PATHOGENICITY STUDIES OF BLUETONGUE VIRUS SEROTYPE 16 IN SHEEP

By
SHAIK JAHANGEER PEERA
B.V.Sc & A.H
RVM/2014-55

MASTER OF VETERINARY SCIENCE
(VETERINARY MICROBIOLOGY)

DEPARTMENT OF VETERINARY MICROBIOLOGY

COLLEGE OF VETERINARY SCIENCE,

RAJENDRANAGAR, HYDERABAD-30

P.V. NARSIMHARAO TELANGANA VETERINARY UNIVERSITY,

HYDERABAD

APRIL, 2017

PATHOGENICITY STUDIES OF BLUETONGUE VIRUS SEROTYPE 16 IN SHEEP

By
SHAIK JAHANGEER PEERA
B.V.Sc & A.H
RVM/2014-55

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 MICROBIOLOGY)

DEPARTMENT OF VETERINARY MICROBIOLOGY
COLLEGE OF VETERINARY SCIENCE,
RAJENDRANAGAR, HYDERABAD-30
P.V. NARSIMHARAO TELANGANA VETERINARY UNIVERSITY,
HYDERABAD
APRIL, 2017

CERTIFICATE

I certify that **SHAIK JAHANGEER PEERA** has satisfactorily prosecuted the course of research and that the thesis entitled **"PATHOGENICITY STUDIES OF BLUETONGUE VIRUS SEROTYPE 16 IN SHEEP"** 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 of thereof has not been previously submitted by him for a degree of any University.

Date**:** **Dr. K. DHANALAKSHMI**

Place: Hyderabad (Major Advisor)

DECLARATION

I, **SHAIK JAHANGEER PEERA** hereby declare that the thesis entitled "**PATHOGENICITY STUDIES OF BLUETONGUE VIRUS SEROTYPE 16 IN SHEEP"** submitted to **P.V.NARASIMHA RAO TELANGANA VETERINARY UNIVERSITY** for the Degree of **MASTER OF VETERINARY SCIENCE** is a result of original research work done by me. It is further declared that the thesis or any part thereof has not been published earlier in any manner.

Date:

Place: Hyderabad (**SHAIK JAHANGEER PEERA**)

CERTIFICATE

This is to certify that the thesis entitled **"PATHOGENICITY STUDIES OF BLUETONGUE VIRUS SEROTYPE 16 IN SHEEP"** submitted in partial fulfillment of the requirements for the degree of **MASTER OF VETERINARY SCIENCE** of the **P.V NARASIMHA RAO TELANGANA VETERINARY UNIVERSITY, HYDERABAD** is a record of the bonafide research work carried out by **SHAIK JAHANGEER PEERA** 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. K. DHANALAKSHMI)

Chairman of the Advisory Committee

Thesis approved by the Student's Advisory Committee.

Chairman: **Dr. K.DHANALAKSHMI** ________________________

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

Member: **Dr. Y. NARASIMHA REDDY** ________________________

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

Member: **Dr.P. KALYANI** ________________________

Assistant professor
Department of Biotechnology
College of Veterinary science
Raejndranagar,Hyderabad-500030.

TABLE OF CONTENTS

CHAPTER NO.	TITLE	PAGE NO.
I	**INTRODUCTION**	1
II	**REVIEW OF LITERATURE**	3
2.1	Bluetongue disease	3
2.1.1	History and distribution	3
2.1.1.1	Prevalence of disease in India	3
2.1.1.2	Prevalence of disease in Andhra Pradesh	4
2.1.2	Host	5
2.1.3	Vector and transmission	6
2.1.4	Clinical signs and Pathogenesis	7
2.2	Bluetongue virus	9
2.2.1	Physico chemical properties of BTV	9
2.2.2	Morphology of BTV	10
2.2.3	Molecular characterization of BTV	10
2.3	Diagnosis	11
2.3.1	Virus isolation	12
2.3.2	Cell culture	13
2.4	Diagnostic techniques	14
2.4.1	Serological diagnostic techniques	14
2.4.1.1	Compliment fixation test	14
2.4.1.2	Agar gel immunodiffusion test	14
2.4.1.3	Enzyme Linked Immunosorbent Assay	14

2.4.1.4	Serum neutralization test	15
2.4.2	Molecular diagnostic techniques	17
2.4.2.1	Genotyping of BTV	17
2.5	Pathogenicity studies of BTV	21
III	**MATERIALS AND METHODS**	
3.1	General Laboratory Materials	23
3.1.1	Glassware	23
3.1.2	Plastic ware	23
3.1.3	Preparation and sterilization of required material	23
3.2	Cell lines	24
3.3	Experimental animals	24
3.4	Housing of Experimental animals	24
3.5	Chemicals and Reagents	25
3.5.1	1X PBS	25
3.5.2	1X TAE	25
3.5.3	Trypsin (0.1%)	25
3.5.4	Crystal violet staining solution	26
3.5.5	Formaldehyde solution	26
3.6	Cell culture media and reagents	26
3.6.1	Schneider's Insect Medium	26
3.6.2	Minimum Essential Medium	27
3.6.3	Tryptose phosphate broth	27
3.6.4	Growth Medium (GM)	27
3.6.5	Maintenance Medium (MM)	27

3.7	Virus	28
3.7.1	Bluetongue Virus Serotype 4 and 16	28
3.7.2	Characterization of BTV isolates	28
3.7.2.1	Extraction of Viral Nucleic acid	28
3.7.2.2	Confirmation of serotype by polymerase chain reaction	29
3.7.2.2.1	Reverse Transcription Polymerase chain Reaction(RT-PCR)	29
3.7.2.2.2	Polymerase chain reaction	30
3.7.2.2.3	Agarose gel electrophoresis	32
3.7.3	Adaptation of BTV-4 & 16 serotypes in KC Cell-lines	33
3.7.4	Clarification of the harvested virus	33
3.7.5	Assay of Virus infectivity	34
3.7.5.1	Subculture	34
3.7.5.2	Infection	34
3.7.5.3	Crystal violet staining	35
3.7.6	BTV Inoculation in Sheep	35
3.7.6.1	Collection of blood samples	35
3.7.6.2	Clinical responses	36
3.7.7	Humoral Immune response to BTV	36
3.7.7.1	Serum Neutralization Test (SNT)	36
3.7.7.2	Competitive Enzyme- Linked Immunosorbent Assay (c-ELISA)	37
IV	**RESULTS**	
4.1	Cell lines	38
4.1.1	Maintenance of KC cells	38
4.1.2	Maintenance of BHK-21cells	38

4.2	Propagation of virus in cell cultures	38
4.3	Characterization of BTV Serotypes	40
4.3.1	Characterization of viral nucleic acid	40
4.3.2	Molecular typing of BTV	40
4.4	Titration of virus	45
4.5	Responses to BTV Inoculation in Sheep	45
4.5.1	Clinical signs	45
4.5.2	Daily body temperatures	46
4.5.3	Haematological parameters	46
4.5.4	Immune response	51
4.5.4.1	c-ELISA	51
4.5.4.2	Serum Neutralization Test	53
V	**DISCUSSION**	55
VI	**SUMMERY AND CONCLUSION**	60
	LITERATURE CITED	62

LIST OF TABLES

Table No.	Particulars	Page No.
1	Peak temperatures of all the animals with respective days of post infection	46
2	c-ELISA Percentage inhibition values for BTV-16 and BTV-4 super infection in Sheep	51
3	Sero conversion results by SNT for BTV-16 and BTV-4 super infection in Sheep	54

LIST OF ILLUSTRATIONS

Figure No.	Particulars	Page No.
1	Monolayer of KC cells (100X)	39
2	Monolayer of BHK-21 cells (100X)	39
3	BTV infected BHK-21 cell monolayer showing CPE –rounding, clumping of cells and detached areas of monolayer (100X).	39
4	Agarose gel electrophoresis of BTV RNA	41
5	Image showing BTV-4 molecular typing	42
6	Image showing BTV-4 molecular typing	43
7	Image showing BTV-16 molecular typing	44
8	BTV -16 Infected Sheep without any oral lesions	47
9	BTV-4 Super infected Sheep without nasal discharges	47
10	Graph depicting daily temperatures of infected sheep primary infected with BTV-16	48
11	Graph depicting daily temperatures of infected sheep super infected with BTV-4	48
12	Comparison of haematological parameters during primary and super infected animals	49
13	Graphical representation of haematology in Deccani sheep during primary infection with the BTV -16	50
14	Graphical representation of haematology in Deccani sheep during primary infection with the BTV -4	50
15	Immune response to BTV-16 during primary infection and super infection by c-ELISA	52
16	Serological relationships between BTV serotypes	59

ACKNOWLEDGEMENTS

All praises are the Almighty who bestowed on me the courage, patience and mental peace so that this piece of work could come to completion.

Fervently and modestly, I extol the genuine co-operation and affection offered to me by my venerable philosopher and guide ***Dr.K.Dhanalakshmi****, Associate Professor , Department of Veterinary Microbiology, 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 humbly place on record my respect and gratitude to the revered member of my advisory committee ***Dr. Y. Narasimha Reddy****, Professor and University Head, Department of Veterinary Microbiology, College of Veterinary Science, Rajendranagar, Hyderabad, for extending his utmost support in each and every aspect of my work continuously and persistent encouragement during every stage of experimentation.*

I am sincerely thankful to ***Dr. P. Kalyani****, Assistant Professor, Veterinary Biotechnology, College of Veterinary Science, Rajendranagar, member of my advisory committee, for her valuable support during my research work.*

I sincerely thank ***Dr.M.Srinivas****, Assistant Professor and* ***Dr.Kavitha****, Assistant Professor, Department of Veterinary Microbiology, College of Veterinary Science, Rajendranagar for their guidance and cooperation in completion of M.V.Sc programme.*

I respectfully acknowledge and express my profound thanks to ***Dr.Mujeeb Athar****, DD,* ***Dr.Sirisha****, VAS****, Bhagyalakshmi****, SRF, TSVBRI, Hyderabad for their cooperation and help throughout my investigation.*

Diction is not enough to express my deep scene of gratitude to my beloved mentors ***Dr. K. Sarjan Rao****, Dean of Student affairs ,SVVU, Tirupathi. and* ***Dr. Jagadesh Babu****, professor and head Department of veterinary public health ,SVVU, Tirupathi, for their inspiration and everlasting affection and moral support .*

The words are inadequate to expressing my deep sense of gratitude and indebtedness to my guru, my well-wisher and my role model, ***Jewel of India, Dr. M. Alpha Raj,*** *Assistant Professor, Department of Veterinary Pharmacology, College of Veterinary Science, Proddatur, for his invaluable and critical suggestions, scholarly guidance, active*

ACKNOWLEDGEMENTS

All praises are the Almighty who bestowed on me the courage, patience and mental peace so that this piece of work could come to completion.

*Fervently and modestly, I extol the genuine co-operation and affection offered to me by my venerable philosopher and guide **Dr.K.Dhanalakshmi**, Associate Professor , Department of Veterinary Microbiology, 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 humbly place on record my respect and gratitude to the revered member of my advisory committee **Dr. Y. Narasimha Reddy**, Professor and University Head, Department of Veterinary Microbiology, College of Veterinary Science, Rajendranagar, Hyderabad, for extending his utmost support in each and every aspect of my work continuously and persistent encouragement during every stage of experimentation.*

*I am sincerely thankful to **Dr. P. Kalyani**, Assistant Professor, Veterinary Biotechnology, College of Veterinary Science, Rajendranagar, member of my advisory committee, for her valuable support during my research work.*

*I sincerely thank **Dr.M.Srinivas**, Assistant Professor and **Dr.Kavitha**, Assistant Professor, Department of Veterinary Microbiology, College of Veterinary Science, Rajendranagar for their guidance and cooperation in completion of M.V.Sc programme.*

*I respectfully acknowledge and express my profound thanks to **Dr.Mujeeb Athar**, DD, **Dr.Sirisha**, VAS**, Bhagyalakshmi**, SRF, TSVBRI, Hyderabad for their cooperation and help throughout my investigation.*

*Diction is not enough to express my deep scene of gratitude to my beloved mentors **Dr. K. Sarjan Rao**, Dean of Student affairs ,SVVU, Tirupathi. and **Dr. Jagadesh Babu**, professor and head Department of veterinary public health ,SVVU, Tirupathi, for their inspiration and everlasting affection and moral support .*

*The words are inadequate to expressing my deep sense of gratitude and indebtedness to my guru, my well-wisher and my role model, **Jewel of India, Dr. M. Alpha Raj,** Assistant Professor, Department of Veterinary Pharmacology, College of Veterinary Science, Proddatur, for his invaluable and critical suggestions, scholarly guidance, active*

persuasion and supervision, which serves as a constant source of inspiration throughout the carrier.

My vocabulary utterly fails in expressing my accolade to my revered parents **Shaik Khasim Peera** and **Shaik Rabiya Bee** who brought me to this stage. I am grateful for their kind blessings, love, patience, overwhelming support and inspiration. I fall short of words for the moral and unrelenting support extended by my family members, brother **shaik Javeed Vali** and who were besides me in all my ups and down.

I extend my gratitude and deep sense of reverence for my love **Dr.Syed Gousia Sony**, for her untiring support and unlimited belief on me.

I would like to thank **Susmitha**, SRF, Department of Veterinary Microbiology for her cooperation during my investigation.

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.

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

I wish to convey my heartfelt thanks to my friends **2K9 Proddatur batchmates**, for what I am today and their continued assistance and encouragement in making my college life a memorable one.

I extol my sincere feelings of gratitude and heartfelt thanks to my seniors **Dr.Hannan Umair, Dr.Satyasamparna, Dr.Shiva Jyothi, Dr.Kartika Lakshmi, Dr.Suresh** who helped me at the time of need and understanding me every time.

I owe my humble and heartfelt thanks to my colleagues **Dr.chikku, Dr.Srikanth, Dr.Samatha, Dr.Jyothsna, Dr.Santosh, Dr.Sirisha, Dr.Yashitha Priya, Dr.Rajshekar** for sharing all the moments during course work and giving ideas to solve the problems and for their help and support during the entire time of my stay.

I feel short of space to express my sincere gratitude to my juniors **Dr.Raju, Dr.Sravan, Dr.Swarupa, Dr.Swathi, Dr.Himaja, Dr.Srinivas, Dr.Swathi** for their help, transcendent suggestions and endless cooperation during the prosecution and presentation of this research work.

I would like to extend my thanks to **Ananthaiah, Sudarshan, Mohan Krishna, Savitri** and **Narasimha,** non-teaching staff of Departments of Veterinary Microbiology and Veterinary Biotechnology.

*I would like to extend my heartfelt thanks to **Mahender** non-teaching staff of Instructional Livestock Farming Complex.*

It is my duty to express sympathy to all Sheep that were infected and sacrificed for the cause of science which made me punctual by timely feeding, watering and blood collection.

*I would like to place on record my gratitude towards **Sri Venkateswara Veterinary University**, Tirupati, Andhra Pradesh for providing me monetary support in the form of stipend to prosecute my Post graduation studies and **P.V. Narsimharao Telangana Veterinary University,** Rajendranagar, Hyderabad for providing all the necessities during my investigation.*

I apologize for the faux pass of the persons who have extended the help in a way or other and deserved such thanks.

(SHAIK JAHANGEER PEERA)

NAME OF THE AUTHOR : **SHAIK JAHANGEER PEERA**

TITLE OF THE THESIS : **PATHOGENICITY STUDIES OF BLUETONGUE VIRUS SEROTYPE 16 IN SHEEP**

DEGREE TO WHICH IT IS SUBMITTED : **MASTER OF VETERINARY SCIENCE**

FACULTY : **VETERINARY SCIENCE**

DISCIPLINE : **VETERINARY MICROBIOLOGY**

MAJOR ADVISOR : **Dr. K. DHANALAKSHMI**

UNIVERSITY : **P.V.NARSIMHA RAO TELANGANA VETERINARY UNIVERSITY, HYDERABAD**

YEAR OF SUBMISSION : **2017**

==

ABSTRACT

The present study was undertaken with the objective of studying the pathogenicity of BTV-16 in Deccani sheep followed by super infection with BTV-4. The study was carried out by infecting 4 seronegative sheep with KC cell line adapted BTV-16 followed by BTV-4 on 30th dpi of BTV-16. The pathogenicity of virus was studied by perceiving clinical symptoms, hematological parameters like TEC, TLC, Hb %, group specific antibody response against type specific antibody by c-ELISA and SNT.

The BTV serotypes 4 and 16 used in the present study were confirmed initially by RT-PCR using serotype specific primers. The virus was propagated in KC cell line and titrated in BHK-21 cells upon which BTV-16 and 4 gave a $TCID_{50}$ value of $10^{6.66}$ and $10^{6.23}$ respectively. The initial infection of four sheep was done with 0.25ml of BTV-16 through

intra dermal route adapted to KC cell line and the control animal received uninfected KC cell suspension. Super infection with 0.6ml of BTV-4 was carried out 30 days after the primary infection.

All selected parameters such as clinical symptoms, hematological parameters like TEC, TLC, Hb %, antibody response against virus by c-ELISA and SNT were compared between initial infection and super infection, and simultaneously with the control. During both primary and super infection, there was a significant increase in TLC whereas TEC and Hb was significantly less as compared to the control. However, there was not much difference in terms of TLC and Hb between primary and super infection. The group specific antibody titers were attained by 7th dpi of primary infection and increased until 28th dpi. Neutralizing antibodies were observed against BTV-16 from day 7dpi continued up to 28dpi and BTV-4 from 7dpi in 1 animal and on day 15 in remaining 2 which continued up to 28 dpsi.

LIST OF ABBREVATIONS

µl	:	Micro liter
°C	:	Degrees Centigrade
AA	:	*Aedes albopictus*
AGID	:	Agar gel immuno diffusion
B-ELISA	:	Blocking Enzyme Linked Immuno Sorbent Assay
BHI	:	Brain Heart Infusion Agar
BHK	:	Baby hamster kidney cells
BT	:	Bluetongue
BTV	:	Bluetongue virus
c-ELISA	:	Competitive Enzyme-linked Immuno Sorbent Assay
CFT	:	Complement Fixation Test
Co2	:	Carbon dioxide
CPE	:	Cytopathic effect
Ctrl	:	Control
DIA	:	Dot Immunobinding Assay
DNA	:	Deoxy ribonucleic acid
dNTP	:	Deoxy nucleoside triphosphate
Dpi	:	Days post infection
Dpsi	:	Days post superinfection
dsRNA	:	Double stranded Ribonucleic acid
ECE	:	Embryonated Chicken Eggs.
EDTA	:	Ethylene Diamine Tetra Acetic acid
EHDV	:	Epizootic Hemorrhagic Disease Virus
EID50	:	Embryo infective dose 50 per cent end point
ELISA	:	Enzyme -linked Immuno Sorbent Assay

FBS	:	Foetal Bovine Serum
FP	:	Forward Primer
G	:	Gram
GM	:	Growth Medium
Hb	:	Haemoglobin
HCl	:	Hydrochloric acid
hrs.	:	Hours
I-ELISA	:	Indirect ELISA
IFNAR	:	Interferon alpha/beta receptor-deficient mice
IU	:	International Unit
KC	:	*Culicoides sonorensis* cell line
KCl	:	Potassium chloride
Kd	:	Kilo Dalton
KH2PO4	:	Potassium di-hydrogen ortho phosphate
L	:	Litre
M	:	Molar
MEM	:	Minimum essential medium
Mg	:	Milligram
Min	:	Minute
Ml	:	Milliliter
MM	:	Maintenance Medium
MOI	:	Multiplicity of infection
Na2HPO4	:	di-Sodium Hydrogen Orthophosphate
NaCl	:	Sodium chloride
NaoH	:	Sodium Hydroxide
NS		Non-structural

PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase Chain Reaction
PFU	:	Plaque forming unit
PI	:	Post Infection
PI	:	Post inoculation / infection
PI	:	Percent Inhibition
PRNT	:	Plaque reduction neutralization test
REPA	:	Restriction enzyme profile analysis
RNA	:	Ribonucleic Acid
RP	:	Reverse Primer
Rpm	:	Revolutions per minute
RPMI	:	Rosewell parker Memorial Institute
RT	:	Reverse Transcription
RT-PCR	:	Reverse Transcriptase Polymerase Chain Reaction
SAC	:	South American Camelids
SDS	:	Sodium dodecyl sulphate
SI	:	Stimulation Index
SNT	:	Serum Neutralization Test
ssRNA	:	Singe stranded RNA
TAE	:	Tris Acetate EDTA
TCID50	:	Tissue culture infective dose 50 percent end point
TEC	:	Total Erythrocyte Count
TLC	:	Total Leukocyte Count
TPB	:	Tryptose Phosphate Broth
VIB	:	Viral Inclusion Bodies

CHAPTER 1

INTRODUCTION

In India large numbers of people are dependent on agriculture as their income source. These farmers rear domestic animals as subsidiary along with agriculture. India has a huge livestock population including cattle, buffaloes, sheep and goat.

India ranks 3rd in sheep population, next to China and Australia and is placed at the 7th position among the top 10 countries of the world in terms of mutton and wool production (FAO 2007). Sheep are susceptible to viral diseases like foot-and-mouth disease, bluetongue, Peste-des-petits-ruminants and sheep pox. Bluetongue is one of the economically important diseases of sheep in India.

Bluetongue (BT) is a vector-borne (*Culicoides sps*) disease of ruminants affecting many species of domestic and wild ruminants such as sheep, goat, cattle, buffalo, white-tailed deer, antelope and sambar, and camelid species such as camels and llamas (Meyer *et al.,* 2009). The disease manifestation can range from asymptomatic to lethal form depending upon the Bluetongue virus (BTV) serotype, species, breed and age of animal (Elbers *et al.,* 2008).

The disease was first reported in late 18th century in the African continent. For many decades the disease was bounded to the African continent. However, the virus crossed its native boundaries and expanded into many tropical, sub-tropical and temperate regions of the world.

BTV belongs to the Orbivirus genus in the family Reoviridae with 27 distinct BTV serotypes (Hofmann *et al.,* 2008; Maan *et al.,* 2011; Zientara *et al.,* 2014). In India, at least 23 serotypes have been recognized (Rao *et al.,* 2014).

Confirmatory diagnosis requires isolation of virus, standard serological methods and several other antigen and nucleic acid detection assays. Competitive ELISA (c-ELISA), indirect ELISA (i-ELISA) and agar gel immunodiffusion (AGID) assay can be used for detection of serogroup-specific antibodies (Pathak *et al*., 2008). Recently, full length sequencing of VP2 gene and virus neutralization test were used to study the antigenic similarity of BTV isolates (Biswas *et al*., 2015). Serogroup-specific sandwich ELISA (s-ELISA) is used for the detection of BTV antigen from blood and tissue samples (Chand *et al*., 2009). Reverse transcription-polymerase chain reaction (RT-PCR) is also used for detection of BTV nucleic acid from blood or tissue samples. A quantitative detection and quantification of viral RNA in a clinical sample is carried out by real-time RT-PCR (Shaw *et al*., 2007).

However, not much information is available on the pathogenicity of the isolated serotypes and the effect of simultaneous and subsequent multi serotype infections which is a common scenario in field conditions especially in BT endemic countries in India. Hence, the current study is designed with the following objectives:

Objectives of investigation

- Adaptation of BTV serotypes 16 and 4 in KC cells.
- Pathogenicity studies of KC cell adapted BTV serotype 16 in sheep.
- To study super infection of BTV 16 infected sheep with serotype 4.

CHAPTER II

REVIEW OF LITERATURE

2.1 BLUETONGUE DISEASE

Bluetongue (BT) is a major disease of sheep and other ruminants in India especially in states of southern peninsula. The disease has its impact on sheep farming in these states by way of severe morbidity and mortality.

2.1.1 History and Distribution

Bluetongue was initially recognized more than a century ago in South Africa in 1876 (Hennings, 1949). By 1960 it was considered enzootic in most of the countries in Africa including Morocco, Chad, Nigeria, Kenya, Tanzania, Rhodesia (FAO, 1960). Subsequently, outbreaks of the disease were reported in many parts of the world (Chaing, 1989). At present 27 serotypes have been reported throughout the world with recent additions of the 25th serotype (Toggenburg orbivirus) from Switzerland in goat, 26th serotype from Kuwait in sheep and goat (Hofmann *et al*., 2008; Maan *et al.*, 2011; Maan *et al*., 2012; Bitew *et al*., 2013) and 27th serotype from Corsica, France in goats (Jenckel *et al*., 2015).

2.1.1.1 Prevalence of disease in India

In India the disease was first reported in Maharashtra (Sapre, 1964). Later it was reported from different states including U.P. (Bhambani and Singh, 1968), Himachal Pradesh (Uppal and Vasudevan, 1980), Haryana (Vasudevan, 1982), Karnataka (Sreenivas *et al*., 1982), Andhra Pradesh (Ramarao, 1982), Rajasthan (Lonkar *et al*., 1983) and Tamilnadu (Janakiraman *et al*., 1991).

Bandopadhyay and Mallick (1983) examined 783 serum samples collected from different parts of the country. BTV antibodies were found in 11.6 per cent of sheep (78/669), 3.7 per cent of cattle (3/81) and 3 per cent (1/33) of goats. Prevalence of BTV antibodies in sheep was also noticed in Haryana (61.11%), Uttar Pradesh (3%), Rajasthan (26.5%) and Andhra Pradesh (2.4%). Breed wise distribution indicated that indigenous sheep had much lower sero prevalence (4/174) than exotic breeds viz. Corriedale (13/38) and Merino (21/73).

In India, Jain *et al.,* (1986) and Mehrotra *et al.,* (1989) isolated BTV in BHK_{21} cell monolayers by inoculating infected sheep blood directly.

Blood samples from Tamil Nadu and Karnataka positive for BTV antigen were used to inoculate chicken embryos to isolate the virus by Nachimuthu *et al.,* (1992) and Ramesh babu *et al.,* (1992).

2.1.1.2 Prevalence of Disease in Andhra Pradesh

In Andhra Pradesh, the BTV (serotype 2) was first isolated by Sreenivasulu *et al.,* (1999). Studies carried out in Andhra Pradesh (A.P.) during 1991 revealed the prevalence of BTV antibodies in 45.71 per cent of sheep, 43.56 per cent of goats, 33.11 per cent of cattle and 20 per cent of buffaloes (Sreenivasulu and Subba Rao, 1999).

Sudheer D (2003) collected 56 blood samples and processed for BTV isolation; ten samples caused cherry red appearance of the embryos on intravenous inoculation. However on further passage in BHK_{21}cell line only one sample exhibited cytopathic effect. This isolate was confirmed as bluetongue virus by capture ELISA system, agarose gel electrophoresis of viral nucleic acid and RNA PAGE later it was typed as BTV-9.

Yugendhar Reddy B (2004) attempted to isolate BTV from total of 92 blood samples collected from animals with pyrexia during bluetongue outbreaks and 17 samples caused

mortality of chicken embryos with cherry red appearance and 5 of them caused cytopathetic effect in BHK_{21} cells. These isolates were later confirmed as BTV serotypes 2, 9 and 15.

2.1.2 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).

All ruminants are susceptible to infection, but clinical disease is mostly seen in sheep. Cattle play an important role in the epidemiology of BTV mainly because of prolonged viraemia (Tweedle and Mellor, 2002).

The disease has also been recorded in axis deer (*Axis axis*), mouflon (*Ovis orientalis musimon*), spanishibex (*Capra pyrenaica*) and captive yak (*Bos grunniens grunniens*) (Rodriguez-Sanchez *et al.,* 2010a,b).

Although BT is mainly a disease found in ruminants and camelids, under certain circumstances it can also be transmitted to carnivores. In dogs, BT has been reported following the use of a BTV-contaminated vaccine (Wilbur *et al.,* 1994; Evermann, 2008).

2.1.3 Vector and transmission

BTV is non-contagious and transmitted biologically by *Culicoides* insects (biting midges), but only a limited number of species are efficient vectors. Species of the genus *Culicoides*, sub-genus *Avarita* are regarded as the vectors of bluetongue viruses (Dyce, 1989). The distribution of the BTV is limited by the presence of competent *Culicoides* vector (Mullens *et al.,* 1995; Tabachnick, 1996).

Several species of *Culicoides* midges have been demonstrated to be vectors of BTV *C. fulvus* and *C. aconi* are proven vectors of BTV in Australia (Standfast *et al.,* 1985) whereas *C. pallidipennis* acts as vector in Kenya (Walker and Boreham, 1976) and *C. varripennis* in USA (Luedke *et al.,* 1967). Du Toit (1962) and Luedke *et al.,* (1967) reported the first implication that cattle might be reservoirs for the infection of the biting agent *Culicoides* during an epizootic of BT.

Cattle are the main amplifying hosts for BTV and important maintenance hosts. The competent *Culicoides* vector species feed more abundantly on cattle. The movement of large masses of *Culicoides* species for long distance by strong wind systems is known to occur (Dyce, 1982; Murray, 1986) and helps in spread of the disease. Rainfall can also transform an insect population from one that is maintaining virus to one of explosive transmission within a few days (Murray, 1986).

Luedke *et al.,* (1967) reported that the peak virus titers in the blood of sheep occur between days 6 and 8 post-infection (PI) which generally coincides with the maximum clinical response.

Erasmus (1985) reported about the virus surveillance in *Culicoides* midges in Africa. 881 viruses were isolated from 4506 specimens in 1979-85. The total number of BTV serotypes isolated per season varied from 11 to 17 with a marked variation in their prevalence.

Two to five serotypes were dominant each season and these were replaced by different highly prevalent serotypes in the next season.

Seasonal variations and other environmental factor such as moisture and wind occurring in temperate climates limit vector-born spread of BT in those areas (Gibbs, 1992).

Du Toit (1944) demonstrated that infected *Culicoides* species transmit the disease to susceptible sheep. Foster and Jones (1979) studied the *C. varripennies* females and noticed 100 fold increase in virus titre by day 15 and maintained until day 35 after oral infection with BTV.

Virus rarely may be excreted in the semen when males are viraemic. Excretion is more likely if there is inflammation of the genital tract, if the animal is aged or if the virus has been laboratory adapted (as in live vaccines or experimental infection). Contaminated semen may infect recipient cows, but these will not initiate a cycle of transmission unless competent insect vectors are abundant. Infection of other ruminant species presumably occurs under similar circumstances. There is much evidence from in vitro and in vivo work that embryos from infected donors washed to International Embryo Transfer Society (IETS) protocols do not transmit the virus. Cross contamination of canine vaccines with BTV during manufacture has resulted in the death of some vaccinated dogs in the United States (Wilbur, 1994).

Rao (2006) reported that BTV can be transmitted mechanically by means of instruments (blood transfer) during periods of viraemia. He also reported the in utero transmission that mainly depends on the stage of development (susceptible between 60 and 140 days gestation), virus strain and immune status of the dam.

2.1.4 Clinical Signs and Pathogenesis

Clinical manifestations of BT in sheep were reviewed by Erasmus (1975a). Clinical signs of disease in sheep vary markedly in severity, influenced by the type or strain of the

infecting virus, as well as by breed (Verwoerd and Erasmus, 2004). In sheep, the disease is manifested as an acute, chronic or subclinical condition.

In sheep BTV infection can cause a disease which is characterized by pyrexia, haemorrhage, inflammation, ulceration and cyanosis of mucous membranes lining the oral and nasal cavities, oedema of the head and neck, torticollis, coronitis and laminitis (Verwoerd and Erasmus, 1994). Oedema of the tongue, lips, submandibulum and cyanotic tongue is found in rare cases. At the end of the pyrexia stage, affected sheep may have coronitis, laminitis or paresis, necrosis of striated muscles, torticollis, dermatitis and breaks in the wool may also develop (Darpel *et al.,* 2007; Elbers *et al.,* 2008, 2009; Kirschvink *et al.,* 2009).

Infection in pregnant ewes may lead to abortion, foetal mummification and the birth of weak calves with potential congenital defects *viz.,* hydrocephalus, cerebral cysts, retinal dysplasia *etc*. (Tweedle and Mellor, 2002).

The most consistently reported gross pathological changes include generalized hyperaemia, oedema and haemorrhage involving lymphoreticular, pulmonary and cardiovascular systems, and the gastrointestinal tract. Erosions and ulceration of the oral cavity and the gastrointestinal tract and necrosis of the skeletal and cardiac musculature are also common findings (Leudke *et al*., 1964; Gard, 1987).

In cattle, Tweedle and Mellor (2002) reported that clinical disease is rare. In the early stages clinical signs are characterized by fever, apathy and depression followed by erosion and necrosis of the oral and nasal mucosae, nasal discharge, excessive salivation, conjunctivitis, lameness and stiffness, ulcerative dermatitis, coronitis, occasional bloody diarrhoea, oedema and hyperaemia. The skin of teats is often inflamed and may crack and peel (Williamson *et al.,* 2008; Elbers *et al.,* 2008, 2009).

Goats are less frequently infected with BTV and rarely show signs of clinical disease.

Affected goats may show sudden drop in milk production, high temperature, oedema of the lips and head, nasal discharge and scabs on the nose and lips, erythema of the skin of udder and small subcutaneous haemorrhagic lesions (Dercksen *et al.,* 2007).

Histological findings include hypertrophy of endothelial capillaries, perivascular oedema and infiltration of skeletal and cardiac muscle with macrophages and lymphocytes, vascular congestion with subsequent tissue infarction leading to epithelial tissue hypoxia and cell desquamation. Chronic cases result in fibrosis and infiltration with mononuclear cells (Tweedle and Mellor, 2002; MacLachlan and Gard, 2009)

2.2 BLUETONGUE VIRUS

BTV is a non-enveloped virus with ten double stranded (ds) RNA segments as genetic material (Verwoerd *et al.,* 1972). Each segment encodes a specific protein. Genome is surrounded by triple layer capsid with icosahedral symmetry (Patel *et al.,* 2014). Virions are composed of 7 discrete structural proteins (VP1 to VP7) organized into two concentric shells, the outer and inner capsids (Prasad *et al.,* 1992). In addition to seven structural proteins, there are four other virus-induced proteins like NSI, NS2, NS3, NS3A and NS4 found in the infected cells (Roy and Gorman, 1990).

While entering into the cell, the bluetongue virus loses outer layers and the remaining core particle enters the cytoplasm which is transcriptionally active ssRNA transcripts of each segment are synthesized from the core particle. This ssRNA transcripts act both as mRNA's for synthesis of viral proteins and as a template for dsRNA production.

2.2.1 Physico-Chemical Properties of BTV

Various physico-chemical and growth properties of BT were reviewed by Sellers (1981). The virus particle is sensitive to acid being inactivated at a pH below 6.0 and it is also slightly sensitive to lipid solvents. The particle is stable at 4° C and -70 ^{o}C but loses infectivity rapidly when frozen at -20 ^{o}C

Mishra and Mehrotra (1996) studied the physico-chemical and biological characteristics of an Indian isolate of BTV. In BHK-21 clone-13 cell line, pinpoint and pin head sized plaques were observed after fixation and staining with crystal violet.

2.2.2 Morphology of BTV

Owen and Munz (1966) studied BTV structure by electron microscopy and described particles with a diameter of 60 nm consisting of 92 capsomers arranged in an icosahedral form. They also reported enveloped forms of virions with an approximate diameter of 100nm.

Bowne and Ritchie (1970) observed the morphological features of BTV and recorded the diameter of particles as 60 to 70 nm.

BTV has buoyant density of 1.36g /cm^3 in cesium chloride and a sedimentation rate of 50S. It consists of a double-layered capsid containing a core particle with a clearly defined capsomer structure and in contrast to reo and rotaviruses; it is surrounded by a structure less diffuse outer layer (Martin and Zweerinck, 1972; Verwoerd *et al*., 1972).

Eaton *et al.,* (1987) examined the BTV infected cells under electron microscope and demonstrated the association of cytoplasmic virus specified tubules, viral inclusion bodies and progeny virus particles with cytoskeleton.

2.2.3 Molecular characterization of BTV

The genes of the bluetongue virus, which code for all the proteins it needs to *replicate* itself inside sheep cells, are in the form of double stranded RNA (ds-RNA). The BTV core contains ten pieces or "segments" of ds-RNA - sufficient information to produce thousands of copies of itself within hours of infecting a cell. Within the Orbivirus genus, there are 19 serogroups which share antigens detectable in complement fixation tests, agar gel immunodiffusion tests and fluorescent antibody tests and there is certain degree of cross reactivity between distinct serogroups. It is a non-enveloped virus with concentric protein layers enclosing ds RNA genome consisting of 10 segments. The outer capsid

consists of two proteins, VP2 and VP5, where VP2 is the major determinant of serotype specificity. Both VP2 and VP5 are

attached to the core particle, although it has been reported that VP5 is more closely associated with the core particle than VP2 (Huismans *et al.,* 1987). The inner core exhibits icosahedral symmetry and is composed of two major proteins (VP3 and VP7), three minor proteins (VP1, VP4 and VP6) (Martin and Zweerink, 1972; Verwoerd *et al.,* 1972). VP7 is a major core protein possessing the serogroup determining antigens.

Ten segments of ds RNA are also located within the core (L1-L3, M4-M6, S7-S10) coding for seven structural proteins and three nonstructural proteins NS1, NS2, NS3 (Mertens *et al.,* 1987; Roy and Gorman 1990)(table no 1). The BTV core appears to be composed of one thousand and eight protein components, made up mainly of two structural proteins VP7 and VP3. It comprises 780 copies of the VP7 protein, which form into trimers arranged as characteristic hexameric and pentameric rings on the surface of the virus core. Below this layer there is a "sub-core" shell formed from 120 copies of the VP3 protein. There are also several enzymes needed by the virus for self-replication (Roy, 1991).

2.3 DIAGNOSIS

A preliminary diagnosis based on clinical signs, post-mortem findings and epidemiological assessment should be confirmed by laboratory examination (Afshar, 1994).

Samples to be examined in the laboratory should include non-coagulated blood (use of EDTA or heparin is preferred), serum, post-mortem tissue samples of spleen, lymph nodes, lungs, liver, bone marrow and in addition, brain tissue collected from fetuses (Afshar, 1994; Tweedle and Mellor, 2002).

Blood samples can be stored at 4 °C for a long time; isolated blood cells in 10% dimethyl sulphoxide require storage at a temperature of –70°C. For transport, serum samples

should be frozen at –20°C and the other samples should be kept on ice (Tweedle and Mellor, 2002).

BT is diagnosed either by virus isolation, detection of nucleic acids or detection of anti BTV antibodies. Virus detection techniques include virus isolation and serotyping by neutralization assays (OIE, 2014). Nucleic acid based detection of different conserved segments (all segments except segment 2 and 6) can be used for diagnosis of BTV, whereas segment 2 based PCR will determine the serotype of the virus (Billinis *et al.,* 2001).

2.3.1 Virus isolation

Specimens for virus isolation include blood from suspected viraemic animals, blood clots after separation of serum, spleen or lymph nodes collected at necropsy of clinical cases or midges.

A number of virus isolation systems for BTV are in common use, but the most sensitive method is the inoculation of embryonated chicken eggs (ECE) (OIE, 2014). Primary inoculations of cell cultures such as the KC cell line (a cell line derived from *Culicoides sonorensis* midges), has been proven to be very sensitive (McHolland and Mecham, 2003). Inoculation of sheep may also be a useful approach, if the titer of virus in sample is very low, in the case of several weeks after virus infection or where laboratory facilities are not available.

Embryonated eggs, 9 to 12 days old are inoculated with the processed sample by intravenous route for BTV isolation. This method is 100- 1000 fold more sensitive than yolk sac inoculation (Dadhich, 2004; Sperlova and Zendulkova, 2011; Biswas *et al.,* 2010).

2.3.2 Cell culture

The first successful attempt to grow BTV in cell cultures was in 1956.BTV can also be isolated in cell lines of insect origin such as the KC line derived from *Culicoides*

sonorensis cells or C6/36 line from *Aedes albopictus* (AA) cells; the mammalian BHK-21 or Vero cell lines can also be used (Mecham, 2006). The cytopathic effect produced by BTV is observed only on cell lines of mammalian origin at three to five days after inoculation and appears as foci of rounded and refractile cells (Clavijo *et al.,* 2000).

Virus isolation can be attempted in BTV susceptible cell cultures such as mouse L, baby hamster kidney (BHK-21), African green monkey kidney (Vero) or *Aedes albopictus* clone C6/36 (AA). The efficiency of isolation is often significantly lower following inoculation of cultured cells with diagnostic samples compared with that achieved in ECE. Successful virus isolation has also been reported using primary isolation in cells derived from *Culicoides sonorensis* free of BTV and *Culicoides* viruses and designated as KC or CuVa cells (McHolland & Mecham, 2003). In case of passage in AA, KC or CuVa cells, additional passages in mammalian cell lines such as BHK-21 or Vero are usually performed. A cytopathic effect (CPE) is not necessarily observed in AA, KC or CuVa cells but appears in mammalian cells. Cell monolayers are monitored for the appearance of CPE for 5 days at 37°C in 5% CO2 with humidity. If no CPE appears, a second passage is made in the mammalian cell culture (OIE, 2014).

Sheep have been variously described to be as efficient as ECE (Foster *et al.,* 1972 and Goldsmit *et al.,* 1975), less efficient than ECE (Breckon *et al.,* 1980) and more efficient than ECE (Luedke, 1969 and Parsonson *et al.,* 1981) for isolation of BTV. However, sheep inoculation is often an impracticable option for many laboratories because of the requirement to maintain the sheep in holding facilities for at least 30 days after inoculation to permit development of the antibody response that provides evidence of virus infection.

2.4 DIAGNOSTIC TECHNIQUES

2.4.1 Serological diagnostic techniques

2.4.1.1 Complement fixation test

The complement fixation test (CFT) is a sensitive and specific test for diagnosis of BTV antibodies and was used until 1980 for diagnosis and certification of animals for export. Pearson *et al.,* (1992) described that CFT allowed detection and quantification of antibodies for 4–12 months after infection but was less reliable after that period and was particularly useful for detecting only recent infections. In most laboratories, ELISA is preferred over the CFT.

2.4.1.2 Agar gel immunodiffusion

AGID replaced complement fixation test as it is easy to perform and is reproducible (Della- porta *et al.,* 1985). Major drawbacks of AGID are lack of sensitivity and documented cross–reactions with other members of orbivirus serogroup (Gustafson and Pearson, 1992).

2.4.1.3 Enzyme linked immunosorbent assay

The ELISA has been used for approximately 40 years and has provided a valuable means of studying numerous antigens and their antibodies. Several different formats have since been applied to the detection of BTV antibody that include the indirect, the antibody blocking, the competitive and IgM capture ELISA. Of significance is the fact that these ELISAs are all serogroup-specific, identifying primarily the highly conserved BTV VP7 of all known serotypes.

Afshar *et al.,* (1989) compared c-ELISA, I- ELISA and AGID for detecting antibodies to BTV in cattle and sheep and concluded that c-ELISA was superior (99.92%) to that of I-*ELISA* (99.85%) and AGID (90%).

Reddington *et al.,* (1991) developed a c-ELISA to detect antibodies to the group antigen of BTV. The test was compared to AGID and SNT and was found to be superior in terms of sensitivity and specificity.

Gustafson and Pearson (1992) evaluated the bluetongue c-ELISA and reported that c-ELISA had a sensitivity and specificity similar to SNT. The AGID and c-ELISA tests showed similar specificity while testing AGID negative samples, but c-ELISA was found to be more specific while testing positive samples and showed greater sensitivity than AGID test.

Naresh *et al.,* (1995) tested the performance of c-ELISA for detecting antibodies against BTV. The performance of c-ELISA relative to dot-ELISA was found to have a sensitivity of 84.61% and specificity of 89.32%.

Ravishankar *et al.,* (2007) used a modified DOT ELISA method for detection of BT virus group specific antibodies in sheep sera and discussed the method of increasing the shelf life of bound antigen.

Bitew *et al.,* (2013) reported that c-ELISA is less subjective than SNT techniques and is able to discriminate BTV from other closely associated Orbivirus, and Epizootic Hemorrhagic Disease virus (EHDV). The ELISA has been used as a valuable means of studying immune properties of numerous antigens and their antibodies of BTV. The current edition of the OIE Manual of Standards for Diagnostic Tests and Vaccines cites the competition ELISA as a prescribed test for the detection of BTV-specific antibodies (OIE, 2014).

2.4.1.4 Serum neutralisation test (SNT)

The SNT is serotype-specific and can be used to differentiate between the antibodies produced against each of the antigenically distinct serotypes of BTV. The methodologies used can vary considerably but the principles remain the same; that is, test sera are reacted

separately with a constant amount of each BTV serotype. The amount of neutralization of virus, relative to a homologous virus control in the absence of any serum, is measured biologically using mammalian cells as an indicator of virus infectivity. The SNT is considered to be highly sensitive and specific in that it does not cross-react with other Orbivirus serogroups. The assay is not usually used for routine testing because it is time-consuming, expensive of reagents and the quality of the test sera may affect the cells.

Patton *et al*., (1994) experimentally immunized deers with BTV-10, BTV-11, BTV-13 and BTV-17 serotypes and raised hyper immune sera. The sera were tested for neutralizing antibodies by AGID, ELISA and SNT and concluded that among all serological tests, SNT is the most sensitive test for diagnosing serotype of BTV.

Singer *et al*., (1998) evaluated PCR, SNT, AGID, Virus isolation (VI) and c-ELISA in free ranging bighorn sheep. The c-ELISA, AGID and SNT had high levels of agreement in determining serogroup exposure in bighorn sheep. Although the c-ELISA and AGID had high sensitivity and specificity, the SNT had perfect specificity but lower apparent sensitivity.

The specificity of VNT in typing BTV was well agreed by the epidemiological studies conducted by Kirkland *et al.,* (2002) in China. The seropositive (by ELISA) sera samples were tested against reference virus types 1, 2, 3, 4, 7, 9, 12, 15, 16, 20, 21, and 2.It shows the presence of BTV serotypes 1, 2, 9, 12, 16, and 23 and the results agreed with the subsequent virus isolation.

Dubay *et al*., (2006) screened sera samples screened by AGID and positive samples were tested for both nonspecific Ab's and cross neutralizing Ab's by SNT. Based on the results the prevalence of serotypes BTV – 11 and BTV – 10 were determined in USA.

Ozguluk (2009) studied seroprevalance of BTV serotypes 9 and 16 in cattle older than 1year age in Turkey. SNT was performed in Vero cells maintained in Dulbecco’s MEM

supplemented with 10% FBS. A total of 740 serum samples were used in 1:10 dilutions in order to detect antibodies against BTV-9 and 16. After 4-5 days incubation it was concluded that among 740 animals, 72 were positive for BTV-9; 87 positive for BTV-16 while 76 animals were found to have antibodies against two serotypes and 505 were found as free of antibodies to both serotypes.

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.

Yilmaz *et al.*, (2012) reported that VN assay is capable of identifying and quantifying antibodies against antigen present in test sample. Its performance was comparable to plaque reduction neutralization test (PRNT) and it requires less sample volume than PRNT and is more suitable for small volumes of samples.

OIE (2014) concluded that SNT is the gold standard test for identification of virus serotypes.

Kamar *et al.,* (2015) monitored seroprevalence of bluetongue isolates in Morocco by SNT. In their study, they analyzed a total of 222 sheep sera and reported total prevalence of BTV as 33.33%, 37.96% and 37.04% for respective serotypes of BTV-1, BTV-4 and BTV-8 in 2009, and 10.53%, 14.91% and 21.93% for serotypes of BTV-1, BTV-4 and BTV-8, respectively in 2012.

2.4.2 Molecular diagnostic techniques

2.4.2.1 Genotyping of BTV

The bluetongue virus with its various serotypes and high antigenic variation among the serotypes creates a confusion in controlling the disease since last two decades. The outstanding technique, amplification of DNA by a polymerase enzyme, invented by the Kary

Mullis in 1983 an American Scientist, provided a solution for most challenging problems of biology (Mullis and Faloona, 1987). With this technique, BTV became one of the most well studied viruses at molecular level. Hence now a days it became a hand tool to type a bluetongue virus isolate using type specific primers against L2 gene of BTV which is more specific, sensitive and less time consuming when compared to serological methods.

Serogroup-specific RT-PCR, sequencing, restriction enzyme profile analysis (REPA) and phylogenetic analyses (targeting conserved genome segments) are now available (Bitew *et al.,* 2013).

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

Mertens *et al.,* (2007) designed primers and standardized RT-PCR assays targetting the segment-2 of the various bluetongue virus isolates that effect Europe. These primers were tested for their efficacy, sensitivity, and specificity in typing of the bluetongue virus isolates.

Maan *et al.,* (2007) cloned and sequenced the full length cDNA copies of each of the 24 reference BTV serotypes. When compared with coding region, the near terminal non-coding sequence is highly conserved with 2.9-64% variation in the 3' and 5.9-64% variation in the 5' NCR. The full length nucleotide sequence of the seg-2 reveals variation among reference strains of all 24 BTV types by 29% to 59% with terminal hexanucleotides as the

fully conserved sequence and VP2 amino acid sequence variation is 22.7% to 72.9% between BTV types. They constructed neighbour-joining tree which indicate 9 evolutionary branching points which correlate with the 10 nucleotypes.

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

Dahia *et al.,* (2004) designed serotype specific primers for amplification of hyper variable regions of VP2 genomes of BTV-1 and BTV-23. They successfully amplified 1240-1844bp region of BTV-1 which consists of two hyper variable regions with no cross amplification of heterologous serotype of BTV. Similarly, they standardized RT-PCR assay for amplification of part of VP2 genome of BTV-23 with no non-specific amplification.

Johnson *et al.,* (2000) tested a total of 132 BTV isolates, from different host species, collected over a period of 24 years by multiplex RT-PCR and concluded that multiplex RT-PCR successfully identified the serotypes of 130 of the isolates and shown to be more reliable and specific than the VN assay.

Anthony *et al.,* (2007) developed duplex RT-PCR assay for the detection of BTV in clinical samples. They concluded that RT-PCR method is more sensitive than cell culture and embryonated egg inoculation assays.

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

A novel tool, real-time quantitative reverse transcription-PCR assay was developed by Hofmann *et al*., (2008) for typing of the BTV serotypes 1, 6 and 8. They designed type specific primers based on the reference sequences of BTV belonging to western group which are labeled with 6-carboxyfluorescein at the 5' end and black hole quencher 1 carboxylic acid at 3' end. The results of the amplification brings out 100% serotype specificity with no cross amplification with heterologous serotypes.

Chauhan *et al*., (2009) developed nested PCR assay using two NS1 gene specific nested primers for the detection of BTV from cell culture adapted BTV-23 and blood samples collected from suspected sheep. Then concluded that PCR based assay provides a valuable tool to study the epidemiology of BTV infection in susceptible domestic livestock.

The outbreak of BT in cows in Netherlands during the year 2008 gave the first report of BTV-6 in Europe which undergone full length characterization by Maan *et al*., (2010). The BT positive was confirmed by real time RT-PCR targeting segment -1. Further, it was typed as BTV- 6 by experimental primers (Mertens *et al.,* 2007) designed against 24 serotypes, in which case amplification was seen only with BTV-6 primers. Also the anti-sera from the infected cow was tested in SNT against all 24 reference strains of BTV and neutralization positive is seen against BTV-6 and BTV-8 (vaccine strain). The full length cDNA sequence

of seg-2 shows close relationship (99.8/99.7% nt/aa dentity) to reference strain of BTV-6 and vaccine strain of BTV-6 from South Africa.

2.5 PATHOGENICITY STUDIES OF BTV

Chander *et al.,* (1990) reported that ability of BT to inflict pathological changes in sheep depends on virulence of particular virus isolate, susceptibility of host and environmental factors.

MacLachlan (2004) reported that sheep that are native to tropical and subtropical regions of the world where BTV is endemic are usually resistant to BT and other factors like nutritional status, immune status and age also influence the severity of BT in individual sheep

Backx *et al.,* (2007) studied clinical signs of BTV serotype 8 infection in sheep and goats by infecting them with infected blood via intravenous route and observed viremia in both sheep and goats.

Darpel *et al*., (2007) studied pathogenicity of BTV serotype 8 in sheep and cattle and concluded that manifestation of clinical signs is dependent on many factors like strain of virus, breed of sheep, individual susceptibility, the immune response of the host and environmental factors. Hence it is unrealistic to expect a simple relationship between the manifestation of clinical signs and the level of viraemia.

MacLachlan *et al*., (2008) inoculated BTV serotype 4 in six sheep which developed severe clinical signs including coronitis, hemorrhage and ulceration of the mucosal lining of the oral cavity and fore stomaches, hemorrhage in the wall of the pulmonary artery, and focally extensive necrosis of skeletal muscle of the neck and increase in numbers of WBC.

Schulz *et al*., (2012) infected South American camelids with BTV serotype 8 and observed very mild clinical signs like anorexia, arched backs, lung sounds, extended recumbency and mild conjunctivitis.

Umeshappa *et al*., (2011) compared intradermal and intravenous inoculation of BTV serotype 23 in sheep and showed that that ID route could be more efficient in enhancing clinico-pathology and viral and immune responses in infections with laboratory passaged BTV-23 in natural host.

Batten *et al*., (2012) assessed the pathogenicity and infection kinetics in six Dorset Poll sheep and observed that sheep infected with both BTV-25 and BTV-26 seem to exhibit similar kinetics of infection with low levels of viral RNA detected and mild or no clinical signs.

Batten *et al*., (2013) assessed the pathogenicity and infection kinetics of BTV-25 and 26 in goats and observed that both seem to exhibit similar infection kinetics with low levels of viral RNA of short duration and mild clinical signs in sheep, and higher levels of viral RNA of longer duration with no clinical signs in goats.

CHAPTER III

MATERIALS AND METHODS

3.1 GENERAL LABORATORY MATERIALS

Molecular grade reagents were used for the preparation of all solutions and buffers. The reagents and lab ware were availed from a number of suppliers including Bangalore GeNei, Himedia (Mumbai), Merck (Mumbai), Sigma Aldrich (Hyderabad) and Invitrogen (USA). All aqueous solutions were prepared using double glass distilled water. Where necessary, solutions were sterilized by autoclaving at 121ºC, 15 lbs pressure for 15 minutes or by membrane filtration using 0.22 µm membrane filter (Millipore Corporation, Bedford, MA, USA).

3.1.1 Glassware

Borosil and Schott Duran glassware were used throughout the study.

3.1.2 Plastic ware

Plastic ware of 'Corning' and 'TPP' brand were used for cell culture studies. The tissue culture flasks T_{75} ($75cm^2$), T_{25} ($25cm^2$) and 2ml, 5ml, 10ml disposable pipette obtained from 'TPP' were used. 96 well plates from 'Corning' brand were used for $TCID_{50}$ and SNT. 'Axygen' and 'Genei' brand microfuge tubes (2 ml, 1.5 ml), PCR tubes (0.2 ml) and micropipette tips were used in the study.

3.1.3 Preparation and sterilization of required material

Glassware were soaked in 5% neutral detergent (Labolene) for 1 hour. Glassware were then scrubbed and rinsed thoroughly under running tap water till all traces of soap were removed. Later, they were soaked and rinsed twice with single distilled water. Finally, they

were rinsed with double distilled (Milli Q) water and kept inverted 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 one and a half hour.

Micropipette tips, microfuge tubes, filter assemblies, deep well plates used for cell culture were sterilized by autoclaving at 121°C/15lb/15 minutes.

3.2 CELL LINES

BHK-21 and KC cell lines were obtained from Ella foundation, Turkapally, genome valley Hyderabad. The sub-culturing of BHK cells was done with a split ratio of 1: 6 using growth medium. These cells were used for propagation and isolation of BTV.

3.3 EXPERIMENTAL ANIMALS

Sheep of Deccani breed maintained in the sheep farm of Instructional Livestock Farming Complex, College of Veterinary Science, Hyderabad were screened for the presence of antibodies to BTV by c-ELISA. Sheep that were seronegative for BTV by c-ELISA showing less than 50 percent inhibition were selected as BTV seronegative and used in the study.

3.4 HOUSING OF EXPERIMENTAL ANIMALS

Five BTV seronegative Deccani sheep between age of 6 months and 1 year were selected for use in present study and were shifted from Instructional Livestock Farming Complex, College of Veterinary Science, Hyderabad to insect proof experimental animal house at College of Veterinary Science, Rajendranagar, Hyderabad.

All the animals were dewormed and held for a period of one week before the start of the experiment for acclimatization. Animals were provided with feed, fodder and water ad-libitum throughout the study for 2 months.

3.5 CHEMICALS AND REAGENTS

Chemicals used for cell-culture work in the present experiment were of 'AnalaR' or 'ExcelaR' grade from Qualigens, Sisco Research Laboratories (Mumbai, India) and Sigma Aldrich (St. Louis, USA). Chemicals used for molecular work in the present study were of molecular grade from Sigma (USA) and SRL (Mumbai, India). DNA molecular size marker was obtained from GeNei, Bengaluru, India.

3.5.1 1X PBS

8g of NaCl, 0.2g of KCl, 2.44g of Na_2HPO_4, 0.24g of KH_2PO_4 were added in a glass bottle. The volume was made up to 1L with Millipore water. The pH of the solution was adjusted to 7.2 and then autoclaved.

3.5.2 1X TAE

Tris base-24.2g, Glacial acetic acid-5.7ml, 0.5M EDTA-10ml were added in glass bottle. The volume was made up to 500ml with distilled water.

3.5.3 Trypsin (0.1%)

0.1g of trypsin(Sigma,T-4799 E.C No 232-650-8), 0.2 g of EDTA, 0.05g of Glucose were added in a sterile glass bottle and the volume was made up to 100ml with autoclaved 1X PBS. It was kept for over-night stirring for proper mixing. The next day the prepared solution was filtered through 0.22µ membrane filter using filter assembly and stored at 4^0C.

3.5.4 Crystal violet staining solution

Crystal violet: 1g

Methanol: 70ml & 1X PBS: 30ml

3.5.5 Formaldehyde solution

10% Formaldehyde: 1ml

1X PBS: 10ml

3.6 CELL CULTURE MEDIA AND REAGENTS

3.6.1 Schneider's Insect Medium

The required chemicals are

1. Schneider's Insect Medium bottle (CAT-S9895; SIGMA-ALDRICH)

2. Sodium bicarbonate

3. NaOH-1N soln

4. HCl-1N soln

5. Calcium chloride solution: 0.6 gm. of calcium chloride is dissolved in 50ml of water for 1L of media.

800ml of autoclaved Millipore water was taken in conical flask. A packet of Schneider medium was added while stirring with the help of magnetic stirrer until all particles were dissolved completely. While stirring 0.4gm of sodium bicarbonate was added to dissolve. The pH is adjusted to 9.2 by using 1N NaOH solution. The solution becomes turbid. Now pH is adjusted to 6.7 using 1N HCl solution. Calcium chloride solution was added in drop wise

manner slowly by rapid mixing to avoid precipitate formation. Finally volume was made up to 1000ml by adding water. To that 100 IU benzyl penicillin and 100µg/ml streptomycin sulphate were added. Medium was filtered with 0.22µ filter and stored at $4^{0}C$ till use. Sterility was checked with Luria Bertani agar.

3.6.2 Minimum Essential Medium

The powdered contents of the sachet meant for 1 liter medium(Earle's MEM, GIBCO, Cat No. 41500-034) were added to about 950ml of sterile glass distilled water and mixed thoroughly, then 2.2g of Sodium bicarbonate was added to the medium and mixed well. The pH of medium was adjusted to 7.2 and volume was made up to 1 liter. To that 100 IU benzyl penicillin and 100µg/ml streptomycin sulphate were added. Then medium was filtered through 0.22µ membrane filter using filter assembly. After filtration the sterility was checked using Luria Bertani agar plating.

3.6.3 Tryptose phosphate broth

2.95 g of Tryptose Phosphate Broth (TPB) powder suspended in 100 ml Millipore water. Sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes. TPB is procured from Himedia (Ref No. AT811-100G).

3.6.4 Growth Medium (GM)

Growth medium was prepared by supplementing MEM with 5%FBS. Foetal bovine serum was procured from Himedia (Ref No. RM1112-500ml).

3.6.5 Maintenance Medium (MM)

The maintenance medium was prepared by supplementing MEM with 1%FBS. Maintenance medium is used during the maintenance period and not for subculture. During this phase cells will survive but rate of division is less. Hence only 1% FBS is used.

3.7 VIRUS

3.7.1 Bluetongue Virus Serotype 4 and 16.

Bluetongue Virus isolates were procured from Ella foundation, Turkapally, genome valley, Hyderabad. These virus isolates are being maintained at the department and were used in the present study.

3.7.2 Characterization of BTV isolates

3.7.2.1 Extraction of Viral Nucleic acid:

2 ml of cell culture fluid taken in 2ml microfuge tube was centrifuged at 13,200rpm for 15mts to obtain pellet. For that pellet 750µl of TRIZOL® was added to the sample. The mixture was vortexed for 1min for proper mixing and incubated in room temperature for 5min.250µl of Chloroform was added to the above mixture. The above mixture was hand mixed to avoid any cellular DNA contamination and incubated in room temperature for 10min.The mixture was centrifuged at 13200 rpm for 15min at 4^0C after the incubation.The aqueous phase was collected carefully to another RNase free 1.5ml microfuge tube without touching the protein layer.Equal amount of ice cold Isopropyl alcohol was added to collected aqueous phase.This mixture was incubated at -20^0C for overnight.The following day this mixture was centrifuged at 13200 rpm for 15 minutes.Supernatant was discarded leaving the small pellet (sometimes its invisible).

1ml of 70% alcohol was added to pellet and centrifuged at 13200 rpm for 10 min.Supernatant was discarded and the pellet was air dried.The pellet was dissolved in 25µl of nuclease free waterThe presence of viral RNA was confirmed by agarose gel electrophoresis.

3.7.2.2 Confirmation of serotype by polymerase chain reaction

3.7.2.2.1 Reverse transcription

RT was carried out using TaKaRa Clonech kit (Cat No.6110A).

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

The reaction mixture consists of RNA mix & RT mix.

RNA mix: RNA -8µl

Water -9.5µl

RNA mix was kept at 94^0C for 10min in PCR and snap cooled on ice.

To the RNA mix RT mix was added with following composition as mentioned below:

RT mix:	Random Hexamer	-1 µl
	RNAse Inhibior	-0.5 µl
	dNTPs	-1 µl
	5X RT buffer	-4 µl
	MuMLV-RT	-1µl

The RNA mix & RT mix were loaded in the thermocycler with following conditions.

Annealing- 25^0C for 10 min

RT Activation- 42^0Cfor 1 hour

RT Inactivation- 70C for 10 min

Before proceeding for PCR cDNA was kept in 4^0C

3.7.2.2.2 Polymerase chain reaction:

Using the cDNA synthesized, PCR was carried out by preparing a master mix for a 12.5µl reaction with the primers described above. The composition of the 12.5 µl PCR mix as given below:

c DNA	2µl
dNTPS	0.5 µl
10x Bufer with $Mgcl_2$	1.25µl
FP(20µM)	0.5µl
RP(20 µM)	0.5 µl
Taq (3U/µl)	0.5 µl
Water	7.25 µl

The above mentioned contents were transferred to a thin walled 200 µl PCR tube on ice and the contents were mixed by vortexing and centrifuged briefly to spin down the contents to the bottom of the tube. The tube was loaded on to an Eppendorf thermal cycler and the cyclic conditions were followed as per the literature provided by Chaitanya 2005.

The cyclic conditions for BTV: 1, 2, 4, 9, 10, 12, 21, 23, 24.

1) Initial denaturation	94^{0}C 3mt
2) Denaturation	94^{0}C 30sec
3) Annealing	55^{0}C 30sec
4) Extension	72^{0}C 1mt
5) Final extension	72^{0}C 10mt
6) Hold it	4^{0}C
No of cycles (for 2,3,4)	35

The cyclic conditions for BTV-16:

1) Initial denaturation	94^{0}C 3mt
2) Denaturation	94^{0}C 30sec
3) Annealing	50^{0}C 30sec
4) Extension	72^{0}C 1mt
5) Final extension	72^{0}C 10mt
6) Hold it	4^{0}C
No of cycles (for 2,3,4)	35

All the primers were obtained in lyophilised form. The primer sets was obtain From IDT. Primers were reconstituted with nuclease free water (Himedia) to get 100pmol/µl stock. Stocks were made into 20pmol/µl working solution and used for for RT-PCR.

Sequence of primers (5′-3′):

BTV 4P2F	5' GTT GGA TCT GAG AAA TGG GT 3'.
BTV 4P2R	5' AAG ACA CGG ATA AGG ATT CG 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'.

3.7.2.2.3 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) and 1KB DNA ladder.

Materials:

1. PCR products
2. Agarose (HI media)
3. 1X TAE buffer
4. Ethidium Bromide (10 mg/ml, Sigma)
5. Gel Loading dye 6x (Prepared manually)
6. DNA Ladder 100 BP ,1KB (Bengaluru, GeNei)

Method:

Agarose gel (1.2%: For product size 1kb) was dissolved in TAE buffer and heated in a microwave oven till it melted completely.After letting it cool to 50ºC, Ethidium Bromide was added to a final concentration of 1µl/10ml. The content was swirled gently to mix after adding it.The molten agarose was poured into a casting tray with positioned comb was allowed to solidify for at least 20 minutes.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.Sample and DNA ladder were loaded with 1x loading dye and electrophoresis was run at 75 volts (5 volts/cm) for 1 hour. The gel was transferred to gel doc having UV trans-illuminator and observed under medium wavelength for desired band.

3.7.3 Adaptation of BTV-4 & 16 serotypes in KC Cell-lines

BTV- 4 & 16 serotypes propagated in BHK cell lines were used as seed virus. 0.3 ml of virus infected tissue culture supernatant mixed with 2.7 ml of Schneider's medium containing 10% FBS was added to the 75 cm^2 tissue culture flask with complete monolayer of KC cells after removing growth medium. Incubating at 25°C for 1hr to allow adsorption of virus. After removing the unabsorbed virus, 25ml of Schneider's medium with 10% FBS was added and incubated at 25°C for 10 days. Flask was observed daily for 3-5 days for development of complete CPE. The virus was harvested after 10 days and stored at 4°C.

3.7.4 Clarification of the harvested virus.

As the BT virus is mostly cell associated, the virus needs to be released from the infected cells so as to get the maximum virus titers. The viral harvest was subjected to rapid freezing and thawing: freezing at –70°C followed by thawing at 37°C for three consecutive times. The harvest was then centrifuged at 4000 rpm for 10 min and supernatant was separated from cell debris.

3.7.5 ASSAY OF VIRUS INFECTIVITY

3.7.5.1 Subculture

T-25 flask having 80-90% confluence was selected for sub culturing. Medium was decanted from the flask and monolayer was washed with 1X PBS. Then the monolayer was treated with 0.5-1 ml of 0.1% trypsin. When cell detachment was observed, the culture bottle was tapped gently to slide the cells into the suspension. 10 ml of growth medium (5%MEM) was then added and flushed with pipette till all cells were individualised. Cell counting was done with the haemocytometer by staining with 0.4% Trypan blue. The number of viable cells was counted by using the formula given below

$$\text{Viable cells / ml} = \frac{\text{Number of viable cells counted} \times \text{Dilution factor}}{\text{Number of 1mm square areas counted}} \times 10000$$

Cell suspension was transferred to a sterile disposable Petri dish. To sterile 96 well flat bottom plate cell suspension was distributed accordingly 1×10^4 cells in 100 µl of media to each well with the multichannel micropipette. The plate was incubated at 37^0C in 5% CO_2 incubator overnight.

3.7.5.2 Infection

The plate with 70-80% confluency was observed and then prepared for infection.

Preparation of samples:

For infection, virus sample was kept on ice (cold chain should be maintained).10 fold serial dilution of virus was made with medium (1% maintenance medium) as a ratio 100 µl (virus) + 900 µl (diluent). The medium was decanted from plate and cell monolayer was infected with 100 µl of diluted virus suspension in four quadruplicate. The plate was

incubated at 37^0C with 5% CO_2 and observed for 5 days to note down the readings. $TCID_{50}$ titre was determined as per Reed and Muench Formula (1938)

3.7.5.3 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). The medium from the plate was discarded and the cells were washed with1X PBS (200µl/well). The cells were fixed in 10% Formaldehyde (200µl/well) and incubated for one hour at room temperature. Formaldehyde was discarded and washed twice with 1X PBS. 1% crystal violet solution (200µl /well) was added and kept on shaker for 1 hour. Crystal violet solution was discarded and the cells were washed thrice with 1X PBS. The plates were dried at room temperature.

3.7.6 BTV INOCULATION IN SHEEP

Five BTV seronegative Deccani breed sheep screened by c-ELISA bearing tag no 209, 214, 215, 244, 223 were selected. Group of four sheep were inoculated with 0.25ml ($10^{6.66}$ $TCID_{50}/_{ml}$) of KC cell adapted live BTV-16 by intradermal route and one control (sheep no 223) with uninfected KC cell culture suspension. Super infection with 0.6ml ($10^{6.23}$ $TCID_{50}$ $/_{ml}$) of KC cell adapted live BTV-4 was done on 30 days post inoculation (dpi). The site of inoculation is just in front of scapula at the shoulder region. The animals were examined daily for clinical signs besides recording rectal temperature for a period of 60 days. They were also tested for immune responses to BTV.

3.7.6.1 Collection of blood Samples

Blood samples were collected on 0, 1, 3, 5, 7, 9, 11, 15, 17, 19, 21, 23, 25, 28, 30, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57 and 59 dpi in Heparin, EDTA and plain vacuutainers (Becton Dickinson) of 5 ml capacity from animals inoculated with virus as well

as control animal. Blood samples collected in EDTA were used for hematology studies on the same day and heparin were used for isolation of virus in KC cell line. Blood samples collected in plain for serum were centrifuged with 4000 rpm for 15 min at 4° C and serum was separated in 1.5 ml microfuge tubes for c-ELISA and SNT. The serum collected was inactivated at 56°C for 30 minutes and stored at -20°C.

3.7.6.2 Clinical responses

The animals inoculated with virus were examined daily for changes in nasal, lingual and oral mucous membranes, inter digital spaces, coronary bands and body temperatures.

3.7.7 HUMORAL IMMUNE RESPONSES TO BLUETONGUE VIRUS

Seroconversion to BTV was determined by SNT and c-ELISA using serum samples collected from the experimental sheep.

3.7.7.1 Serum Neutralization Test (SNT)

Serum neutralization test was carried out to detect antibodies against BTV-4 for first month and for both BTV-4 and BTV-16 for second month. Briefly, the method was as follows. 1:10 serum dilution was prepared in maintenance medium in sterile deep well plate with the volume of 250 µl (25µl serum+225µl maintenance medium). 250 µl of medium containing 100 $TCID_{50}$ virus per 50 µl was added to each well. The serum virus mixtures were incubated for 1 hr at 37°C with 5% CO_2. 100µl of incubated mixture was added to well with BHK monolayer in 96 well microtiter plate in quadruplicates. Four wells were kept as virus controls and four wells as cell controls in each plate. The plates were incubated for 5 days at 37°C with 5% CO_2 and CPE readings were noted from 3-5 days. The plates were stained as described in 3.8.5.3 on 5th day.

3.7.7.2 Competitive Enzyme- Linked Immunosorbent Assay (c-ELISA).

Bluetongue Virus Antibody Test Kit (M/S Veterinary diagnostic technology, Inc.USA) was employed to screen the serum samples for group specific antibodies to BTV by c-ELISA.

cELISA was done using the bluetongue antibody test kit supplied by the Veterinary Diagnostic Technology, Inc., according to the kit protocol .Briefly, Diluting and washing buffers were prepared in 1x concentration in deionized water. Control and test serum were prepared by diluting them 1:5 in dilution buffer. Precoated plates were washed with PBS twice. 50µl of test and negative, weak positive, strong positive controls were added to duplicate wells. After adding test and control sera, 50µl of the diluted (1:100) monoclonal antibody was added to each well. The plates were incubated at room temperature for two hours covered with a lid. Just before the incubation period ends, 1:300 dilution of the conjugate was prepared in 1x diluting buffer. At the end of incubation period, the reactants were poured off from the wells and washed with wash buffer for thrice. Diluted conjugate (100µl) was added to each well and incubated at room temperature for one hour. Buffer substrate solution was prepared by dissolving the contents of buffer substrate capsule in 100 ml of deionized water. Each OPD tablet is dissolved in 5 ml of this buffer substrate solution. After preparing the OPD buffer substrate solution, washed the well 5 times with washing buffer. Now 100µl of substrate OPD solution was added to all wells and the plate was incubated for 10 minutes in dark. After 10 minutes, 50µl of stop reagent (3N sulfuric acid) was quickly added to all the wells. The OD values for each well were taken at 490 nm wavelength using ELISA miroplate 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 $\geq$50% inhibition were considered as seropositive.

CHAPTER IV

RESULTS

The focus of current research work was on the pathogenicity studies of BTV serotype 16 along with super infection of BTV serotype 4 in sheep. These Bluetongue Virus isolates were procured from Ella foundation, Turkapally, genome valley, Hyderabad. These virus isolates are being maintained at the department and were used in the present study.The details of the results are presented below.

4.1 Cell lines

Culicoides cells (KC) and Baby Hamster Kidney cells (BHK-21) were used for this research purpose.

4.1.1Maintenance of KC cells

On seeding with KC cells, the cell culture flasks with 80% confluency (Figure no1) were observed within 24hrs. These 25cm² cell culture flasks were used for the infection purpose.

4.1.2 Maintenance of BHK-21cells

On seeding with BHK-21 cells, the cell culture flasks with 90% confluency (Figure 2) were observed within 36 hrs under optimum conditions (5% CO_2 at 37ºC).These cell culture flasks were used for the infection purpose.

4.2 Propagation of virus in cell cultures

BHK-21 adapted BTV serotypes 4 and 16 were infected onto KC cell monolayer. Infected KC cells did not exhibit distinct cytopathic effect unlike mammalian cells which showed distinct cytopathic effect (rounding, clumping and detached monolayer)(figure no 3) as BTV particles are released primarily by virus-induced cell lysis; while in KC cells they bud from the plasma membrane and establish a persistent infection.

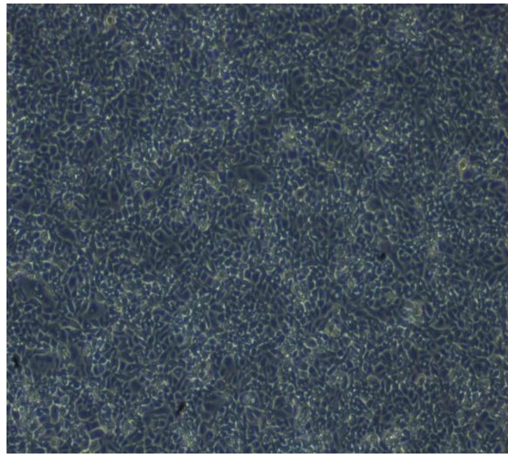

Figure no 1: Monolayer of KC cells (100X)

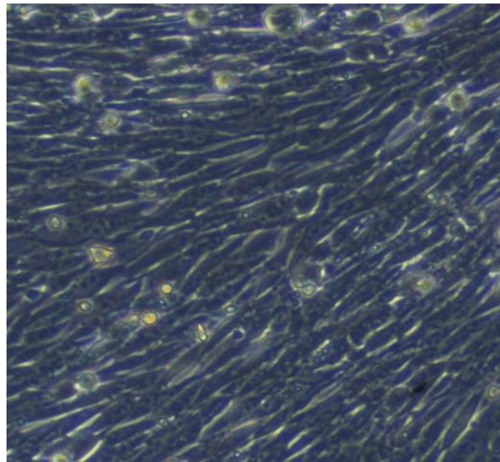

Figure no 2: Monolayer of BHK-21 cells (100X)

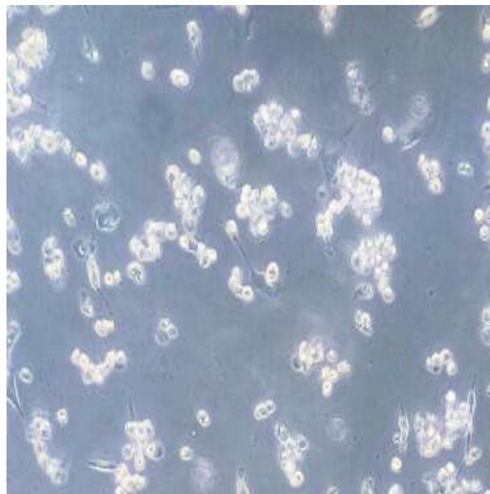

Figure no 3: BTV infected BHK-21 cell monolayer showing CPE – rounding, clumping of cells and detached areas of monolayer (100X)

4.3 CHARACTERIZATION OF BTV SEROTYPES

4.3.1 Characterization of viral nucleic acid

Double stranded RNA isolated from BHK-21 cell cultures infected with BTV serotype 16 and BTV serotype 4 were analysed by Agarose gel electrophoresis. Both the isolates clearly showed the segmented pattern of genome with nine clearly resolved bands and two indistinct bands of BTV (Figure 4).

4.3.2 Molecular typing of BTV

BT viruses used in this study were typed using type specific primers by RT –PCR .It was observed that amplification was only seen using primers specific for BTV -4 and BTV-16, for BTV -4 and BTV- 16 isolates, respectively. No amplification was observed using primers specific for BTV-1,2E,9,10,12,13,21,23,24 which are commonly circulating in India (Figure 5,6&7).

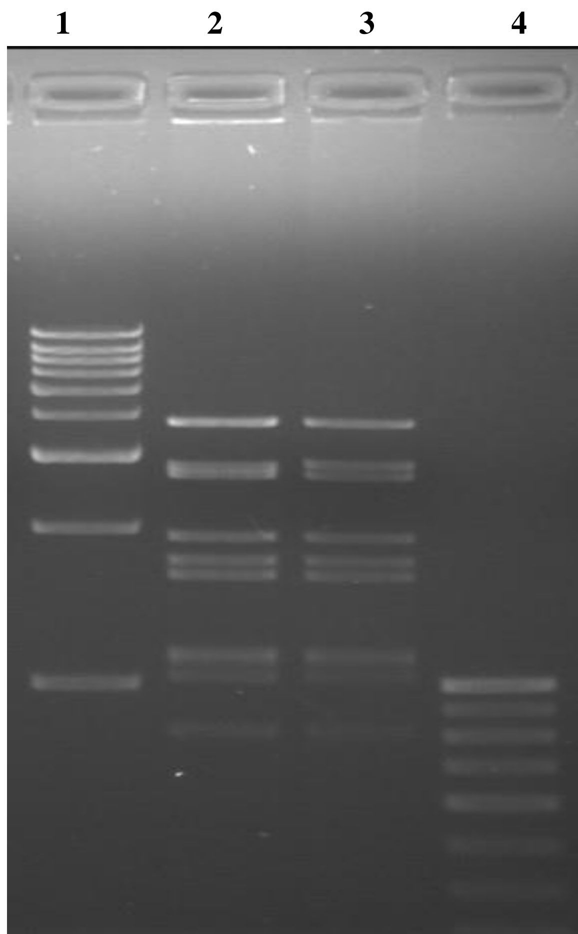

Figure no 4: AGAROSE GEL ELECTROPHORESIS OF BTV RNA

Lane 1: 1 Kb ladder

Lane 2: BTV 16 RNA

Lane 3: BTV 4 RNA

Lane4: 100 bp ladder

MOLECULAR TYPING OF BTV -4

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

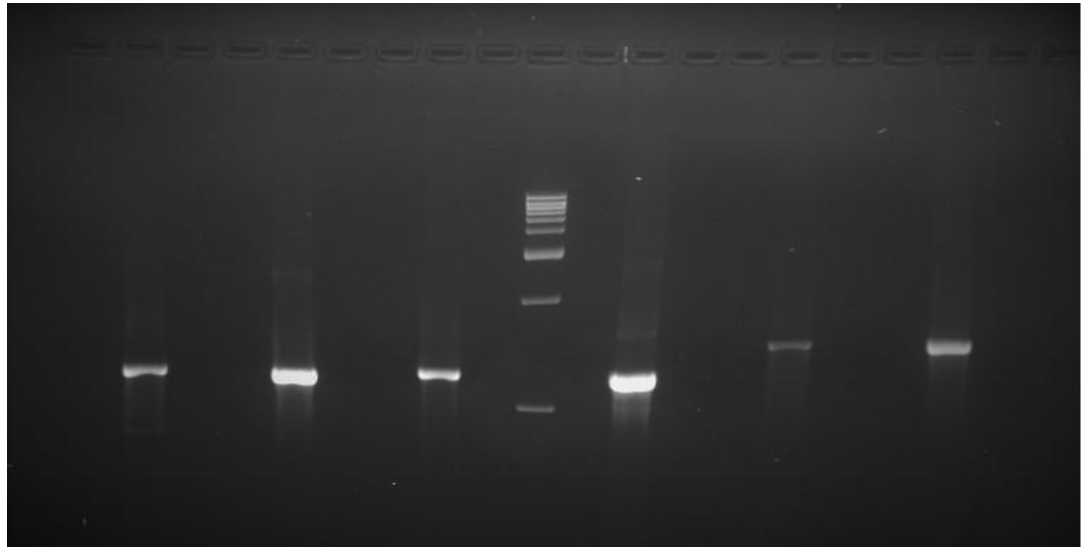

Figure no 5: Typing of BTV- Agarose gel electrophoresis of RT-PCR products

lane1: BTV 1 PCR product

lane 2: BTV 1 positive control 1172 BP

lane3: BTV 1 negative control

lane 4: BTV 2 E PCR product

lane5: BTV2 E positive control 1172 BP

lane6: BTV2 E negative control

lane7: BTV 9 PCR product

lane8: BTV 9 positive control 1200BP

lane9: BTV 9 negative control

lane10: 1KB ladder

lane11: BTV 16 PCR product

lane12: BTV 16 positive control 1200BP

lane13: BTV 16 negative control

lane14: BTV 21 PCR product

lane15: BTV 21 positive control 1388 BP

lane16: BTV21 negative control

lane17: BTV23 PCR product

lane18: BTV 23 positive control 1370BP

lane19: BTV23 negative control

lane20: empty

1 2 3 4 5 6 7 8 9 10 11 12 13

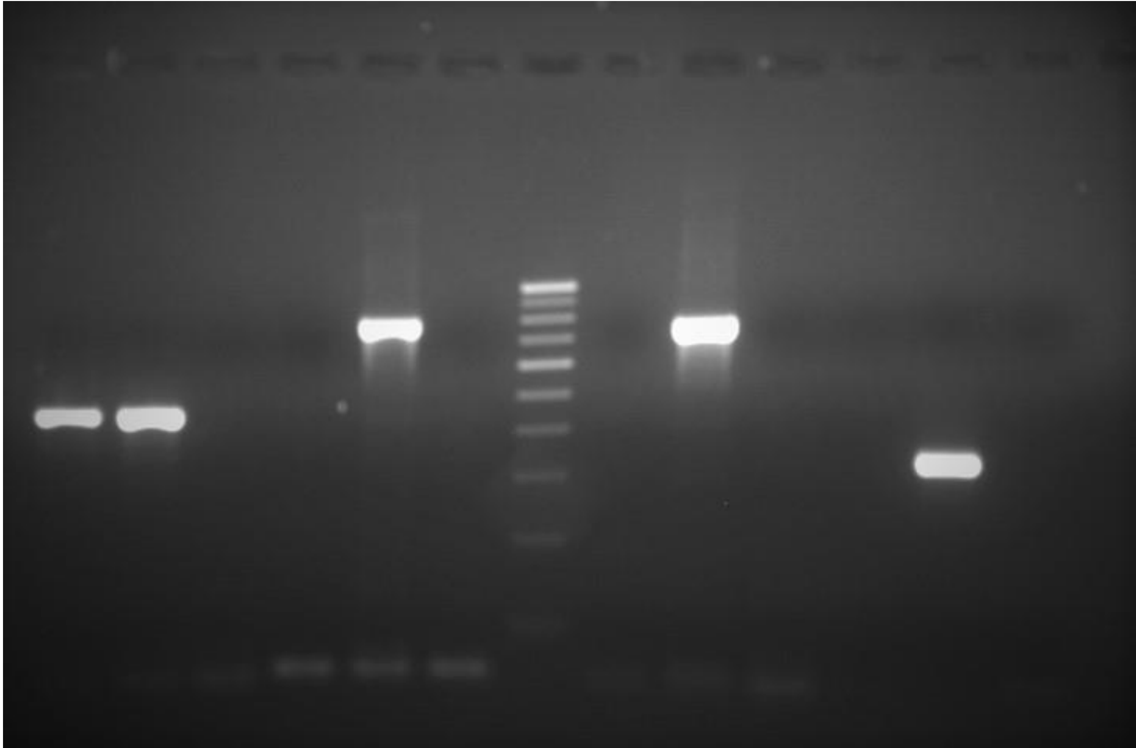

Figure no 6: Typing of BTV -Agarose gel electrophoresis of RT-PCR products

lane1: BTV 4 PCR product

lane 2: BTV 4 positive control 445 BP

lane3: BTV 4 negative control

lane 4: BTV 10 PCR product

lane5: BTV 10 positive control 800 BP

lane6: BTV2 10 negative control

lane7: 100 BP ladder

lane8: BTV 12 PCR product

lane9: BTV 12 positive control 750BP

lane10: BTV12 negative control

lane11: BTV 24 PCR product

lane12: BTV 24 positive control 319 BP

lane13: BTV 24 negative control

MOLECULAR TYPING OF BTV-16

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

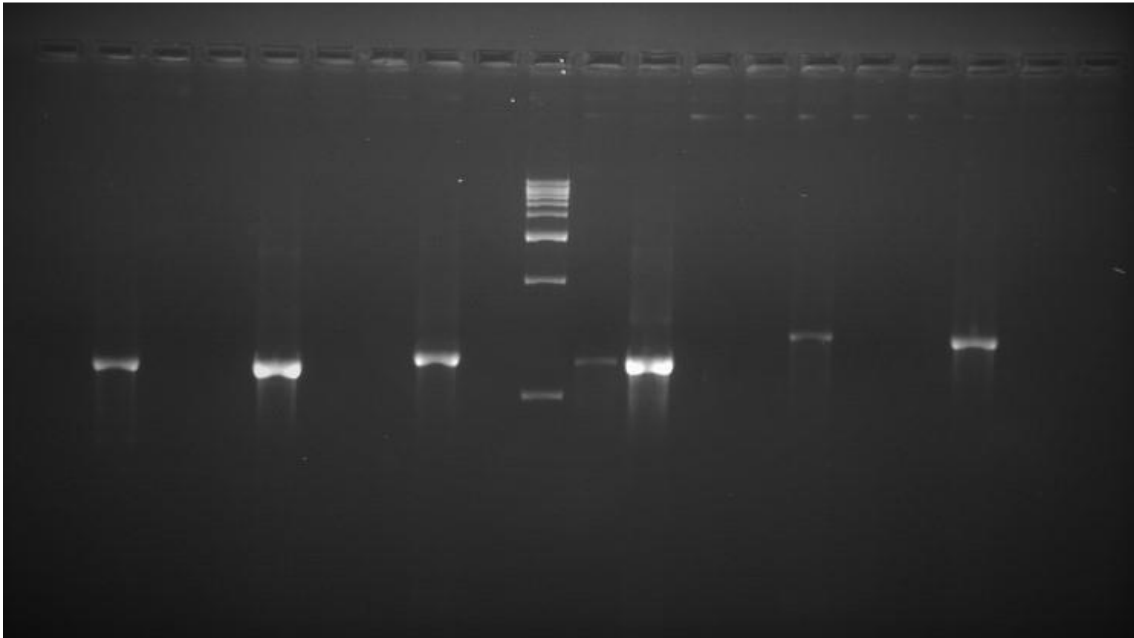

Figure no 7: Typing of BTV -Agarose gel electrophoresis of RT-PCR products

lane1: BTV 1 PCR product

lane 2: BTV 1possitive control 1179 BP

lane3: BTV 1 negative control

lane 4: BTV 2E PCR product

lane5: BTV2E positive control 1172 BP

lane6: BTV2E negative control

lane7: BTV 9 PCR product

lane8: BTV 9 positive control 1200BP

lane9: BTV 9 negative control

lane10: 1KB ladder

lane11: BTV 16 PCR product

lane12: BTV 16 positive control 1200BP

lane13: BTV 16 negative control

lane14: BTV 21 PCR product

lane15: BTV21 positive control 1388 BP

lane16: BTV21 negative control

lane17: BTV23 PCR product

lane18: BTV 23 positive control 1370BP

lane19: BTV23 negative control

lane20: empty

4.4 TITRATION OF VIRUS

Tissue culture infective dose was determined for both BTV-16 and 4 serotypes in Baby Hamster Kidney - 21 (BHK-21) cells. The $TCID_{50}$ of BTV-16 was determined to be $\log_{10}$ 6.66 $TCID_{50\ Per}$ ml and that of BTV-4 to be $\log_{10}$ 6.23 $TCID_{50\ Per}$ ml.

4.5 RESPONSES TO BTV INOCULATION IN SHEEP

A total of Twenty Deccani breed sheep of age 6-8 months were screened with c-ELISA for the presence of BTV. Five sheep which were found to be sero-negative were recruited for the study. On day 0, four animals were infected with BTV-16 serotype ($\log_{10}$ 6.66 $TCID_{50}$ per ml; intradermal) and one animal was injected with uninfected KC cells (control).After then on 31st day animals were super infected with BTV-4 serotype ($\log_{10}$ 6.23 $TCID_{50}$/ml; intradermal).The animals were monitored for 60 days during which the clinical signs, temperatures, haematology and sero-biochemical parameters were studied.

4.5.1 Clinical signs

No clinical signs except pyrexia were observed on primary infection with BTV-16 and super infection with BTV-4 (Figure No 8 and 9).

4.5.2 Daily Body Temperature

The body temperatures of each animal were recorded every day for sixty days after inoculation of virus. All sheep showed raise in rectal temperatures 5-6 dpi in BTV-16 infection ranging 104.6 - 105.4ºF and 4-8 dpi in BTV-4 super infection ranging 103.8-104.9ºF whereas the control animals exhibited no pyrexia. The gradual changes in temperature were showed in graphical presentation for individual animals in (Figure no 10&11) and peak temperatures of all infected animals were shown in Table No 2.

Table No 2: Peak temperatures of all the animals with respective days of post infection

Sheep no	BTV-16		BTV-4(super infection)	
	Dpi	Temperature ºF	Dpsi	Temperature ºF
209	5	105.4	4	104.4
214	6	105.2	5	104.5
215	6	105.2	9	104.9
244	5	104.6	8	103.8

Dpi- days post infection dpsi- days post super infection

4.5.3 Haematological parameters

Primary Infection

Infection with BTV-16 (15.57±1.20 thousand/cc) significantly (P<0.005) increased TLC in affected animals compared to control (12.07±1.19 thousand/cc). However, TEC of infected animals (9.42±0.72 million/cc) was significantly (P<0.005) lower than control (11.11±1.19 million/cc). Similarly, haemoglobin concentration in BTV-infected animals (8.51±0.75) significantly (P<0.005) declined than control animal (9.39±0.77 g %).

Super infection

The TLC in super infected animals (16.43±1.31 thousand/cc) remained significantly (P<0.005) higher than control animal (11.81±0.92 thousand/cc). A significant (P<0.005) decrease in TEC was observed in BTV-4 infected animals (10.17±0.54 million/cc) compared to control (12.09±1.17 thousand/cc). Similarly, the haemoglobin concentration in super infected animals (8.95±0.43 g %) was significantly (P0.005) lower than control (10.13±0.77 g %) (Figure no 12,13&14).

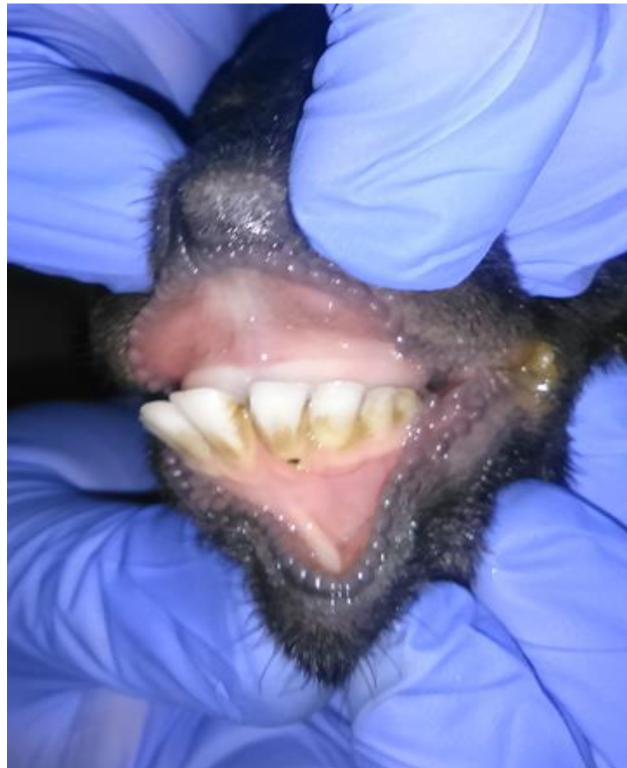

Figure No 8 : BTV-16 infected sheep without any oral lesions

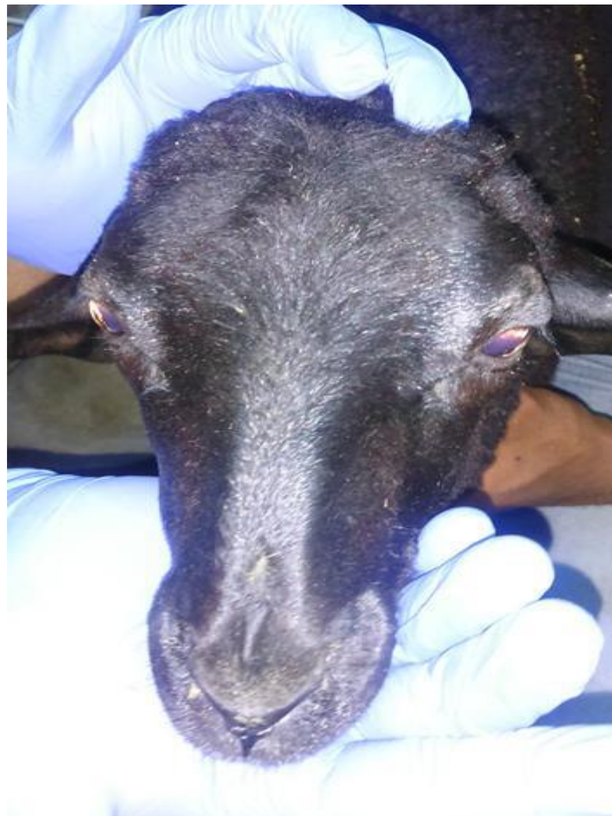

Figure No 9: BTV-4 super infected sheep without nasal discharges

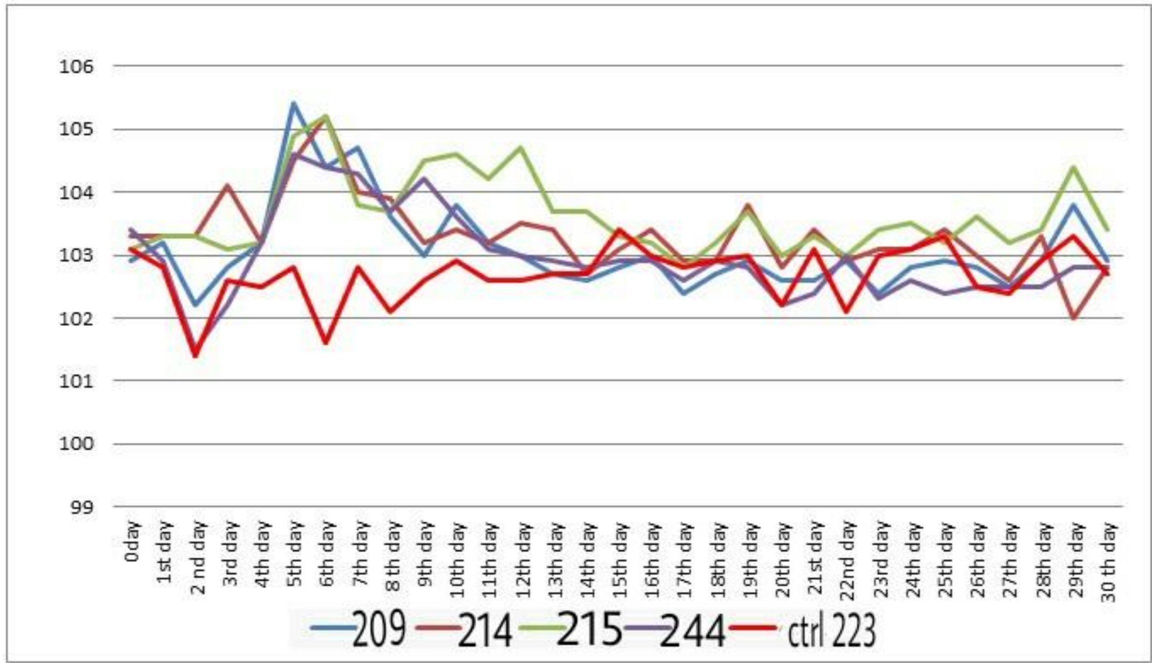

Figure No 10: Graph depicting daily temperatures of infected sheep primary infected with BTV-16.

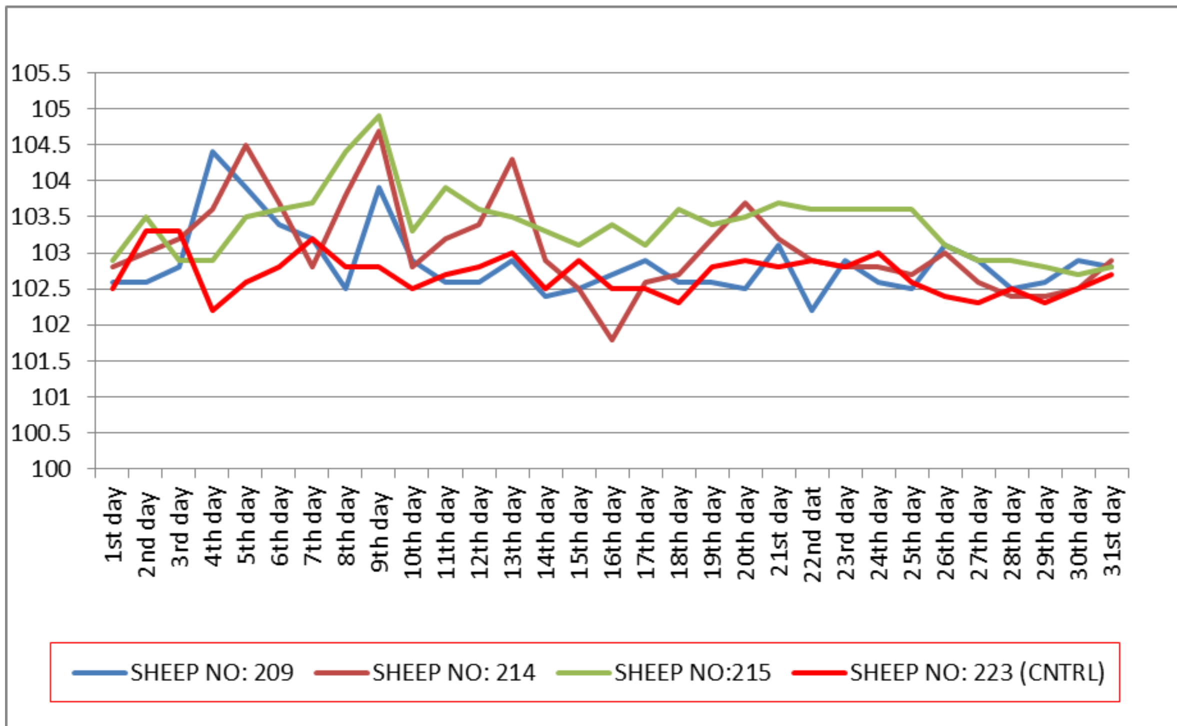

Figure No 11: Graph depicting daily temperatures of sheep super infected with BTV-4.

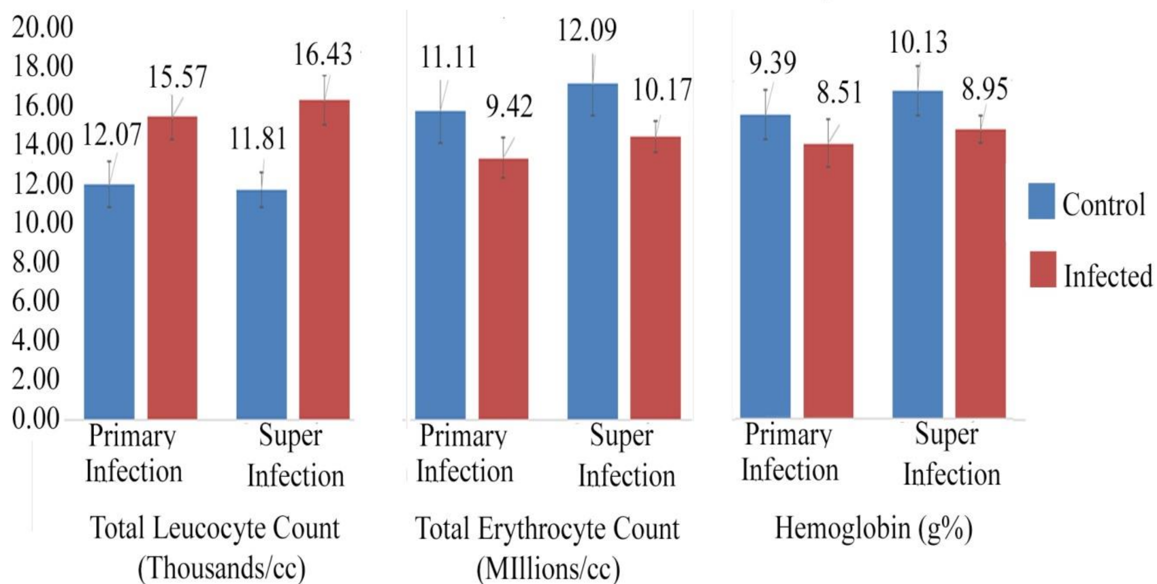

Figure No 12: Comparison of haematological parameters during primary and super infected animals

During primary Infection with BTV-16 (15.57±1.20 thousand/cc) significantly ($P<0.005$) increased TLC in affected animals compared to control (12.07±1.19 thousand/cc). However, TEC of infected animals (9.42±0.72 million/cc) was significantly ($P<0.005$) lower than control (11.11±1.19 millions/cc). Similarly, haemoglobin concentration in BTV-infected animals (8.51±0.75) significantly ($P=0.004$) declined than control animal (9.39±0.77 g%).

The TLC in super infected animals (16.43±1.31 thousand/cc) remained significantly ($P<0.005$) higher than control animal (11.81±0.92 thousand/cc). A significant ($P<0.005$) decrease in TEC was observed in BTV-4 infected animals (10.17±0.54 million/cc) compared to control (12.09±1.17 thousand/cc). Similarly, the haemoglobin concentration in super infected animals (8.95±0.43 g%) was significantly ($P<0.005$) lower than control (10.13±0.77 g%).

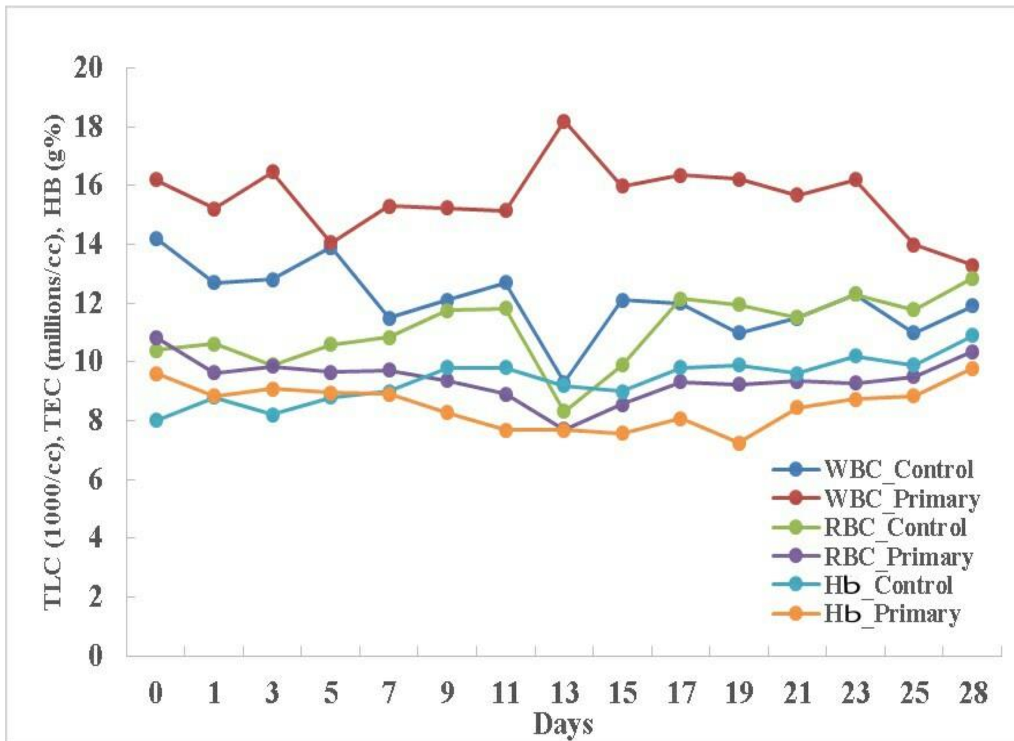

Figure No 13: Graphical representation of haematology in Deccani sheep during primary infection with the BTV -16

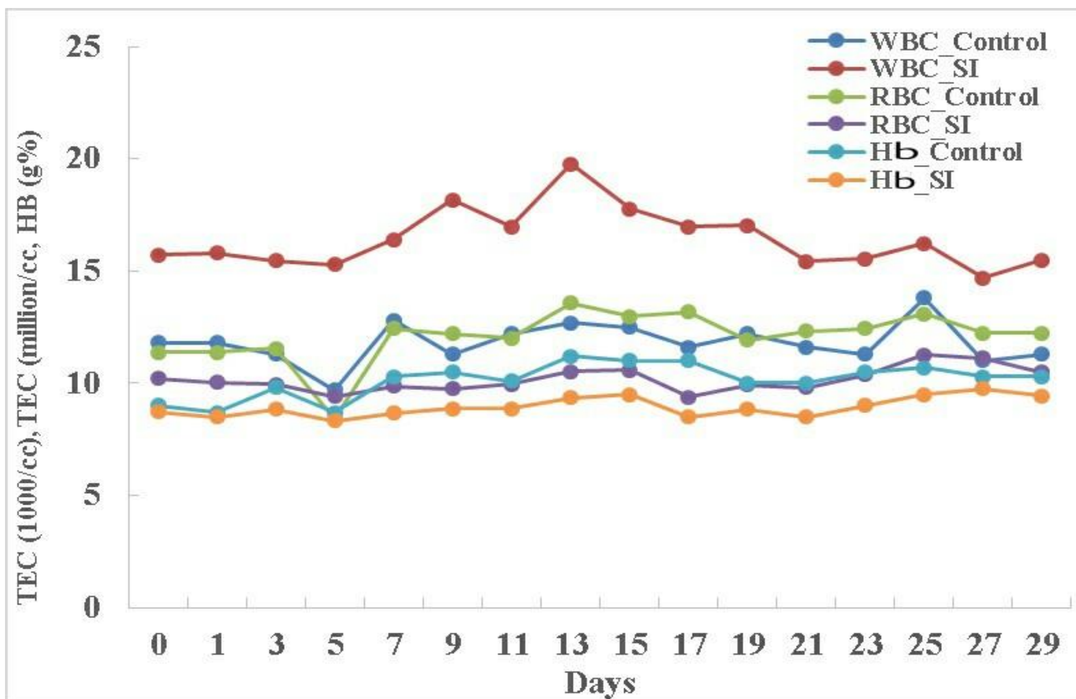

Figure No 14: Graphical representation of haematology in Deccani sheep during primary infection with the BTV -4

4.5.4 Immune response

4.5.4.1 c-ELISA

c-ELISA was used to monitor non-neutralizing group specific antibodies. Animals showing more than 50 percent inhibition (PI) were considered as positive for BTV antibody as per protocol. The mean serum antibody titres (% inhibition) against BTV estimated using c-ELISA are presented in Table No3. The antibody titters PI (>50%) was attained by BTV-16 infected animals by day 7 (67.07±14.32). The antibody titters from day 11 of primary infection (91.95±12.41) to day 28 of primary infection (96.61±15.92) remained significantly higher than day 7. (figure No 15

Table No 3: c-ELISA PERCENTAGE INHIBITION VALUES FOR BTV-16 AND BTV- 4 SUPER INFECTION IN SHEEP

Dpi	Sheep tag no				
	209	214	215	244	223
0	20.06861	27.27273	24.87136	34.64837	35.84906
3	35.16295	46.3122	22.29846	32.07547	31.21784
7	62.6072	84.21955	71.22813	50.22813	33.6346
11	81.13208	109.777	89.57993	87.30703	37.90738
17	89.7084	110.4631	106.6895	82.67581	33.96226
23	78.7307	110.2916	109.777	87.65009	31.38937
28	81.6623	110.3578	104.7865	89.4585	31.43784
15dpsi	99.87437	99.74874	100.8794	Sacrificed	19.72362
29dpsi	96.23116	98.87437	99.74874	Sacrificed	27.88945

The values in the table indicate the % inhibition. Above 50% inhibition considered as positive for BTV antibodies.

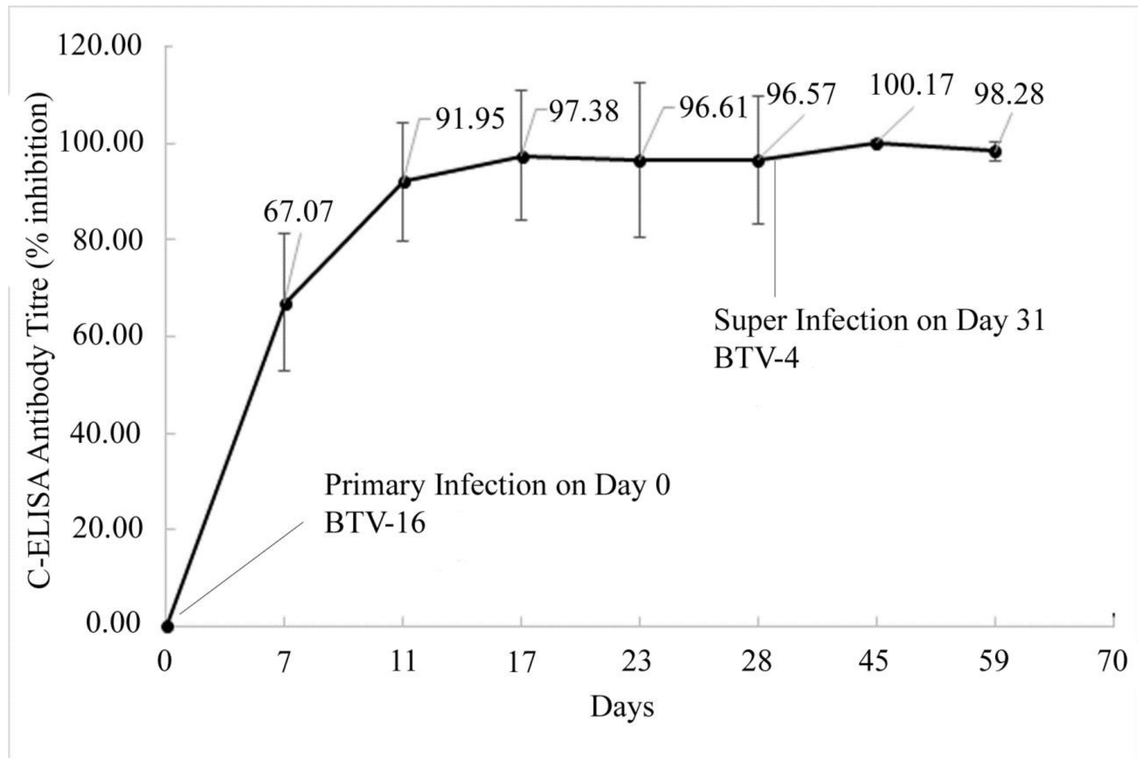

Figure No 15: Immune response to BTV-16 during primary infection and super infection by c-ELISA.

.

4.5.4.2 Serum Neutralization Test

SNT was carried out in BHK-21 cells to study the presence of type specific neutralizing antibodies to BTV serotype 16 for two months and BTV serotype 4 for second month Absence of CPE in infected wells of BHK-21 indicates the presence of neutralizing antibodies in the test serum.

In primary infection with BTV-16, the serum from infected animals could neutralize BTV-16 by 7dpi which continued up to 28 dpi and 59dpi. In super infected animals with BTV-4, the serum could neutralize BTV-4 by 7th day of the super infection in one sheep out of three animals and in the remaining two animals by 15dpsi and which continued up to 29 dpsi. The control sheep did not show any neutralizing antibodies to either BTV-16 or BTV-4 (Table No 4).

Table No 4: SERO CONVERSION RESULTS BY SNT FOR BTV16 INFECTION AND BTV4 SUPER INFECTION IN SHEEP

Dpi	Serotype	Sheep tag no				
		209	214	215	244	223 (control)
7	BTV 16	+	+	+	+	-
15	BTV 16	+	+	+	+	-
21	BTV 16	+	+	+	+	-
28	BTV 16	+	+	+	+	-

BTV -4 Super infection

D psi	Serotype	209	214	215	223 Control
7	BTV 16	+	+	+	-
	BTV 4	-	+	+	-
15	BTV 16	+	+	+	-
	BTV 4	+	+	+	-
21	BTV 16	+	+	+	-
	BTV 4	+	+	+	-
29	BTV 16	+	+	+	-
	BTV 4	+	+	+	-

Dpi- days of post inoculation; dpsi -days of post-super infection

(+) –Presence of neutralizing antibodies

(-) – Absence of neutralizing antibodies

CHAPTER V

DISCUSSION

Bluetongue virus (BTV) is a segmented double-stranded RNA (dsRNA) virus of the Orbivirus genus of the Reoviridae family. It infects both domestic and wild ruminants. Blood-feeding insects of the *Culicoides* genus act as a vector to transmit the virus between ruminant hosts. In this study, the effect of prior exposure to BTV-16 on subsequent BTV-4 infection was studied in Deccani sheep.

In this study, BTV- 4 and BTV- 16 were successfully adapted and grown in KC cell line, which is of insect origin from *Culicoides sonorensis* cells. BTV was also reported to be grown in mammalian cell lines such as BHK-21, CPAE or Vero cell lines (Wechsler and Holland, 1988; Mecham, 2006). The cytopathic effect of BTV can be observed only on mammalian cell lines at three to five days after inoculation appearing as foci of rounded and refractile cells (Clavijo *et al.,* 2000).

In this study, BTV were serotyped using RT-PCR. Nucleic acid amplification technology is considered as the gold standard for the specific identification of microbial genomic targets in biological samples (Polci *et al.,* 2007). Real-time -PCR methods have already been developed for genotyping of BTV1, BTV2, BTV4, BTV6, BTV8, BTV9, BTV11 and BTV16 (Hoffman *et al.,* 2009; Mann *et al.,* 2012). Earlier works also demonstrated that a direct identification of BTV was possible with RT-PCR that allows for serotyping (MacLachlan *et al.,* 1994).

The concentration of BTV in the inoculum is an important factor in experimental induction of BTV in animals. In this study 0.25ml ($TCID_{50}$ of 6.66 Log10 cells mL^{-1}) of BTV-16 and 0.6ml (6.23 Log10 cells mL^{-1}) of BTV- 4 was used to induce primary and super

infections in sheep. Earlier, it was demonstrated that BT can be induced in sheep using titre as low as $10^{1.4}$ $TCID_{50}$ (Van Gennip *et al.,* 2012). In one study, Eschambauer *et al.,* (2010) infected sheep with BTV -8 using a $TCID_{50}$ of 2×10^4 and 5×10^5 cells/mL to experimentally induce viremia. In another study, Darpel *et al.,* (2016) used a $TCID_{50}$ of 10^7 cells /mL for infection of sheep with BTV.

The establishment of viremia in BTV inoculated animals is evident by an increase in body temperature above 104°F or 40°C (Baylis, 2008). In this study all sheep showed raise in rectal temperatures 5-6 dpi in BTV-16 infection ranging 104.6 - 105.4ºF; and 4-8 dpi in BTV-4 super infection ranging 103.8-104.9ºF; whereas the control animals exhibited no pyrexia. The rectal temperature results were similar to that of Darpel *et al*., (2007), Backx *et al.,* (2007), MacLachlan *et al.,* (2008), Umeshappa *et al.,* (2011) and Batten *et al.,* (2012). In this study no other clinical signs were observed on primary infection and super infection with BTV-16 and BTV-4, respectively. However, in a related study done in the department, sheep were experimentally infected with BTV-4 and prominent clinical signs like salivation, hyperaemia and excoriation of lips and nasal discharges were noticed. However, on super infection with BTV-16 apparent clinical signs were not observed. This observation needs further scrutiny since no cross reactivity has been reported between BTV-16 and BTV-4 so far. Saegerman *et al*., (2008) reported that BTV serotypes 1, 2, 3, 4, 6, and 10 have a high pathogenic index and high epidemic potential.

Experimental inoculation of sheep with BTV is generally reported to result in mild clinical signs (Bonneau *et al*., 2002; DeMaula *et al*., 2002; Verwoerd and Erasmus, 2004). In the current study upon infection with BTV-16 no clinical signs were observed except moderate pyrexia. In addition, super infection with virulent BTV-4 also exhibited no prominent clinical signs. This may be because of unusual cross reactivity between these two

isolates (Abdul Muzeer Shaik, 2016). Further, viraemia in infected animals has a prolonged course, but is not persistent (Barratt-Boyes and MacLachlan, 1994; Bonneau *et al.,* 2002). Its duration depends on the longevity of erythrocytes (MacLachlan and Garg, 2009), species and breed of the infected animal. Viraemia lasts 14 to 54 days in sheep and 19 to 54 days in goats (Luedke and Anakwenze, 1972; Koumbati *et al*., 1999).

Haematology of BTV-16 infected sheep revealed leucocytosis. Erythrocyte and haemoglobin concentration decreased significantly compared to control. While, during BTV-4 super infection, leucocytosis was observed with significant decrease in erythrocyte count. In early viraemia, BTV is associated with all blood elements, while at later stages of viraemia it exclusively associates with erythrocytes (MacLachlan *et al*., 2009; MacLachlan, 2004). Virus particles appear to be sequestered in invaginations of the erythrocyte membrane (Brewer and MacLachlan, 1994; MacLachlan, 2004), allowing prolonged viraemia even in the presence of neutralizing antibodies (Richards *et al*., 1988; Brewer and MacLachlan, 1994). The findings in haematology corroborate with Mellor *et al*., (2009) who reported that during BTV infection, initially there is leucopoenia, mainly lymphopaenia followed by leucocytosis. McConnel *et al.,* (1983) reported a transient leucopoenia (44% reduction) at 8 days post-challenge with virulent BTV 10, 11, 13 and 17. McColl and Gould (1994) also found similar changes in peripheral blood leukocyte concentrations of naïve sheep infected with virulent BTV 23.

The antibody titres in experimentally inoculated animals were determined by c-ELISA. It was observed that antibody could be detected against BTV-16 by day 7pi and persisted throughout the experimental period. Serogroup-specific antibodies against BTV can be detected by a competitive ELISA test targeted to the VP7 protein, which is a rapid method permitting determination of serum or plasma antibody as early as the 6th post-infection day

(Koumbati *et al*., 1999). In addition, serogroup-specific antibodies can be identified by an agar-gel immune-diffusion test, which, however, may produce cross-reactions with other orbiviruses (Afshar *et al*., 1989). Similarly, serum neutralisation test has been reported to have highest specificity and sensitivity of all the tests although being expensive and time-consuming (Reddington *et al*., 1991).

Serotype specific neutralizing antibodies to BTV -16 after primary infection detected from 7dpi, whereas after super infection with BTV-4 were detected from 7 dpsi in one (animal tag no 209) sheep out of three and in the other two by 15dpsi. Jeggo *et al*., (1983) observed similar results with neutralizing antibodies against BTV-5 on 15 dpi. Batten *et al.,* (2012) observed similar results with complete absence of development of neutralizing antibodies in one sheep out of six sheep infected with BTV-26. Martinella *et al*., (2016) reported that BTV-1 super infection in calves that were experimentally infected with BTV-8 showed low titres of neutralizing antibodies to BTV-1 by SNT. Though in the current study titration was not done in SNT, the time taken for onset of neutralizing antibodies for BTV- 4 super infection was 7 days in one sheep (animal tag no 209) and 15 days for remaining two sheep (animal tag no 214, 215) when compared to 7 days in BTV-16 primary infection.

According to the findings of Maan *et al.,* (2007), the BTV serotypes 4 and 16 are distantly related (figure no 16). In this study, it was observed that primary infection with BTV-16 likely conferred resistance against BTV- 4 serotype. This could be a result of cross reactive antibodies induced by BTV – 16 which protected against BTV - 4. Similar observation were made in earlier studies by Jeggo *et al.,* (1983) who reported that animals infected with BTV-4 or BTV-6 showed a transient heterotypic neutralising antibody response against number of types. Schwartz *et al*., (2008) also reported that BTV infection produces long lasting antibody titres with limited cross protection against heterologous serotypes

(Schwartz *et al*., 2008). This phenomenon of cross protection could be explained by the sequence identity of amino acids (70-78%) of VP2 and nucleotide sequence (67-71%) of Seg-2 of various BTV serotypes (Maan *et al*., 2007). Hence, further studies at serological and genotypic level are required to elucidate this phenomenon of cross reactivity.

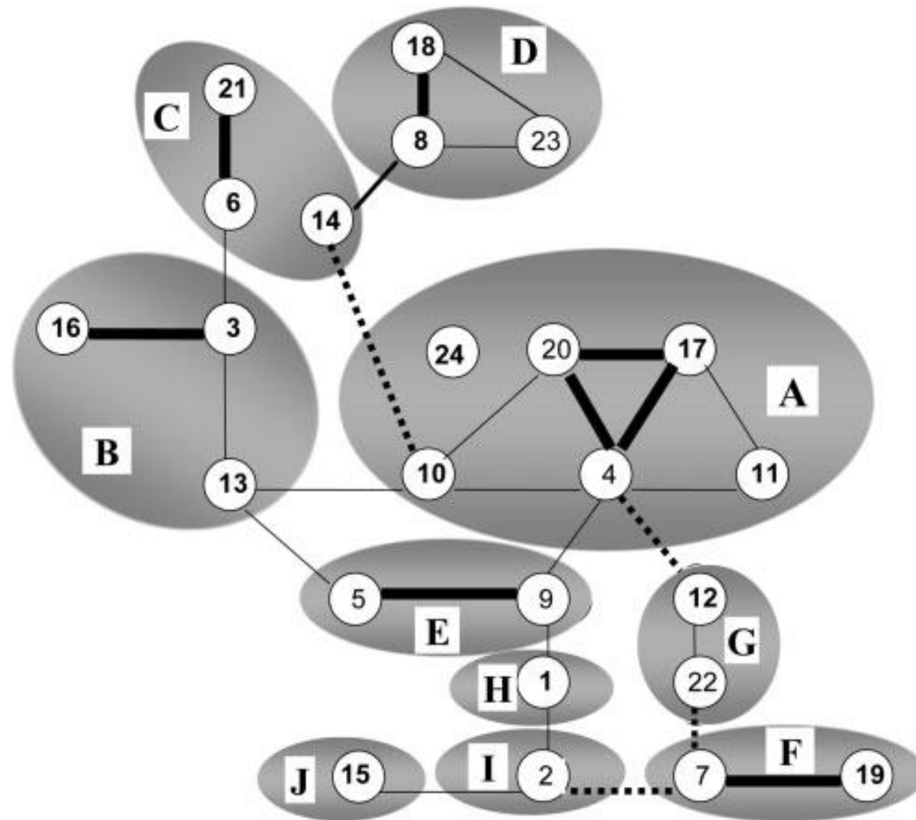

Figure no 16: Serological relationships between BTV serotypes

The thicker lines shown between serotypes indicate stronger serological relationships, as detected in plaque-reduction assays. The thinner lines represent relationships that are only evident as cross- or heterotypic antibody responses in cross-protection assays. The black dotted lines represent interrelationships that are very weak.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The effect of prior exposure to less pathogenic BTV-16 on subsequent super infection with pathogenic BTV-4 was studied in Deccani sheep. The results of the study are summarized below

BTV- 4 and BTV- 16 were successfully adapted to grow in KC cell line, isolated and characterized by RT-PCR.

Tissue culture infective dose was determined for both BTV-16 and 4 serotypes in Baby Hamster Kidney - 21 (BHK-21) cells. The $TCID_{50}$ of BTV-16 was determined to be $\log_{10}$ 6.66 $TCID_{50}$/ ml and that of BTV-4 to be $\log_{10}$ 6.23 $TCID_{50}$/ ml. These doses were further used to infect experimental animals.

No clinical signs except pyrexia were observed on primary infection and super infection with BTV-16 and BTV-4, respectively.

The body temperatures of each animal were recorded every day for sixty days after inoculation of virus. All sheep showed raise in rectal temperatures 5-6 dpi in BTV-16 infection ranging 104.6 - 105.4ºF and 4-8 dpi in BTV-4 super infection ranging 103.8-104.9ºF whereas the control animals exhibited no pyrexia.

In both primary and super infection, TLC was significantly ($P<0.05$) increased while TEC and HB were significantly ($P<0.05$) decreased compared to control.

The serum antibody titres (% inhibition) showed a protective antibody (>50%inhibition) titre against BTV-16 by day 7 (67.07±14.32) and increased significantly ($P<0.05$) from day 11 (91.95±12.41) to day 28 (96.61±15.92)post infection.

In primary infection, type specific neutralization against BTV-16 virus was observed by day 7pi and continued up to day 28. Similarly, in super infection, it was observed by day 7 in one sheep and in two more animals by day 15 which continued up to day 29 post-super infection.

In conclusion further studies on serological cross reactivity between BTV- 4 and 16 are warranted.

LITERATURE CITED

Abdul Muzeer Shaik 2016 Pathogenicity studies of Bluetongue virus serotype 4 in sheep.Thesis submitted to PV Narasimha Rao Veterinary University, Hyderabad, Telangana.

Afshar A 1994 Bluetongue: Laboratory diagnosis. Journal of Comaparitive Immunology, Microbiology and Infectious Diseases, Vol.17: 221-241.

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.

Anderson J, Merten P P C and Herniman K A J 1993 A competitive ELISA for the detection of anti-tubule antibodies using a monoclonal antibody against bluetongue virus non- structural protein NSI. Journal of virological methods 43:167-176.

Anthony S, Jones H, Darpel K E, Elliott H, Maan S, Samuel A and Mertens P P C 2007 A duplex RT-PCR assay for detection of genome segment 7 (VP7 gene) from 24 BTV serotypes. Journal of virological methods 141:188-197.

Backx A, Heutink C G, Van Rooij E M and Van Rijn P A 2007 Clinical signs of bluetongue virus serotype 8 infection in sheep and goats. Veterinary Record, 161(17), 591.

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 and Mallick B B 1983 Serological prevalence of bluetongue antibodies in India. Indian Journal of Animal Science 53: 1355-1356.

Barbara S Drolet, Lindsey M Reister, Tara D Rigg, auline Nol, Brendan K Podell, James O Mecham, Kurt C VerCauteren, Piet A van Rinj, William C Wilson and Richard A Bowen 2013 Experimental infection of white-tailed deer (*Odocoileus virginianus*) with Northern European bluetongue virus serotype 8. Veterinary Microbiology 166: 347-355.

Barratt-Boyes S M and MacLachlan N J 1994 Dynamics of viral spread in bluetongue virus infected calves. Veterinary Microbiology 40, 361-371.

Batten C A, Henstock M R, Bin-Tarif A, Steedman H M, Waddington S, Edwards L, and Oura C A L 2012 Bluetongue virus serotype 26: Infection kinetics and pathogenesis in Dorset Poll sheep. Veterinary microbiology 157, no. 1: 119-124.

Batten C A, Henstock M R, Steedman H M, Waddington S, Edwards L and Oura C A L 2013 Bluetongue virus serotype 26: infection kinetics, pathogenesis and possible contact transmission in goats. Veterinary microbiology 162, no. 1: 62-67.

Baylis M, O'Connell L and Mellor P S 2008 Rates of bluetongue virus transmission between Culicoides sonorensis and sheep. Medical and Veterinary Entomology 22, 228-237.

Bhambani B D and Singh P P 1968 Bluetongue disease in India. Indian Veterinary Journal 45: 370-371.

Billinis C, Koumbati M, Spyrou V, Nomikou K, Mangana O, Panagiotidis C A and Papadopoulos O 2001 Bluetongue virus diagnosis of clinical cases by a duplex reverse transcription-PCR : a comparison with conventional methods. Journal of Virology Methods 98:77- 89.

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 Virology 155(12):2041-2046.

Biswas S K, Chand K, Rehman W, Reddy Y N, Mondal B 2015 Segment-2 sequencing and cross-neutralization studies confirm existence of a neutralization resistant VP2 phenotypic variant of bluetongue virus serotype 1 in India. Veterinary Microbiology 176: 358-364.

Bitew M, Nandi S, Ravishankar C, Somvanshi R 2013 Serological and molecular evidence of bluetongue in sheep and goats in Uttar Pradesh India. African Journal of Biotechnology 12(19):2699-2705.

Bonneau K R, DeMaula C D, Mullens B A and MacLachlan N J 2002 Duration of viraemia infectious to *Culicoides sonorensis* in bluetongue virus-infected cattle and sheep. Veterinary Microbiology 88, 115-125.

Bowne J G and Ritchie A E 1970 Some morphological features of bluetongue virus. Virology 40: 903-911.

Bréard E, Sailleau C, Coupier H, Mure-Ravaud K, Hammoumi S, Gicquel B, Hamblin C, Dubourget P, 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 2003, Nov-Dec; 34(6): 777-89. PubMed PMID: 14746772.

Breckon R D, Luedke A J and Walton TE 1980 Bluetongue virus in bovine semen: viral isolation. American Journal of Veterinary Research 41: 439-442.

Brewer A W and MacLachlan N J 1994 The pathogenesis of bluetongue virus infection of bovine blood cells in vitro: ultrastructural characterization. Archives of virology, 136(3-4), 287-298.

Chand K, Biswas S K, De A, Sing B, Mondal B 2009 A polyclonal antibody-based sandwich ELISA for the detection of bluetongue virus in cell culture and blood of sheep infected experimentally. Journal of Virology Methods 160: 189-192.

Chaing B L 1989 An outbreak of bluetongue in imported sheep. Proceedings of First Congress Veterinary Association Malaysia pp.49-51.

Chander S, Prasad G and Jain N C 1990 Clinical, haematological and immunological studies on sheep experimentally inoculated with 2 strains of bluetongue virus type 1. Indian Journal of Virology 6, no. 1/2: 23-31.

Chauhan H C, Kher H N, Chandel B S, Dadawala A I, Jain L, Agrawal S M and Bhadaniya A 2009 Evaluation of Group specific Nested PCR for detection of Bluetongue virus. Vet World 2: 179-82.

Clavijo A, Heckert R A, Dulac G C and Afshar A 2000 Isolation and identification of bluetongue virus. Journal of virological methods, 87(1), 13-23.

Dadhich H 2004 Bluetongue An overview of recent trends in diagnostics. Veterinaria Italiana 40:564-566.

Dahia S, Prasad G, Minakshi and Ramesh C Kovi 2004 Typing of bluetongue virus serotype 1 and 23 By RT-PCR. Indian Journal of Biotechnology Vol 4 : 373-377.

Daniels P W, Sendow I, Pritchardl I, Sukarsih and Eaton B T 2004 Regional overview of bluetongue viruses.

Darpel K E, Batten C A, Veronesi E, Shaw A E, Anthony S, Bachanek-Bankowska K, Kgosana L, Bin-Tarif A, Carpenter S, Muller-Doblies U U, Takamatsu H H, Mellor P S, Mertens P P C and Oura C A L 2007 Clinical signs and pathology shown by British sheep and cattle infected

with bluetongue virus serotype 8 derived from the 2006 outbreak in northern Europe. Veterinary Record 161: 253–261.

Darpel, James Barber, Andrew Hope, Anthony J. Wilson,Simon Gubbins, Mark Henstock, Lorraine Frost, Carrie Batten, Eva Veronesi, Katy Moffat,1 Simon Carpenter, Chris Oura, Philip S. Mellor, and Peter P. C. Mertens. 2016 Using shared needles for subcutaneous inoculation can transmit bluetongue virus mechanically between ruminant hosts. Sci Rep. 2016; 6: 20627.

Della-porta A J, Parsonson I M and McPhee D A 1985 Problems in the interpretation of diagnostic tests due to cross-reactions between orbiviruses and broad serological responses in animals. Bluetongue and related orbiviruses ed. Barber T L 445-453.

DeMaula C D, Leutenegger C M, Bonneau K R and MacLachlan N J 2002 The role of endothelial cell-derived inflammatory and vasoactive mediators in the pathogenesis of bluetongue. Virology 296, 330-337.

Dercksen D, Groot Nibbelink N, Paauwe R, Backx A, van Rijn P and Vellema P 2007 First outbreak of bluetongue in goats in The Netherlands (in Dutch). Tijdschrift Diergeneeskunde 132: 786–790.

Dubay S A, Rosenstock S S, Stallknecht D E and deVos Jr J C 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(1), 159-163.

Du Toit R M 1944 The transmission of bluetongue and African horse sickness by Culicodes. Onderstepoort journal of veterinary science and animal industries 19:7-16.

Du Toit R M 1962 The role played by bovines in transmission of bluetongue in sheep. Journal of South Africa veterinary medical Association 33:483-490.

Dyce A L 1982 In Arbovirus Research in Australia. Proceedings of the third symposium, edited by T D St. George, B H Kay, CSIRO and QIMR, Brisbane 35.

Dyce A L 1989 In Arbovirus Research in Australia Proceedings of fifth symposium edited by M F Uren, J Block, L H Manderson, CSIRO and QIMR, Brisbane 135.

Eaton B T Hyatt A D and White J R 1987 Association of bluetongue virus with the cytoskeleton. Virology 157: 10.

Elbers A R W, Backx A, Meroc E, Gerbier G, Staubach C, Hendrickx G, van der Spek A and Mintiens K 2008 Field observations during the bluetongue serotype 8 epidemic in 2006 I. Detection of first outbreaks and clinical signs in sheep and cattle in Belgium, France and the Netherlands. Preventive Veterinary Medicine 87: 21–30.

Elbers A R W, van der Spek A N and van Rijn P A 2009 Epidemiologic characteristics of bluetongue virus serotype 8 laboratory-confirmed outbreaks in The Netherlands in 2007 and a comparison with the situation in 2006. Preventive Veterinary Medicine 92: 1–8.

Erasmus B J 1975a Bluetongue in sheep and goats. Australian Veterinary Journal 51: 165-170.

Erasmus B J 1975b The control of bluetongue in and enzootic situation. Australian Veterinary Journal 51: 209-210.

Erasmus B J 1985 The history of bluetongue. Progress in clinical and Biological Research, 178:7-12.

Eschbaumer M, Hoffmann B, Moss A, Savini G, Leone A, Konig P, Zemke J, Conraths F and Beer M 2010 Emergence of bluetongue virus serotype 6 in Europe—German field data and experimental infection of cattle. Vet Microbiol 143, 189-195.

Evermann J F 2008 Accidental introduction of viruses into companion animals by commercial vaccines. Veterinary Clinics of North America-Small Animal Practise 38: 919–929.

FAO-OIE-WHO 1960 Animal Health Year Book. Food and Agricultural Organisation, Rome, Italy.

Foster N M, Luedke A J and Metcalf H E 1972 Bluetongue in sheep and cattle: efficacy of embryonating chicken eggs in viral isolations. American Journal of Veterinary Research 33: 77-81.

Foster N M and Jones R H 1979 Multiplication rate of bluetongue virus in the vector culicoides varripennis (Dipetra: ceratopogonidae) infected orally. Journal of Medical Entomology 15: 302-303.

Garcia-Sastre, A., 2011. 2 methylate or not 2 methylate: viral evasion of the type I interferon response. Nat Immunol 12, 114-115.

Gard G P 1987 Studies of bluetongue virulence and pathogenesis in sheep. Technician Bulletin No. 103 Department of Industries and Development, Australia.

Gibbs E P J 1992 Epidemiology of orbiviruses-blue-tongue: Towards 2000 and the search for patterns. Bluetongue, African Horsesickness, and Related Orboviruses, 65-75.

Goldsmit L, Barzilai E and Tadmor A 1975 The comparative sensitivity of sheep and chicken embryos to bluetongue virus and observations on viremia in experimentally infected sheep. Australian Veterinary Journal 51: 190-196.

Gustafson G A and Pearson J E 1992 Evaluation of the bluetongue competitive enzyme linked immunosorbent assay. In bluetongue, African horse sickness and related Orbiviruses- Proceeding of the second International symposium.

Henning M W 1949 Animal diseases in South Africa. *Animal diseases in South Africa*, (2nd Edition).

Hofmann M A, Renzullo S, Worwa M, G Thuer B 2008 Genetic characterization of Toggenburgorbivirus, submitted to MAFSU, Nagpur, India. A new bluetongue virus, from goats, Switzerland. Emerging and Infectious Diseases. 14: 1855-1861.

Hofmann M A, Renzullo S, Mader M, Chaignat V and Wowa G 2008 Genetic characterization of Toggenburg orbivirus a new bluetongue virus from goats Switzerland. Emerging Infectious Diseases 12: 1855-1861.

Hofmann M A, Renzullo S, Mader M, Chaignat V, Worwa G and Thuer B 2008 Genetic characterization of toggenburg orbivirus, a new bluetongue virus, from goats, Switzerland. Emerging Infectious Diseases, 14(12), 1855-1861.

Hoffmann B, Beer M, Reid S M, Mertens P, Oura C A, Van Rijn P A, Slomka M J, Banks J, Brown I H, Alexander D J and King D P 2009 A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. Veterinary microbiology, 139(1), 1-23.

Huismans H, Van der Walt N T, Clote M and Erasmus B T 1987 Isolation of capsid protein of BTV that induces a protective immune response in sheep. Virology 157: 172-179.

Jain N C, Sharma R and Prasad G 1986 Isolation of bluetongue virus from sheep in India. Veterinary Record 119: 17-18.

Janakiraman D, Venugopalan A T, Ramaswamy V and Venkatesan R A 1991 Serodiagnostic evidence of prevalence of bluetongue virus serotypes among sheep and goats in Tamil Nadu. Indian Journal of Animal Science 61: 497-498.

Jayakar Johnson 2006 Sero epidemiology of bluetongue in Andhra Pradesh. M.V.Sc. Thesis submitted to Acharya N.G. Ranga Agricultural University.

Jenckel M, Bréard E, Schulz C, Sailleau C, Viarouge C, Hoffmann B and Zientara S 2015 Complete coding genome sequence of putative novel bluetongue virus serotype 27. Genome announcements 3: e00016-15.

Jeggo M H, Gumm I D and Taylor W P 1983 Clinical and serological response of sheep to serial challenge with different bluetongue virus types. Research in Veterinary Science 34, 205-211.

Johnson D J, Wilson W C and Paul P S 2000 Validation of a reverse transcriptase multiplex PCR test for the serotype determination of US isolates of bluetongue virus. Veterinary microbiology 76: 105-115.

Kamar D, Mehdi E, Chafiqa L and Ouafaa F F 2015 Bluetongue virus in Morocco from 2004-2012. Journal of Animal Health Production 3: 48-53.

Kirkland P D, Zhang N, Hawkes R A, Li Z, Zhang F, Davis R J, Sanders D A, Li H, Zhang K, Ben J, He G F, Hornitzky C L and Hunt N T 2002 Studies on the epidemiology of bluetongue virus in China. Epidemiology and Infection 128: 257–263.

Kirschvink N, Raes M and Saegerman C 2009 Impact of a natural bluetongue serotype 8 infection on semen quality of Belgian rams in 2007. Veterinary Journal 182: 244– 251.

Koumbati M, Mangana O, Nomikou K, Mellor P S and Papadopoulos O 1999 Duration of bluetongue viraemia and serological responses in experimentally infected European breeds of sheep and goats. Veterinary Microbiology 64, 277-285.

Krishna Mohan Reddy Y, Koteeswaran A and Roy P 2003 Advanced Molecular Techniques in the diagnosis of BT. Article came in www.indiaveterinarycommunity.com.

Lonkar P S ,Sharma M M, Sreenivastava C P, Subey S C, Maru A and Karla D B 1983.Epedemiology of Bluetongue in sheep at an organized farm in semiarid part of Rajasthan, India. Indian journal of comparative Microbiology Immunology and Infectious Diseases 6: 188-192.

Luedke A J, Bowne J G, Jochim M M and Doyle C 1964 Clinical and pathologic features of bluetongue in sheep. American Journal of Veterinary Research 25: 963-970.ttle by Culicoides varipennis. American Journal of Veterinary research. Vol.28:457-460.

Luedke A J, Jones R H and Jochim M M 1967 Transmission of Bluetongue between sheep and cattle by *Culicoides variipennis*. American journal of veterinary research, 28(123): 457-460.

Luedke A J 1969 Bluetongue in sheep: viral assay and viremia. American Journal of Veterinary Research 30: 499-509.

Luedke A J and Anakwenze E I 1972 Bluetongue virus in goats. American Journal of Veterinary Research 33, 1739-1745.

Maan S, Maan NS, Samuel AR, Rao S, Attoui H, Mertens PP 2007a Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. Journal of General Virology. 2007 Feb;88(Pt 2):621-30. PubMed PMID: 17251581.

Maan S, Rao S, Maan N S, Anthony S J, Attoui H, Samuel A R, Mertens P P C 2007b Rapid cDNA synthesis and sequencing techniques for the genetic study of bluetongue and other dsRNA viruses. Journal of Virological Methods, 143 (2), pp. 132-139.

Maan S, Maan N S, Van Rijn P A, Van Gennip R G, Sanders A, Wright I M, Batten C, Hoffmann B, Eschbaumer M, Oura C A L and Potgieter A C 2010 Full genome characterisation of bluetongue virus serotype 6 from the Netherlands 2008 and comparison to other field and vaccine strains. PloS one, 5(4), e10323.

Maan S, Maan N S, Nomikou K, Veronesi E, Bankowska B K, Manjunatha N, Attoui H and Mertens P P C 2011 Complete Genome characterization of a novel 26th Bluetongue virus serotype from Kuwait. PLoS One 6: e2614.

Maan S, Maan N S, Nomikou K, Batten C, Antony F, Belaganahalli M N, Samy A M, Reda A A, Al-Rashid S A, Batel M E, Oura C A L and Mertens P P C 2011 Novel bluetongue virus serotype from Kuwait. Emerging Infectious Diseases 17:886-889.

Maan N S, Maan S, Belaganahalli M N, Ostlund E N, Johnson D J, Nomikou K and Mertens PPC 2012 Identification and differentiation of the twenty six bluetongue virus serotypes by RTPCR amplification of the serotype-specific genome segment 2. PLoS ONE 7(2):e32601.

Martin S A and Zweerink H J 1972 Isolation and characterization of two types of bluetongue virus particles. Virology 50: 495 – 506.

MacLachlan N J, Jagels G, Rossitto P V, Moore P F and Heidner H W 1990 The pathogenesis of experimental bluetongue virus infection of calves. Veterinary Pathology 27,223-229.

MacLachlan N J 2004 Bluetongue: pathogenesis and duration of viraemia. Veterinaria Italiana 40, 462-467.

Maclachlan N J, Crafford J E, Vernau W, Gardner I A, Goddard A, Guthrie A J and Venter E H 2008 Experimental reproduction of severe bluetongue in sheep. Veterinary Pathology Online, 45(3), 310-315.

MacLachlan N J, Gard G 2009 Clinical signs and pathology. In: Mellor P, Baylis M, Mertens PPC (eds.): Bluetongue Academic Press, London 285–293.

McColl K A and Gould A R 1994 Bluetongue virus infection in sheep: haematological changes and detection by polymerase chain reaction. Australian veterinary journal, 71(4), 97-101.

McConnell S, Martens J G, Miller T D and Morrill J C 1983 Bluetongue in Texas cattle: a serological study [Viral disease]. Southwestern Veterinarian (USA).

McHolland L E and Mecham J O 2003 Characterization of Cell Lines Developed from Field Populations of Culicoides sonorensis (Diptera: Ceratopogonidae). Journal of Medical Entomology 40(3): 348-351.

Mecham J O 2006 Detection and titration of bluetongue virus in *Culicoides* insect cell culture by an antigen-capture enzyme-linked immunosorbent assay. Journal of virological methods. Volume 135, Issue 2, Pages 269–271.

Mehrotra M L, Shukla D C and Kataria J M 1989 Isolation and identification of viral agent resembling bluetongue virus in an outbreak in sheep. Indian Journal of Animal Sciences 59(9): 1072-1074.

Mellor P S, Baylis M and Mertens P P C 2009 Bluetongue. (Elsevier, Academic Press, London).

Mertens P P C, Burroughs J N and Anderson L 1987 Purification and properties of virus particles, infectious sub viral particles and cores of bluetongue virus serotype 1 and 4 Virology 157: 375-386.

Mertens P P C, Maan N S, Prasad G, Samuel A R, Shaw A E, Potgieter A C, Anthony S J, Maan S 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: 2811-2823.

Meyer G, Lacroux G, Leger S, Top S and Goyeau K 2009 Lethal Bluetongue virus serotype 1 infection in llamas. Emerging Infectious Diseases 15: 608-610.

Mishra S and Mehrotra M L 1996 Physico chemical and biological characterization of a Indian isolate of bluetongue virus (BTV) Indian Journal of Comparative Microbiology Immunology and Infectious Diseases 17 (2): 109 – 113.

Monteys V S I and Ardanaz M S 2003 Culicoides midges in Catalonia (Spain), with special reference to likely bluetongue virus vectors. Medical and Veterinnary Entomology 17 (3): 288-293.

Mullens B A., Tabachnick W I, Holbrook F R and Thompson L H 1995 Effects of temperature on virogensis of bluetongue virus serotype 11 in Culioides varripennis sonorensis. Medical and Veterinary Entomology 9:71-6.

Mullis K B, Faloona F A 1987 Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods in Enzymology 155:335-50. PubMed PMID: 3431465.

Murray M D 1986 In Arbovirus Research in Australia-Proceedings of the Fourth symposium. Eds. St George, T. D., Kay, B. H and Block, J. CSIRO and QIMR, Brisbane pp. 232.

Nachimuthu K, Thangavelu A, Dhinakar Raj G and Venkatesan R A 1992 Isolation of bluetongue virus from sheep. Indian Journal of Animal Science 62: 112-114.

Naresh A and Prasad G 1995 Relative Superiority of C-ELISA for detection of bluetongue virus antibodies. Indian Journal of Experimental Biology 33 (11): 880-2.

Office international des epizooties Terrestrial manual 2014, bluetongue.

OIE Terrestrial Manual 2014 Bluetongue. Chapter 2.1.3.

Owen N C and Munz E K 1966 Observations on a strain of bluetongue virus by electron microscopy. Onderstepoort Journal of Veterinary Research 33: 9-14.

Ozgunluk I 2009 A serologic investigation of blue tongue virus serotypes (BTV-9, BTV-16) in cattle in the Southeastern Anatolia project area in Turkey. Journal of Animal and Veterinary Advances 8: 2613-2616.

Parsonson I M, Della-Porta A J, McPhee D A, Cybinski D H, Squire K R, Standfast H A and Uren MF 1981 Isolation of bluetongue virus serotype 20 from the semen of an experimentally infected bull. Australian Veterinary Journal 57: 252-253.

Patel, Avnish and Polly Roy 2014 The molecular biology of Bluetongue virus replication. Virus research 182: 5-20.

Pathak K B, Biswas S K, Tembhurne P A, Hosamani M, Bhanuprakash V, Prasad G, Singh R K, Rasool T J and Mondal B 2008 Prokaryotic expression of truncated VP7 of Bluetongue virus (BTV) and reactivity of the purified recombinant protein with all BTV type-specific sera. Journal of Virology Methods 152: 6-12.

Patton J F, Work T M, Jessup D A, Hietala S K, Oliver M N and Maclachlan N J 1994 Serologic detection of Bluetongue virus infection of black-tailed deer: comparison of serum neutralization, agar gel immunodiffusion, and competitive ELISA assays. Journal of wildlife diseases 30: 99-102.

Pearson J E, Gustafson G A, Shafer A L and Alstad A D 1992 Diagnosis of bluetongue and epizootic haemorrhagic disease. In Bluetongue, African horse sickness and related orbiviruses (T.E. Walton & B.I. Osburn, eds). Proceedings of Second International Symposium, Paris 17-21 June 1991. CRC Press, Boca Raton pp: 533-546.

Polci A, Cammà C, Serini S, Di Gialleonardo L, Monaco F and Savini G 2007 Real-time polymerase chain reaction to detect bluetongue virus in blood samples. Veterinaria Italiana 43(1), 77-87.

Prasad G, Jain N C and Gupta Y 1992 Bluetongue virus infection in India: a review. Revue scientifique et technique (International Office of Epizootics) 11: 699711.

Ramarao G 1982 A study on bluetongue disease is Mahaboobnagar district of Andhra Pradesh. In proceedings of the National Work shop on bluetongue, IVRI Izatnagar, pp 66-69.

Ramesh babu N G, Byregowda S M, Bragitha A J and Gopal T 1992 Isolation and preliminary characterization of bluetongue virus from sheep. Indian Veterinary Journal 69: 1071-1074.

Rao V D P 2006 Epidemiology of Bluetongue: Strategies for control of Bluetongue 26-34.

Rao P P, Hegde N R, Reddy Y N, Krishnajyothi Y, Reddy Y V, Susmitha B, Gollapalli S R, Putty K and Reddy G H 2014 Epidemiology of Bluetongue in India. Journal on Transboundary and Emerging Diseases.

Ravishankar chintu, Krishnan Nair G, Mini M and Jayaprakasan V 2007 A modified DOT ELISA for detection of Bluetongue virus antibodies in sheep sera. Indian Veterinary Journal 84: 119-120

Richards R G, Maclachlan N J, Heidner H W and Fuller F J 1988 Comparison of virologic and serologic responses of lambs and calves infected with bluetongue virus serotype 10. Veterinary microbiology, 18(3-4), 233-242.

Reddington J J, Reddington G M and MacLachlan N J 1991 A competitive ELISA for detection of antibodies to the group antigen of bluetongue virus. Journal of Veterinary Diagnosis and Investigation 3 (2): 144-7.

Rodriguez-Sanchez A, Gortazar C, Ruiz-Fons F, Sanchez-Vizcaino J M 2010a Bluetongue virus serotypes 1 and 4 in red deer, Spain. Emerging Infectious Diseases 16: 518– 520.

Rodriguez-Sanchez B, Sanchez-Cordon P J, Molina V, Risalde M A, Perez de Diego A C, Gomez-Villamandos J C, Sanchez-Vizcaino J M 2010b Detection of bluetongue serotype 4 in mouflons (Ovis aries musimon) from Spain. Veterinary Microbiology 141: 164–167.

Roy P and Goorman M 1990 Bluetongue virus. Current Topics in Microbiology and Immunology 162: 1-200.

Roy P 1991 Towards the control of emerging bluetongue disease. Oxford Virology Publications, London.

Saegerman C, Berkvens D and Mellor P S 2008 Bluetongue epidemiology in the European Union. Emerging Infectious Diseases 14, 539-544.

Sapre S N 1964 An outbreak of bluetongue in goats and sheep. Veterinary Review 15: 78-80.

Schulz, Claudia, Michael Eschbaumer, Miriam Rudolf, Patricia König, Markus Keller, Christian Bauer, Matthias Gauly, Christoph G. Grevelding, Martin Beer and Bernd Hoffmann 2012. Experimental infection of South American camelids with bluetongue virus serotype 8. Veterinary microbiology 154, no. 3: 257-265.

Schwartz-Cornil I, Mertens P P C, Contreras V, Hemati B, Pascale F, Breard E, Mellor P S, MacLachlan N J and Zientara S 2008 Bluetongue virus: virology, pathogenesis and immunity. Veterinary Research 39.

Sellers R F 1981 Blutongue and related diseases. In virus Diseases of food animals.

Shaw A E, Monaghan P, Alpar H O, Anthony S, Darpel K E, Batten C A, Guerico A, Alimena G, Vitale M, Bankowska K, Carpenter S, Jones H, Oura C A L, King D P, Elliott H, Mellor P S and Mertens P P C 2007 Development and initial evaluation of a real-time RT-PCR assay to detect bluetongue virus genome segment 1. Journal of Virology Methods 145:115-126.

Singer R S, Boyce W M, Gardner I A, Johnson W O and Fisher A S 1998 Evalaution of bluetongue virus diagnostic tests in free-ranging bighorn sheep. Preventive Veterinary Medicine 35 (4): 265-82.

Sperlova A and Zendulkova D 2011 Bluetongue A review. Journal of Veterinary Medicine 56: 430452.

Sreenivas R P, Moogi V M, Ananth M, Gopal T and Keshavamurthy B S 1982 Bluetongue disease outbreak among sheep and goats of Karnataka. National Seminar on sheep and goat Diseases. CSWRI Avikanagar, Rajastan, pp 15.

Sreenivasulu D and Subba Rao M V 1999 Seroepidemiology of bluetongue disease in Andhra Pradesh, India. Indian Journal of Animal Sciences 69 (5): 292 – 294.

Sreenivasulu D, Subba Rao M V and Gard G P 1999 Isolation of bluetongue virus serotype 2 from native sheep in India. Veterinary Record 144: 452-453.

Standfast H A, Dyce A L and Muller M J 1985 Vectors of bluetongue viruses in Australia. Progress in clinical and biological research (178): 177-186.

St George T D 1981 In Bluetongue and Related Orbiviruses. Eds. Barber, T L and Jochim, M M Alan R Liss. Inc, New York pp. 519.

Sudheer D 2003 Characterization of bluetongue virus isolates. M.V.Sc thesis, Acharya N.G Ranga Agricultural University, Hyderabad.

Tabachnick W S 1996 Culicoides varripennis and bluetongue virus epidemiology in the United States. Annual review of entomology 41:23-43.

Toussaint J F, Sailleau C, Breard E, Zientara S and De Clercq K 2007 Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. Journal of virological methods, 140(1), 115-123.

Toussaint J F, Sailleau C, Mast J, Houdart P, Czaplicki G, Demeestere L, Vandenbussche F, van Dessel W, Goris N, Bréard E, Bounaadja L, Thiry E, Zientara S and De Clercq K 2006 Bluetongue in Belgium. Emerging Infectious Diseases, 13(4):614.

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.

Umeshappa, Channakeshava Sokke, Karam Pal Singh, Rudragouda Channappanavar, Kavita Sharma, Roopa Hebbandi Nanjundappa, Meeta Saxena, Rajendra Singh, and Anil Kumar Sharma 2011

A comparison of intradermal and intravenous inoculation of bluetongue virus serotype 23 in sheep for clinico-pathology and viral and immune responses. Veterinary immunology and immunopathology 141: 230-238.

Uppal P K and Vasudevan B 1980 Occurrence of bluetongue in India. Journal of comparative Microbiology, Immunology and Infectious Diseases 1: 18-20.

Van Gennip R G, Van de Water S G, Potgieter C A, Wright I M, Veldman D and Van Rijn P A 2012 Rescue of recent virulent and avirulent field strains of bluetongue virus by reverse genetics. PloS one, 7(2), e30540.

Vasudevan, B., 1982. A note on bluetongue incidence among sheep in India. In proceedings of the National Work Shop on bluetongue. IVRI, Izatnagar. Pp 129-145.

Verwoerd D W, Els H J, De Villiers E M and Huismans H 1972 Structure of the bluetongue virus capsid. Journal of Virology 10: 783-794.

Verwoerd D W and Erasmus B J 1994 Bluetongue in: Coetzer J A W, Thomson G R, Tustin R C, editors. Infectious diseases of livestock with special reference to Southern Africa. Vol.1. Oxford University Press, Cape Town 443 – 459.

Verwoerd D W & Erasmus B J 2004 Bluetongue *In*: Infectious Diseases of Livestock, Second Edition, Coetzer.

Walker A R and Boreham P F L 1976 Blood feeding of culicodes (Diptera ceratopogonidae) in Kenya in relation to the epidemiology of bluetongue and ephemeral fever. Bulletin Entomology Research 66:181-188.

Ward M P 1994 The epidemiology of bluetongue virus in Australia - a review. Australia Veterinary Journal 71: 3-7.

Wechsler S J and Holland L E 1988 Susceptibilities of 14 cell lines to bluetongue virus infection Journal of Clinical Microbiology 26:2324-2327.

Wilbur L A, Everman J F, Levings R L, Stoll I R, Starling D E, Spillers C A, Gustafson G A and McKeira A J 1994 Abortion and death in pregnant bitches associated with a canine vaccine contaminated with bluetongue virus. Journal of the American Veterinary Medical Association 204: 1762-1765.

Williamson S, Woodger N, and Darpel K 2008 Differential diagnosis of bluetongue in cattle and sheep. Practise 30: 242–251.

Yilmaz Volkan and Aykut Ozkul 2012 Serotypic identification of local Bluetongue virus isolates using Plaque Reduction Neutralization (PRN) and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Techniques. Ankara Üniversitesi Veteriner Fakültesi Dergisi 9: 35-40.

Yugendhar Reddy B 2004 Isolation,Sero-epidemiology and Molecular Characterization of Bluetongue virus. M.V.Sc thesis, Acharya N.G Ranga Agricultural University, Hyderabad.

Zientara S, E Bréard, C Sailleau 2004 Bluetongue diagnosis by reverse transcriptase-polymerase chain reaction ,Veterinaria Italiana 2004.

Zientara S, Sailleau C, Viarouge C, Höper D, Beer M, Jenckel M, Hoffmann B, Romey A, Bakkali-Kassimi L, Fablet A, Vitour D and Bréard E 2014 Novel bluetongue virus in goats, Corsica, France. Emerging Infectious Diseases 20: 2123-2125.