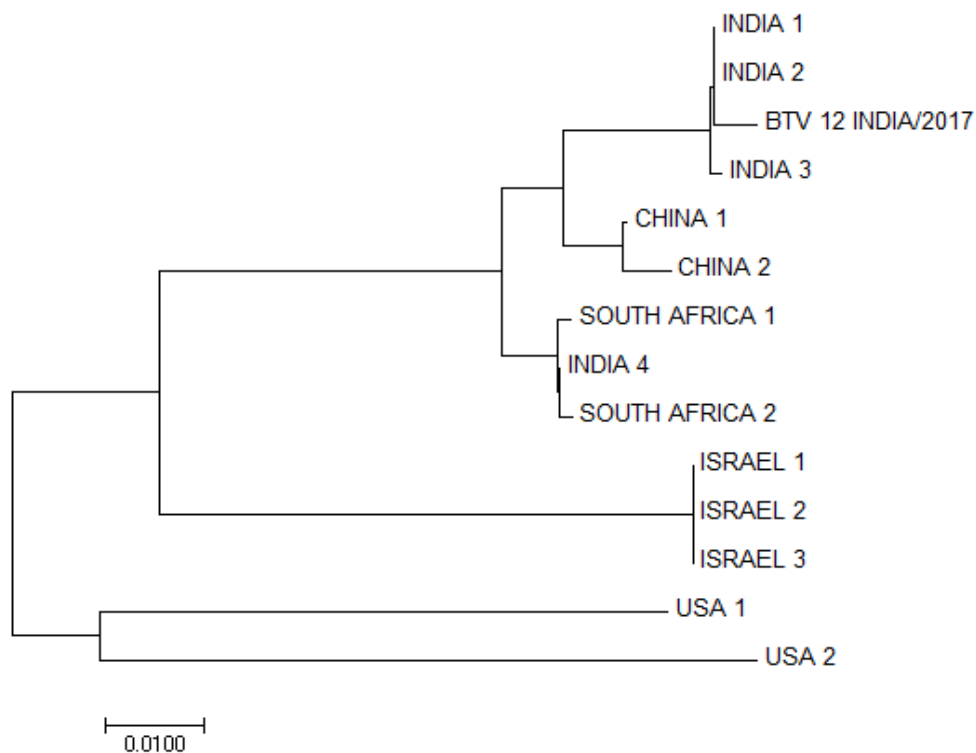
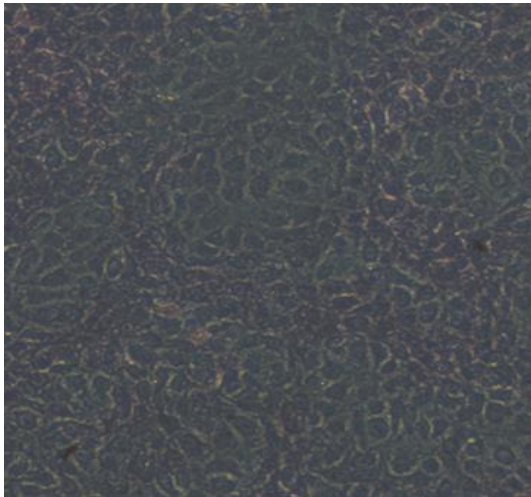


FIG10:PHYLOGENETIC ANALYSIS OF BTV-12 INDIA/ 2017

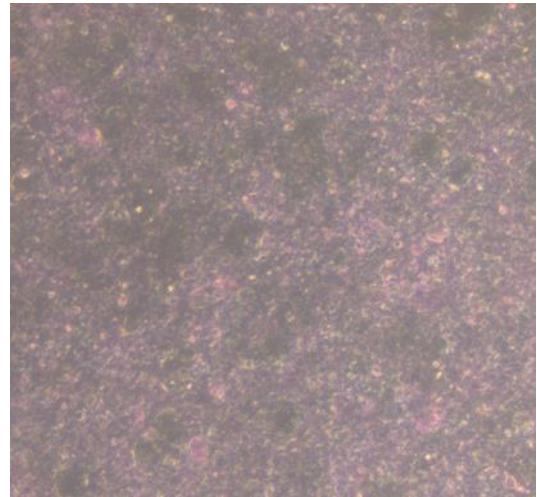
The evolutionary history was inferred using the Neighbor-Joining method (Saitou *et al.*). The optimal tree with the sum of branch length = 0.28396251 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*) and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 688 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

4.10 BTV12 IN VERO CELLS

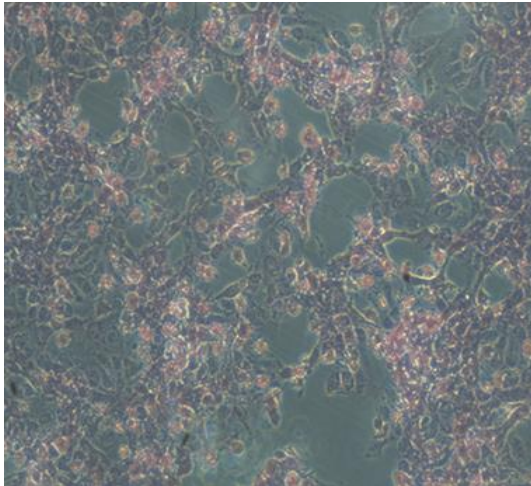
Cytopathic effect of BTV12 in Vero cell was studied by H&E staining of infected Vero cells at 12, 24, 36, 48, 72hrs post infection (PI) for understanding the kinetics of infection. During 24 hrs of initial infection, there was no observed CPE (Fig11.A& B) but 48hrs later, characteristic CPE with swollen spindle shaped cells which aggregated together into small and large clumps were observed (Fig11.C& D). By 72hrs of PI, complete detachment of the monolayer took place (Fig11.E &F).



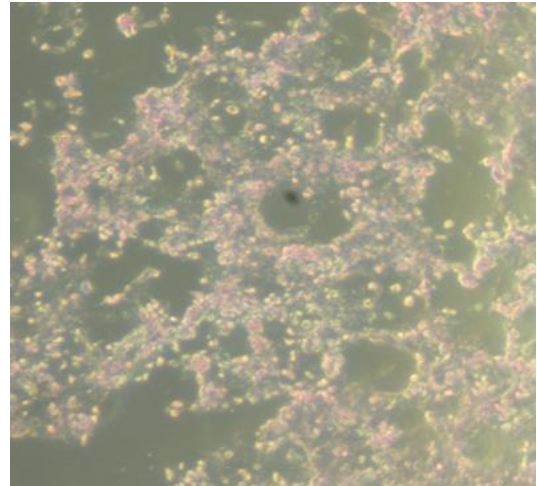
A



B



C



D

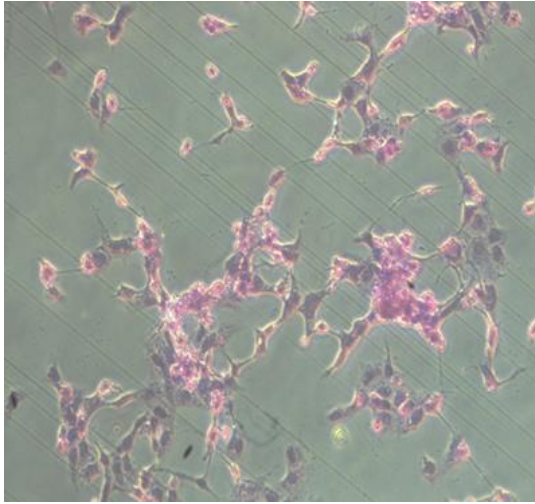
**FIG 11:CYTOPATHIC EFFECT OF BTV 12 IN VERO CELL LINE
(H& E STAINING)**

Fig A: Monolayer after 24hrs PI (100X);

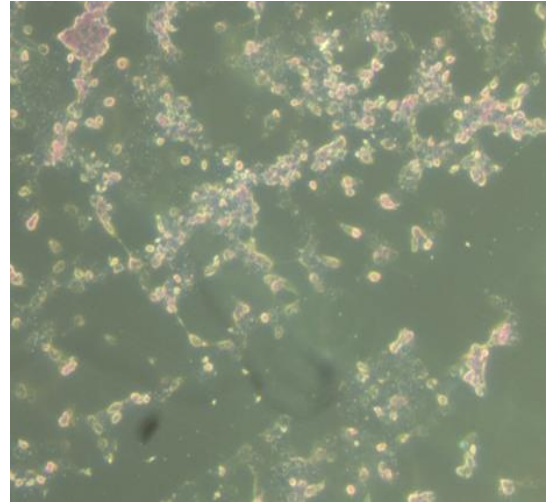
Fig B : Monolayer after 24hrs PI (40X).

Fig C: Rounding and clumping of infected monolayer after 48 hrs. PI (100X).

Fig D :Rounding and clumping of infected monolayer after 48 hrs.PI (40X).



E



F

**FIG 11:CYTOPATHIC EFFECT OF BTV 12 IN VERO CELL LINE
(H & E STAINING)**

Fig E:Complete detachment of infected monolayer after 72 hrs. PI (100X).

Fig F: Complete detachment of infected monolayer after 72 hrs. PI (40X).

CHAPTER V

DISCUSSION

5.1 PROPAGATION OF BLUETONGUE VIRUS ISOLATE

Bluetongue virus serotype 12 (BTV-12) isolated during 2016 was used in this study. This isolate was maintained on BHK-21 cell line previously but now it has been adapted to Vero cell line. The pattern of CPE observed was rounding and granulation of cells, cluster formation, detachment of cells and finally sloughing off the monolayer. Similar pattern of CPE was also observed by Ghalib *et al.* (1984), Bhat *et al.* (1996) and Babu *et al.* (2005). These results suggest the characteristic CPE of BTV infection.

After being adapted to Vero cells, infectivity titre was assayed and was found to be $10^{5.5}$ TCID₅₀/ml at 4th passage. But as it is passaged further four times in Vero cells, as a part of plaque purification, the titre at the end of 8th passage titre was found to be $10^{6.116}$ TCID₅₀/ml. These results were in correlation with Reddy (2016) who observed Vero adapted BTV-4 titre increased from $10^{-4.46}$ / ml to $10^{-6.46}$ / ml in 8 passages.

5.2 PLAQUE PURIFICATION

In the present study, agarose overlay method was used to plaque purify the BTV-12 in Vero cells for three times. Serial 10-fold dilutions of stock virus was made and 10^{-2} to 10^{-6} dilutions were infected on to monolayer of each well of 6-well plate. After 3 days of incubation suspected plaque areas of minute size were noticed which became more prominent by 4th day, a finding which can be correlated with the observations of Howell *et al.* (1967), Dulbecco (1952) and Reddy (2016).

Regarding the morphology, plaques observed in this study were almost clear, circular in shape with similar sizes (1-1.5 mm). However, increase in size was observed on prolonged incubation. The small size of plaques might be due to increased agarose concentration (1.2%) in overlay as observed by Howell et al. (1967).

5.3 MOLECULAR CONFIRMATION

Agarose gel electrophoresis of RNA isolated from BTV infected Vero cells revealed segmented genome pattern with 8 clear and 2 indistinct bands. Segmented pattern of genome is a characteristic feature of BTV nucleic acid as reported by many researchers (Verwoerd et al., 1971) and (Roy 1992).

For serotype confirmation, RT-PCR was conducted using available primers of BTV types (BTV-1, 2, 4, 9, 10, 12, 16, 21, 23 and 24). 750bp PCR product was observed only with BTV-12 specific primers. No amplification was observed with primers specific for other serotypes. Amplification of positive cDNA of each serotype yielded expected size of products with respective primers. These results are in accordance with the conclusions of Prasad et al. (2013), Reddy et al. (2015) and Krishnaji et al. (2016) regarding VP2 based serotype-specific RT-PCR.

5.4 ANIMAL INOCULATION

Sheep acts as the natural host for bluetongue virus. Lambs are more susceptible to BT than the adults. Hence, 6-8 months old lambs were chosen for the study. These sheep were screened for seronegativity by ELISA (Afshar et al, 1989).

Sheep was inoculated with 1ml of tissue culture containing $10^{6.116}$ /ml TCID₅₀ of plaque purified BTV-12 virus. It developed pyrexia on 6dpi (104.3⁰ F) which persisted for 4 days with mild clinical signs. However, pyrexia was not observed with the booster dose, which might be due to development of neutralizing antibodies with a

titre of 1/160 by 15th day. Parker et al. (1975) considered a titre of 1/20 or more as positive for neutralization. Control animal was found to be normal as before inoculation, during this entire study (101.9⁰F- 103.4⁰F) and didn't show any clinical signs. Similar observations were made previously by Jeggo et al. (1983) and Reddy (2016) where they mentioned that experimental inoculation of sheep with cell culture adapted BTV results in mild clinical manifestations.

5.5 SERUM NEUTRALIZATION STUDIES

SNTs were carried out for serotype confirmation as it was considered as gold standard for typing and also to study neutralization behaviour of plaque purified BTV-12 hyper immune serum (HIS). This was found to neutralize plaque purified BTV-12. Titre was observed to increase from 1/40 to 1/320 during 7th day to 28th dpi. Development of neutralizing antibodies appeared earlier and with high titre compared to the experiments of Jeggo et al. (1983) and Reddy (2016). This may be because of virus, its passages and other factors like culture conditions etc.

In cross-neutralization studies, BTV-12 hyper immune serum didn't neutralize BTV- 2, 4, 9, 10, 16, 21, 23 and 24 types. But, despite plaque purification, HIS of BTV-12 was found to neutralize BTV-1 serotype. Test was repeated for confirmation but gave same results. It is interesting to note that the phylogenetic analysis of partial VP2 of the tested serotypes in the current study revealed close relationship between BTV 12 and BTV 1 (Fig 12). This needs further studies as no such cross reaction between BTV-1 and BTV-12 are reported so far. Since the viruses are evolving independently in different geographical areas. This warrants cross neutralization studies between BTV-1 and BTV-12 isolates from different geographical origin.

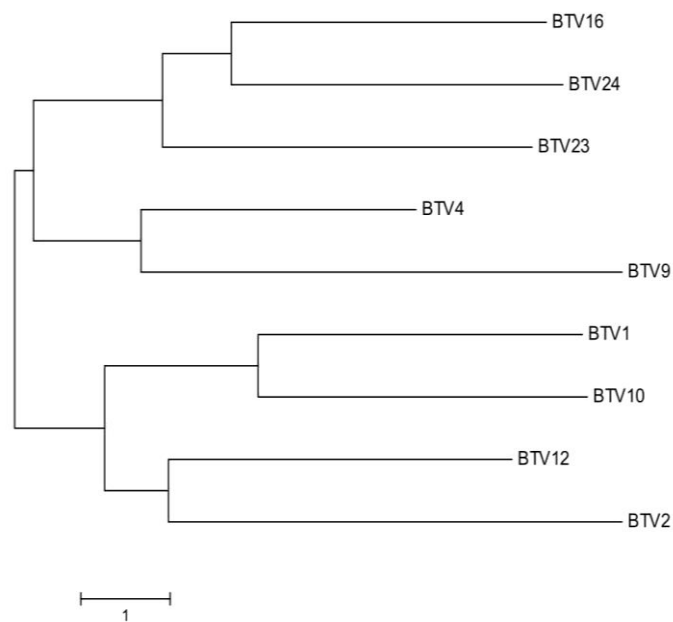


FIG. 12

Phylogenetic tree shows relationship between the partial VP2 gene of BTV 12 and VP2 of other predominantly circulating BTV serotypes in India. BTV-1 (GENE BANK ACC.NO. [KU234258.1](#)) BTV-2 (GENE BANK ACC.NO. AB686224.1) BTV-4 (GENE BANK ACC.NO. [KF560418.1](#)) BTV-9 (GENE BANK ACC.NO. [KP821093.1](#)) BTV-10 (GENE BANK ACC.NO. [KP339245.1](#)) BTV16 (GENE BANK ACC.NO. [KP339225.1](#)) BTV23 (GENE BANK ACC.NO. [AY643507.1](#)) BTV24 (GENE BANK ACC.NO. [KT945048.1](#))

Given that VP2 is the major protein involved in eliciting neutralizing antibody response (Hassan *et al.*, 1999), the cross reactivity observed in the current study can be attributed to the evolutionary dynamics of BTV VP2 as in Fig 12. Even more intriguing is the finding that no cross reactivity of BTV 12 was observed with even closely related BTV 2 and BTV 10. A two way cross neutralization test would have provided better insights into understanding this phenomenon. This cross neutralization behaviour of BTV serotypes is in agreement with previous observations of Zulu and Venter (2014) who observed some cross or heterotypic antibody response in cross protection assays between BTV-9, 10 and 11 each with BTV-4. Reddy (2016) also observed some cross or heterotypic antibody response in cross protection assays between BTV-9 and 10 each with BTV-4. There were no details regarding sequence similarities.

5.6 VP2 GENE SEQUENCING AND PHYLOGENETIC ANALYSIS OF BTV12 VP2

The sequences of partial segment 2 of BTV 12 isolate obtained after sequencing and multiple sequence alignment generated by comparing the nucleotide sequence of BTV 12 with other sequences of bluetongue virus downloaded from NCBI-Gene Bank.

Phylogenetic tree reconstruction by neighbour joining method (NJ) using MEGA7 software was done to study the evolutionary relationship between BTV12 of the current study and other bluetongue viruses. It revealed that BTV12 (BTV12 INDIA/2017) was very closely (99%) related to BTV 12 isolates: India 1, India 2 and India 3. Its sequences were 97% similar to BTV12 isolates: India 4, China 1 and China 2. While 96% similarity was seen with South Africa 1 and South Africa 2. It had 90% similarity with Israel 1, Israel 2, Israel 3 and 87-88% similarity with USA 1 and USA 2. Similar results were observed by Prasad *et al.* (2013) wherein he observed novel BTV10 isolate share maximum 100% nucleotide sequence identity with four USA isolates and

one of the Indian isolate and more than 95% nucleotide identity with six other USA isolates and one Indian isolate. Krishnajyothiet *al.* (2016) also observed BTV-24 (IND2010/10) isolate share 94% sequence identity with the reference strain of BTV24 (RSArrrr/24), 92% with an Israeli isolate and 89% with Central and South American isolates. Phylogenetic analysis of all these sequences indicated that IND2010/10 was more related to South African and Israeli isolates than to the Central and South American isolates.

5.7GROWTH CHARACTERIZATION OF BTV-12 IN VERO CELLS

BTV 12 infected Vero cell line was observed by H&E staining at several time points post infection (12, 24, 36, 48, 72hrs.). During 24 hrs of initial infection there is no observed CPE but 48hrs later characteristic CPE with swollen spindle shaped cells which were aggregated together forming small and large clumps was observed. By 72hrs of PI, complete detachment of the monolayer took place. These results are in agreement with the observations made by Balam(2011) and Subhadra (2014). They observed CPE with swollen spindle shaped cells and complete detachment of monolayer took place by 72 hrs PI.

CONCLUSION: BTV-12 was successfully plaque purified in Vero cells and on infection into seronegative sheep elicited mild clinical signs. Further antibody response was noticed from 7 dpi. BTV-12 hyper immune serum was also found to cross neutralize BTV-1. Sequence analysis of the VP2 PCR products revealed close identity between the sequences of BTV-12 of the current study and other BTV-12 sequences available in NCBI-Gene Bank. In this study cross reaction observed between BTV-1 and BTV-12 needs further investigations.

CHAPTER- VI

SUMMARY

Bluetongue (BT) is an infectious, non-contagious disease of ruminants and camelids transmitted by culicoides biting midges. It is caused by bluetongue virus. BTV, is a segmented double-stranded RNA virus of genus Orbivirus under family Reoviridae and is placed in the Office International des Epizooties (OIE) of diseases (Sperlova and Zendulkova, 2011). In the present study, BTV12 serotype was taken for plaque purification and for pathogenicity studies in sheep and ability of BTV-12 antiserum to cross neutralize with other available serotypes (BTV-1, 2, 4, 9, 10, 16, 21, 23 and 24) was also studied. Sequence analysis of partial VP2 segment of BTV-12 and growth characters of BTV-12 in Vero cell line was also taken up.

First, BHK-21 adapted BTV 12 was infected in to Vero cell lines get higher titre for plaque purified. Titre of Vero adapted BTV-12 was $10^{4.5}$ /100 μ l which increased to $10^{5.116}$ /100 μ l after 4 passages more as a part of plaque purification. Thus, plaque purified BTV-12 was then used for molecular, serological confirmation and characterization.

For molecular typing, extraction of RNA was done from plaque purified BTV12 infected Vero cell culture supernatant and subjected to 1% agarose gel electrophoresis for segmented RNA pattern typical of BTV. Then, the BTV-12 was checked against available type specific primers of BTV types (BTV-1, 2, 4, 9, 10, 12, 16, 21, 23, 24) in PCR and it was confirmed as BTV-12. PCR typed BTV-12 cell culture fluid was inoculated in to BTV seronegative sheep (one) and control animal was inoculated with uninfected Vero cells.

BTV inoculated animals exhibited pyrexia (103.8°F - 105.1°F), along with nasal discharges on day 13dpi. Sera of blood collected from control and BTV inoculated animals on 0, 7, 14, 21, and 28 dpi was subjected to cELISA. It was revealed that control animal remained as seronegative during entire the study; while BTV inoculated animal seroconverted from 7 dpi. and remained seropositive throughout the experimental study.

Serum neutralization studies using BTV-12 immune serum from infected sheep revealed antibody of 1/40 on 7dpi. in inoculated animal. This increased to 1/320 by 28 dpi. In cross neutralization studies, unexpectedly, BTV-12 hyper immune serum cross neutralized the available plaque purified BTV-1 serotype but not BTV-2, 4, 9, 10, 16, 21, 23 and 24.

Further, partial VP2 gene of BTV- 12 was sequenced and analysed. Phylogenetic analysis revealed that BTV-12 of the current study had closest sequence similarities with the Indian and South Africa isolates of BTV-12 available in Gene Bank. Sequence comparisons of partial VP2 with other available BTV VP2 sequences revealed similarities with serotypes BTV -2, 10 and 1.

Infection of BTV12 in Vero cell line revealed that CPE started to appear by 48hrs post infection with complete detachment of the monolayer taking place by 72hrs post infection.

Thus, BTV-12 was plaque purified thrice in Vero cells and confirmed by molecular and serological techniques. Further, cross neutralization of available plaque purified BTV-1 serotype by immune serum of BTV-12 was also found to exist. The isolate had close sequence Similarities with Indian and South African isolates of BTV-12 available in Gene bank with regard to partial VP2 gene sequence.

LITERATURE CITED

- Afshar A, Thomas F C, Wright P F, Shapiro J L and Anderson J 1989. Comparison of competitive ELISA, indirect ELISA, standard AGID tests for detecting bluetongue virus antibodies in cattle and sheep. *Veterinary record* 124:136-141
- Akita G Y, Chinsangaram J, Osburn B I, Ian Conescu M and Kaufman R 1992 Detection of bluetongue virus serogroup by polymerase chain reaction. *Journal of Veterinary Diagnostic Investigation* 4(4): 400-405.
- Babu B N, Sreenivasulu D and Rao V D P 2005 Fluorescence inhibition test for identification of bluetongue virus serotypes. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases* 26(1) 15-18.
- Balam D, Daggupati S and Maddireddy H 2011 Studies of the Antigenic relationships between Bluetongue virus serotypes 2, 9 and 15 isolated in Andhra Pradesh, India. *Veterinary World* 4: 444-448.
- Bandyopadhyay S K, Kataria R S and Tiwari A K 1998 Detection of bluetongue virus genome segment 6 sequences by RT-PCR. *Indian Journal of Experimental Biology* 36(10): 1034-1037.
- Bekker J G, Dekock G and Quinlan J B 1934 The occurrence and identification of bluetongue in cattle the so-called pseudo foot and mouth disease in South Africa. Onderstepoort *Journal of Veterinary Research* 2: 393-407.
- Bhat R M, Srivastava R N and Prasad G 1996 Adaptation and growth pattern of bluetongue virus serotype-1 in an insect cell line. *Indian Journal of Animal Science* 66 (2): 113-119.

- Biswas S K, Chand K, De A, Pandey L K, Mohapatra J K, Prasad G and Mondal B 2010 Isolation of bluetongue virus serotype 1 (BTV-1) from goats and its phylogenetic relationship to other BTV-1 isolates worldwide based on full-length sequence of genome segment-2. *Archives of virology* 155: 2041-2046.
- e0156110.
- Blacksell S D and Lunt R A 1993 Serotype identification of Australian bluetongue viruses using a rapid fluorescence inhibition test. *Journal of virological methods*, 44(2-3) 241-250.
- Bouianger P and Frank J F 1975 Serological methods in the diagnosis of bluetongue. *Australian Veterinary Journal* 51:185-189.
- Bowne R A, Howard T H and Pickett B W 1985 Seminal shedding of bluetongue virus in experimentally infected bulls In *Bluetongue and related Orbiviruses* (Eds) Barber T L Jochim M M New York, Alan Research. Liss 91.
- Braverman Y, Rubina M and Frisk K 1981 Pathogens of veterinary importance isolated from mosquitoes and biting midges in Israel. *Insect Science and its Applications* 2: 157-161.
- Bréard E, Sailleau C, Coupier H, Mure-Ravaud K, Hammoumi S, Gicquel B and Zientara S 2003 Comparison of genome segments 2, 7 and 10 of bluetongue viruses serotype 2 for differentiation between field isolates and the vaccine strain. *Veterinary research*, 34(6) 777-789.
- Chand K, Biswas S K, Pandey A B, Muthuchelvan D, and Mondal B 2015 Bluetongue in India: A review *AdvAnim Vet Sci* 3(11) 605-612.

- Cowley J A and Gorman B M 1989 Cross-neutralization of genetic reassortants of bluetongue virus serotypes 20 and 21. *Veterinary Microbiology* 19: 37–51.
- Dahiya S, Prasad G and Kovi R C 2005 Typing of bluetongue virus serotype 1 and 23 by RT-PCR.
- Dangler C A, deMattos C C and Osburn B I 1990 Identifying bluetongue virus ribonucleic acid sequences by the polymerase chain reaction. *Journal of Virological Methods* 28: 281-292.
- Daniels P W, Sendow I, Pritchard I, Sukarsih and Eaton B T 2004. Regional overview of bluetongue viruses.
- Dal Pozzo F, Saegerman C and Thiry E 2009 Bovine infection with bluetongue virus with special emphasis on European serotype-8. *Veterinary Journal* 182(2): 142-151.
- De Campos Nogueira A H, De Stefano E, Nunes Martins M D S, Okuda L H, Lima M D S, Garcia T D S and Pituco E M 2016 Prevalence of Bluetongue virus serotype 4 in cattle in the State of Sao Paulo Brazil. *Veterinariaitaliana*, 52(3-4) 319-323.
- Della-Porta A J, Sellers R F, Herniman K A J, Littlejohns I R, Cybinski, D H, George T S and Corbould A 1983 Serological studies of Australian and Papua New Guinean cattle and Australian sheep for the presence of antibodies against bluetongue group viruses. *Veterinary microbiology* 8(2) 147-162.
- DeMaula C D, Bonneau K R and MacLachlan N J 2000 Changes in the outer capsid proteins of bluetongue virus serotype ten that abrogate neutralization by monoclonal antibodies. *Virus research* 67: 59-66.

- Dubay S A, Rosenstock S S, Stallknecht D E and deVos Jr JC 2006 Determining prevalence of bluetongue and epizootic hemorrhagic disease viruses in mule deer in Arizona (USA) using whole blood dried on paper strips compared to serum analyses. *Journal of wildlife diseases* 42: 159-163.
- Dulbecco R 1952 Production of plaques in monolayer tissue cultures by single particles of an animal virus. *Proceedings of the National Academy of Sciences* 38: 747-752.
- DuToit R 1944 The transmission of bluetongue and African horse-sickness by *Culicoides* Onderstepoort *Journal of Veterinary Science and Animal Husbandry* 19: 7-16.
- Eaton B T, Hyatt A D and White J R 1988 Localization of the nonstructural protein NS1 in bluetongue virus-infected cells and its presence in virus particles. *Virology* 163(2) 527-537.
- Eschbaumer M, Hoffmann B, König P, Teifke J P, Gethmann J M, Conraths F J and Beer M 2009 Efficacy of three inactivated vaccines against bluetongue virus serotype 8 in sheep. *Vaccine* 27: 4169-4175.
- French T J, Inumaru and Polly Roy 1989 Expression of two related NS proteins of BTV type 10 in insect cells by a recombinant baculovirus production of polyclonal ascetic fluid and characterization of the gene product in BTV-infected BHK cells. *Journal of virology* 63: 3270-3278.
- Gambles R M 1949 Bluetongue of sheep in Cyprus. *Journal of Comparative Pathology* 59:176-190.

- Ghalib H W, Cherrington J M and Osburn B I 1985 Virological, clinical and serological responses of sheep infected with tissue culture adapted bluetongue virus serotypes 10, 11, 13 and 17. *Veterinary microbiology* 10(2) 179-188.
- Ghiasi H, Purdy M A and Roy P 1985 The complete sequence of bluetongue virus serotype 10 segments 3 and its predicted VP3 polypeptide compared with those of BTV serotype 17. *Virus Research* 3: 181-190.
- Gibbs E P J and Greiner E C 1988 Bluetongue and Epizootic haemorrhagic disease. In the Arboviruses: Epidemiology and Ecology Vol II Ed.: T P Monath, Boca Raton and CRC Press 39-70.
- Gollapalli S R K, Mallavarapu S, Uma M, Rao P P, Susmitha B, Prasad P U V S and Reddy Y N 2012 Sequences of Genes Encoding Type-Specific and Group-Specific Antigens of an Indian Isolate of Bluetongue Virus Serotype 10 (BTV-10) and Implications for their Origin. *Transboundary and emerging diseases* 59(2) 165-172.
- Gould A R and Pritchard L I 1990 Relationships amongst bluetongue viruses revealed by comparisons of capsid and outer coat protein nucleotide sequences. *Virus research*, 17(1), 31-52.
- Goutham Roy and Mehrota ML 1999 Biological and serological characterization of two Indian isolates of bluetongue virus. *Indian journal of comparative microbiology, Immunology, Infectious Disease*. Volume 20 no2 (July to December):96-101.
- Grubman M J, Appleton J A and Letchworth III G J 1983 Identification of bluetongue virus type 17 genome segments coding for polypeptides associated with virus neutralization and intergroup reactivity. *Virology* 131(2) 355-366.

- Harbola P C, Chaudhary P G, Lalkrishna, Siriguppi B S and Kole R S 1982 Incidence of bluetongue in sheep in Maharashtra. *Indian Journal of Microbiology Immunology and Infectious diseases* 3:121-127.
- Hassan S S and Roy P 1999 Expression and functional characterization of bluetongue virus VP2 protein: role in cell entry. *Journal of virology* 73(12) 9832-9842.
- Hassan S H, Wirblich C, Forzan M and Roy P 2001 Expression and functional characterization of bluetongue virus VP5 protein: role in cellular permeabilization. *Journal of virology* 75: 8356-8367.
- Hemadri D, Maan S, Chanda M M, Rao P P, Putty K , Krishnajyothi Y and Maan N S 2016 Dual Infection with BTV and First-Time Isolation of Serotype 5 in India. *Transboundary and Emerging Diseases* (2016).
- Hewat, Elizabeth A, Timothy F, Booth and Polly Roy 1992 Structure of bluetongue virus particles by cryoelectron microscopy. *Journal of structural biology* 109: 61-69.
- Howell P G, Verwoerd D W and Oellermann R A 1967 Plaque formation by bluetongue virus. *The Onderstepoort journal of veterinary research* 34: 317-332.
- Huismans H and Els H J 1979 Characterization of the tubules associated with the replication of three different orbiviruses. *Virology* 92:397-406.
- Huismans H and Erasmus B J 1981 Identification of the serotype-specific and group-specific antigens of bluetongue virus Onderstepoort. *Journal of Veterinary Research* 48: 51-58.
- Huismans H, van Dijk A A and Bauskin A R 1987a In vitro phosphorylation and purification of a nonstructural protein of bluetongue virus with affinity for single-stranded RNA. *Journal of virology* 61(11) 3589-3595.

- Huismans H, van der Walt N T, Cloete M and Erasmus B J 1987b Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. *Virology* 157:172-179.
- Huismans H, van Dijk A A and Els H J 1987 c Uncoating of parental bluetongue virus to core and subcore particles in infected L cells. *Virology* 157(1) 180-188
- Hutcheon D 1902 Malarial catarrhal fever of sheep. *Vet Rec* 14 629-633.
- Hyatt A D, Zhao Y and Roy P 1993 Release of bluetongue virus particles from insect cells is mediated by BTV nonstructural protein NS3/NS3A. *Virology* 193:592-603.
- Hwang G Y, Chiou J F, Yang Y Y and Li J K 1993 High-sequence conservation among the United States bluetongue virus cognate M2 genes which encode the non-structural NS1 tubule protein. *Virology* 192(1): 321-327.
- Islam M M, Anjum S, Modi, R J and Wadhwani K N 2016. Scenario of livestock and poultry in india and their contribution to national economy. *International Journal of Science, Environment and Technology* 5(3) 956-65.
- Jeggo M H, Wardley R C and Taylor W P 1984 Role of neutralising antibody in passive immunity to bluetongue infection. *Research in veterinary science* 36: 81-86.
- KrishnaJyothi Y 2007 Development and evaluation of multivalent inactivated bluetongue vaccine. M.V.Sc Thesis., *Sri Venkateshwara Veterinary University Tirupathi*.
- Krishnajyothi Y, Maan S, Kandimalla K, Maan N S, Tutika R B, Reddy Y V, and Ahmed S M 2016 BTV-24 from India—An Exotic Serotype to Australasia. *Transboundary and emerging diseases*, 63(4), 360-364.

Kumar N, Barua S, Riyesh T, Chaubey K K, Rawat K D, Khandelwal N and Singh M K 2016 Complexities in isolation and purification of multiple viruses from mixed viral infections viral interference persistence and exclusion. *PloS one* 11(5).

Kumar S, Stecher G, and Tamura K (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33:1870-1874.

Luedke A J, Jochim M M, Bowne J G and Jones R H 1970 Observations on latent bluetongue virus infection in cattle. *Journal of the American Veterinary Medical Association* 156: 1871-1879.

Maan S, Maan N S, Singh K P, Samuel A R, Mertens P P C 2004 Development of reverse transcriptase–polymerase chain reaction based assays and sequencing for typing European strains of bluetongue virus and differential diagnosis of field and vaccine strains. *Veterinaria Italiana* 40(4): 552-561.

Maan S, Maan N S, Samuel A R, Rao S, Attoui H and Mertens P P C 2007 Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. *Journal of General virology* 88(2) 621-630.

Maan S, Maan N S, Ross-Smith N , Batten C A, Shaw A E , Anthony S J, Samuel AR, Darpe K E , Veronesi E , Oura C A and Singh KP 2008 Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. *Virology* 377: 308-318.

Maan N S, Maan S, Belaganahalli M N, Ostlund E N, Johnson D J, Nomikou K and Mertens P P 2012a Identification and differentiation of the twenty six BTV by RT–PCR amplification of the serotype-specific genome segment 2. *PloS one* 7(2): 32601.

- Maan N S, Maan S, Guimera M, Nomikou K, Morecroft E, Pullinger G and Mertens P P 2012b The genome sequence of a reassortant bluetongue virus serotype 3 from India. *Journal of virology* 86: 6375-6376.
- Maan, S, Maan N S, Belaganahalli M N, Potgieter A C, Kumar V, Batra K, and Mertens P P (2016) Development and evaluation of real time RT-PCR assays for detection and typing of Bluetongue virus. *PloS one* 11 (9) e1063014.
- Maclachlan N J, Drew C P, Darpel K E, and Worwa G 2009 The pathology and pathogenesis of bluetongue. *Journal of comparative pathology* 141(1) 1-16.
- Mcphee D A, Parsonson I M and Della Porta A J 1982 Comparative studies on the growth of Australian bluetongue virus serotypes in continuous cell lines and embryonated chicken eggs. *Veterinary Microbiology* 7: 401 – 410.
- Meenaambagai T V, Prabhakar T G, Govindarajan R, Nachimuthu K, Kotesawaran A 2006. A Comparative study of the inclusion bodies in BHK-21 and Vero cells infected with bluetongue virus. *Tamilnadu journal of Veterinary Virology and Animal Sciences*. 2:6 248-250.
- Meiswinkel R, Baldet T, De Deken R, Takken W, Delécolle J C and Mellor P S 2008 The 2006 outbreak of bluetongue in northern Europe the entomological perspective. *Preventive veterinary medicine* 87: 55-63.
- Mellor P S 1990 The replication of bluetongue virus in Culicoides vectors. *Current Topics of Microbiology and Immunology* 162: 143–161.
- Mellor P S 2000 Replication of arboviruses in insect vectors. *Journal of Comparative Pathology* 123: 140–146.

- Mertens P P C, Brown and DV Sangar 1984 Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode. *Virology* 135: 207-217.
- Mertens P P C, Pedley S, Cowley J, Burroughs J N, Corteyn A H, Jeggo M H and Gorman B M 1989 Analysis of the roles of bluetongue virus outer capsid proteins VP2 and VP5 in determination of virus serotype. *Virology* 170: 561-565.
- Mertens P P C, Burroughs J N and Wade-Evans A M 1992 Analysis of guanylyl transferase and transmethylese activities associated with bluetongue virus core and recombinant baculovirus expressed core like particles In: Walton T E, Osburn B I (Eds) *Bluetongue, African horse sickness and related Orbiviruses* p p 404-415.
- Mertens P P, Diprose J, Maan S, Singh K P and Attoui H 2004 Bluetongue virus replication, molecular and structural biology. *Veterinaria Italiana* 40:426-437.
- Mertens P P C, Maan N S, Prasad G, Samuel A R, Shaw A E, Potgieter A C and Maan 2007 Design of primers and use of RT-PCR assays for typing European bluetongue virus isolates: differentiation of field and vaccine strains. *Journal of General Virology*, 88(10) 2811-2823.
- Monaco F, De Luca N, Spina P, Morelli D A O, Liberatore I, Citarella R and Savini G 2004 Virological and serological response of cattle following field vaccination with bivalent modified-live vaccine against bluetongue virus serotypes 2 and 9. *Veterinariaitaliana*, 40(4) 657.
- Moultan J E 1961 Pathology of bluetongue of sheep in California. *Journal of the American Veterinary Medical Association* 138: 493-498.

- O. I. E. Manual of standard Diagnostic Tests and Vaccines 4th Edition, 2006.
- Owens R J, Limn C and Roy P 2004 Role of an arbovirus nonstructural protein in cellular pathogenesis and virus release. *Journal of Virology* 78: 6649-6656.
- Prasad G, Minakshi, Sushilamaan and Malik Y 1998. Genomic diversity in Indian isolates of bluetongue virus serotype1. *Indian Journal of Microbiology* 38:161-163.
- Prasad M, Ranjan K, Kumar P and Prasad G 2013 Segment 2 based characterization of a novel Indian Bluetongue virus isolate. *Veterinary World* 6(5).
- Pritchard L I and Gould A R 1995 Phylogenetic comparison of the serotype-specific VP2 protein of bluetongue and related orbiviruses. *Virus research*, 39(2-3), 207-220.
- Rao P P, Hegde N R, Reddy Y N, Krishnajyothi Y, Reddy Y V, Susmitha B and Reddy G H 2014 Epidemiology of BT in India. *Transboundary and emerging diseases* 63: 151-164.
- Reddy M S 2016 Plaque purification of bluetongue virus serotype-4 (BTV-4) and its characterization.
- Reddy Y V, Krishnajyothi Y, Susmitha B, Devi B V, Brundavanam Y, Gollapalli S R K and Rao P P 2015 Molecular Typing BTV Isolated Over a Decade in South India. *Transboundary and emerging diseases* 10.1111/tbe: 2320.
- Roy P, Fukusho A, Ritter G D and Lyon D 1988 Evidence for genetic relationship between RNA and DNA viruses from the sequence homology of a putative polymerase gene of bluetongue virus with that of vaccinia virus conservation of RNA polymerase genes from diverse species. *Nucleic Acid Research* 16(24): 11759-11767.

- Roy P and Gorman B M 1990 Bluetongue virus Current Topics in Microbiology and Immunology 162: 1-200 Heidelberg, Springer – Verlag.
- Roy P 2008 Bluetongue virus: dissection of the polymerase complex. *Journal of general Virology* 89(8): 1789-1804.
- Sairaju V, Susmitha B, Rao P P, Hegde N R, Meena K and Reddy Y N 2013 Type-specific seroprevalence of bluetongue in Andhra Pradesh, India during 2005–2009. *Indian Journal of Virology* 24(3): 394-397.
- Saitou N and Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4:406-425.
- Sandhya P 2007 Evaluation of trivalent inactivated vaccine for bluetongue at farm level. M.V. Sc Thesis. *Sri Venkateshwara Veterinary University, Tirupathi*.
- Santman-Berends I M, Hage J J, van Rijn P A, Stegeman J A and van Schaik G 2010 Bluetongue virus serotype-8 (BTV-8) infection reduces fertility of Dutch dairy cattle and is vertically transmitted to offspring. *Theriogenology* 74(8): 1377-1384.
- Sapre S N 1964 an outbreak of bluetongue in goats and sheep *Vet. Rev* 15: 78-80.
- Schwartz-Cornil I, Mertens P P, Contreras V, Hemati B, Pascale F, Bréard E and Zientara S 2008 Bluetongue virus: virology pathogenesis immunity *Veterinary research* 39(5) 1.
- Sellers R F 1981 Bluetongue and related diseases. In *Virus diseases of food animals* vol II (Eds) E P J Gibbs London Academic Press 567-584.
- Sekar P, Ponmurugan K and Gurusubramanian G 2009 Comparative Susceptibility of BHK 21 and Vero Cell Lines to Bluetongue Virus (BTV) Isolate Pathogenic for Sheep Internet *J Microbiology* 7: 1-5.

- Squire K R E 1989 Serological reactions in sheep and cattle experimentally infected with three Australian isolates of bluetongue virus. *Australian veterinary journal*, 66(8) 243-246.
- Sperlova A and Zendulkova D 2011 BT a review. *Vet Med-Czech* 56(9): 430-452.
- Spreull J 1905 Malarial catarrhal fever (bluetongue) of sheep in South Africa. *Journal of Comparative Pathology and Therapeutics* 18 321-337.
- Sreenivasulu D, Subba Rao M V, Reddy Y N and Gard G P 2004 Overview of bluetongue disease viruses, vectors, surveillance and unique features: the Indian sub-continent and adjacent regions. *Veterinariaitaliana* 4: 73-77.
- Stäuber N , Martinez-Costas J, Sutton G, Monastyrskaya K and Roy P Bluetongue virus VP6 protein binds ATP and exhibits an RNA-dependent ATPase function and a helicase activity that catalyze the unwinding of double-stranded RNA substrates, *J Virol.* (1997) 71:7220–7226
- Subhadra S, Kumar S, Suryanarayana V V and Sreenivasulu D 2014 Comparison of bluetongue virus detection and quantitation methods in south India. *The Journal of Infection in Developing Countries* 8: 1307-1312.
- Susmitha B, Sudheer D, Rao P P, Uma M, Prasad G, Minakshi P, & Reddy Y N 2012 Evidence of BTV-21 divergence. *Virus gene* 44(3): 466-469.
- Tamura K, Nei M, and Kumar S (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences (USA)* 101:11030-11035.
- Theiler A 1906 Bluetongue in sheep. Transvaal Department of Agriculture Annual report for 1904-1905: 110-121.

- Tweedle N and Mellor PS 2002 Technical review–bluetongue: The virus hosts and vectors Version 1.5 Report to the Department of Health Social Services and Public Safety UK (DEFRA) 25.
- Urakawa T and Roy P 1988 Bluetongue virus tubules made in insect cells by recombinant baculoviruses expression of NS1 gene of bluetongue virus serotype 10. *Journal of Virology* 62: 3919-3927.
- Urakawa T, Ritter D G and Roy P 1989 Expression of largest RNA segment and synthesis of VP1 protein of bluetongue virus in insect cells by recombinant baculovirus association of VP 1 protein with RNA polymerase activity. *Nucleic Acid Research* 17: 7395-7401.
- Verwoerd D W, Els H J, De Villiers E M and Huisman H 1972 Structure of the bluetongue virus capsid. *Journal of Virology* 10: 783-794.
- Walker A and Davis F G 1971 A preliminary survey of the epidemiology of bluetongue in Kenya. *Journal of Hygiene (Cambridge)* 69: 47-61.
- Yamaguchi S, Fukusho A and Roy P 1988 Complete sequence of VP2 gene of the bluetongue virus Australian serotype 1 (BTV-1). *Nucleic Acids Research* 16: 2725.
- Yamakawa M, Krasnyck V and Roy P 1994 Phylogenetic relationships of the VP2 protein of a virulent isolate of bluetongue virus (BTV-23) compared to those of 6 other BTV serotypes. *Virus research*, 34(1)81-92.
- Zulu G B and Venter E H 2014 Evaluation of cross-protection of bluetongue virus serotype 4 with other serotypes in sheep. *Journal of the South African Veterinary Association* 85: 01-02.

