UNIVERSITY OF GLASGOW

Isolation and Molecular characterization of Bluetongue Virus from Southern India

by

Sarita Yadav

A thesis submitted in fulfilment for the degree of Doctor of Philosophy in Virology

College of Medical, Veterinary and Life Sciences

May 2018

Abstract

BT is endemic in India, particularly in the South and BTV strains belonging to twelve BTV-serotypes (BTV-1, 2, 3, 4, 5, 9, 10, 12, 16, 21, 23 and 24) have been isolated since 2001. A pentavalent inactivated BTV vaccine containing serotypes 1, 2, 10, 16 and 23, is currently being used in the South. This study focusses on the isolation and the typing of BTV strains from Southern India, 2014-15, then generation of full length sequence data, primarily for BTV genome Seg-2 to provide molecular epidemiology information concerning the strains circulating in the region.

Suspected field outbreaks of BTV from Telangana, Andhra Pradesh, Karnataka and Tamil Nadu states in Southern India during 2014-2015 were attended to collect well documented blood and necropsy clinical samples from mixed population of small ruminants and cattle. Of the 447 field samples (EDTA blood, spleen, lymph node and saliva), 236 gave positive results for BTV RNA by serogroup-specific real-time RT-PCR targeting Seg-9 and Seg-10 for 2014 and 2015 samples respectively. A total of 141 BTV virus isolations were made from 141 serogroup-specific qRT-PCR positive samples, by infecting KC and BHK cells.

The isolated 63 BTVs were serotyped by qRT-PCR assay targeting Seg-2 of the BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29, confirming the current co-circulation of 7 BTV serotypes, including BTV-1, BTV-2, BTV-4, BTV-5, BTV-9, BTV-10 and BTV-12 in 62 BTV isolates. Dual and triple BTV co-infections with these serotypes were detected in 22 and 4 BTV isolates, respectively.

None of the serotypes present in the pentavalent vaccine were identified in any of the 22 BTV isolates from vaccinated animals, suggesting that the vaccine was indeed effective against the homologous serotype field strains.

Among the 7 serotypes identified by Seg-2 based ORF sequence analysis of 46 BTV isolates (BTV-1, BTV-2, BTV-4, BTV-5, BTV-9, BTV-10 and BTV-12), 4 serotypes BTV-1, BTV-2, BTV-4 and BTV-9, belonged to eastern topotypes, whereas 3 serotypes, BTV-5, BTV-10 and BTV-12 were identified as western topotypes. For 4 BTV isolates, the serotype detected by qRT-PCR was different of that detected by NGS. The full genome sequencing and analyses is still ongoing in Glasgow. This data will assist in understand the epidemiology of the BT in India and implementation of effective BTV control to ensure the use of appropriate BTV serotype and strains in the available BT vaccine in India.

Abbreviations

µL	Microlitre
aa	Amino acid
AGE	Agarose gel electrophoresis
AGID	Agar gel immuno-diffusion
AHSV	African horse sickness virus
AMV	Avian myeloblastosis virus
ATCC	American Type Culture Collection
BHK	Baby hamster kidney
BHQ	Black Hole Quencher
bp	Base pair
BT	Bluetongue
BTV	*Bluetongue virus*
cDNA	Complementary DNA
CF	Complement fixation
CPE	Cytopathic effects
Ct	Cycle threshold
dd NTP	Dideoxy nucleotides triphosphates
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
ds	Double-stranded
EDTA	Ethylene diamine tetra acetic acid
EEV	*Equine Encephalosis Virus*
EHDV	*Epizootic Hemorrhagic Disease Virus*
ELISA	Enzyme linked immune sorbent assay
FAM	6-Carboxyfluorescein
FBS	Foetal bovine serum
FLAC	Full-length amplification of cDNA
FMD	foot and mouth disease
FRET	Fluorescence resonance energy transfer

GTPV	*Goat Pox Virus*
HPLC	High performance liquid chromatography
ICAR	Indian Council for Agriculture Research
ICTV	International Committee for the Taxonomy of Viruses
ID	Identification
IND	India
ISVP	Infectious subviral particles
IVRI	Indian Veterinary Research Institute
Kb	Kilobase
Kbp	Kilo base pair
kDa	Kilo Dalton
LiCl	Lithium chloride
mL	Millilitre
MLV	Modified live virus
NCR	Noncoding region
NFW	Nuclease free water
NGS	Next generation sequencing
NIVEDI	National Institute of Veterinary Epidemiology and Disease Informatics
nM	Nanomolar
NPM	Nextera PCR master mix
NS	Non-structural
nt	Nucleotide
°C	Degree Celsius
OIE	Office International des Epizooties
ORC	*Orbivirus* Reference Collection
ORF	Open reading frame
ORFV	*Orf Virus*
P	Phosphorus
PAGE	Polyacrylamide gel electrophoresis
pM	Picomolar
PPR	Peste des Petite Ruminant

PPRV	*Peste des Petite Ruminant Virus*
q	Quantitative
RGD	Arginine-glycine-aspartate
RNA	Ribonucleic acid
rpm	Revolutions per minute
RSB	Resuspension buffer
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
S	Sulphur
SBL	Sequencing by ligation
SBS	Sequencing by synthesis
Seg	Segment
SMRT	Single molecule real-time sequencing
SNT	Serum neutralization tests
SPAT	Single primer amplification technique
spp	Species
SPPV	*Sheep Pox Virus*
ss	Single stranded
TAE	Tris acetate EDTA
TC	Transcriptase complex
UV	Ultraviolet
v/v	Volume by volume
VIBs	Viral inclusion bodies
VLP	Virus-like particles
VNT	Virus neutralization tests

Table of contents

Abstract……i

Abbreviations……ii

Table of contents……v

List of Tables……..xv

List of Figures……xviii

Acknowledgements……..xxii

Declaration of Authorship……xxiv

Chapter 1: General Introduction 1

1.1 Introduction 1

1.2 Taxonomy 2

1.2.1 dsRNA viruses 2

1.2.2 The family *Reoviridae* 3

1.3 The genus *Orbivirus* 3

1.4 The structure of the Bluetongue virus particle 5

1.5 The BTV genome 7

1.6 BTV structural proteins 8

1.6.1 Outer capsid proteins (VP2 and VP5) 8

1.6.2 Major core proteins (VP3 and VP7) 9

1.6.3 Minor enzymatic core proteins (VP1, VP4 and VP6) 10

1.7 BTV non-structural proteins (NS1, NS2, NS3, NS4 and NS5) 11

1.8 The BTV replication cycle 13

1.8.1 Attachment and entry 13

1.8.2 Uncoating 14
1.8.3 Transcription 14
1.8.4 Replication 15
1.8.5 Assembly and release 15
1.9 BTV pathogenesis 16
1.9.1 Clinical signs 16
1.9.2 Pathogenesis 18
1.10 Evolution and molecular epidemiology of BTV 20
1.10.1 BTV serogroup, serotypes and topotype 21
1.11 DNA sequencing 23
1.11.1 The Maxam and Gilbert method 23
1.11.2 Sanger's dideoxy sequencing technology 23
1.11.3 Next generation sequencing 24
1.11.3.1 Illumina (MiSeq, NextSeq 500, and HiSeq) 24
1.11.3.2 Ion Torrent (Life Technologies) 25
1.11.3.3 Roche pyrosequencing platforms 25
1.11.3.4 SOLiD sequencing 25
1.11.3.5 Third generation sequencing 25
1.11.4 Sequencing approaches for dsRNA viruses 26
1.12 BTV control 26
1.12.1 Attenuated vaccines 27
1.12.2 Inactivated vaccines 28

1.12.3 Virus-like particles 28
1.13 BTV diagnosis 29
1.13.1 Virus isolation 29
1.13.2 Serological assays 30
1.13.2.1 Group-specific antibody detection assays 30
1.13.2.2 Serotype-specific antibody and antigen detection assays 31
1.13.3 Molecular assays 32
1.13.3.1 Group-specific RT-PCR assays 33
1.13.3.2 Serotype-specific RT-PCR assays 34
1.14 Bluetongue epidemiology in India 34
1.14.1 Geographical distribution of bluetongue disease in India 34
1.14.2 Influence of climate of India on bluetongue disease 35
1.14.3 Landless, small and marginal farmers in India 39
1.14.4 Livestock population in India 40
1.14.5 Contribution in the national economy 41
1.14.6 Susceptibility to BT of sheep and goat breeds in Southern India 41
1.15 Serotypes of BTV in India 42
1.16 BTV vaccination in India 43
1.17 Aims and objectives of Project 46
Chapter 2: Materials and Methods 48
2.1 Cell culture 48
2.1.1 Cell culture media 48

2.1.2 Cell lines 49
2.1.2.1 Mammalian cell lines 49
2.1.2.2 Insect cell lines 49
2.1.3 Cell culture technique 49
2.1.3.1 Mammalian cell culture 49
2.1.3.2 Insect cell culture 50
2.1.4 Cryopreservation of cells: 50
2.1.5 Revival of cryopreserved cells 50
2.2 Reagents for RNA extractions 51
2.3 Blood sample collection 51
2.4 Agarose gel electrophoresis 51
2.5 Primers for qRT-PCR 52
2.5.1 Serogroup-specific oligonucleotide primers and probe 52
2.5.2 Serotype-specific oligonucleotide primers and probe 52
2.6 Primers for full-length amplification of cDNA (FLAC) 53
2.6.1 Anchor-primers for ligation 53
2.6.2 Primer for amplification of cDNA 53
2.7 Enzymes and kits 53
2.7.1 Ligation, Reverse transcription, cDNA synthesis / purification 53
2.7.2 Next Generation Sequencing-Illumina sequencing, kits / reagents 54
2.8 Description of the study area 54
2.9 Field outbreaks of BT 55

2.10 Collection of field samples 55

2.11 Viruses..... 57

2.12 Preparation of clinical sample..... 57

2.13 Total RNA extraction from clinical samples 58

2.14 Laboratory diagnosis by quantitative real-time RT-PCR 58

2.14.1 Serogroup-specific qRT-PCR 59

2.15 Virus propagation..... 60

2.15.1 Virus propagation in KC cells..... 60

2.15.2 Virus propagation in BHK-21 cells 61

2.16 Storage of BTV field isolates..... 61

2.17 BTV dsRNA extraction from virus-infected cell culture 62

2.18 Type-specific TaqMan based qRT-PCR..... 63

2.19 Agarose gel electrophoresis 64

2.20 RT-PCR for full-length cDNA amplification (FLAC) 64

2.20.1 Anchor-primer ligation 65

2.20.2 Heat denaturation of dsRNA..... 65

2.20.3 Reverse transcription (RT)..... 65

2.20.4 Amplification of cDNA 65

2.21 Next-generation-Sequencing/Illumina-sequencing of Indian BTV isolates..... 66

2.21.1 DNase Treatment of BTV dsRNA samples 66

2.21.2 Anchor-primer ligation and purification of dsRNA 70

2.21.3 Synthesis and amplification of cDNA 70

2.21.4 Amplified cDNA purification 71

2.21.5 DNA Quantification 71

2.21.6 Library preparation 71

2.21.7 ‘Tagmentation’ of input DNA 71

2.21.8 cDNA Library Indexing and PCR amplification 72

2.21.9 Ampure XP dsDNA purification 72

2.21.10 Library Quantification 73

2.21.11 Pooling of sequencing library, denaturation and dilution 73

2.22 Processing and assembly of the raw sequencing data 74

2.23 Sequence data phylogenetic analysis of Seg-2 74

Chapter 3: Field outbreaks of bluetongue disease in Southern India during 2014-15 76

3.1 Introduction 76

3.2 Results 78

3.2.1 Field outbreaks in 2014 78

3.2.2 BT Field outbreaks in 2015 79

3.2.2.1 Telangana, 2015 79

3.2.2.2 Andhra Pradesh, 2015 79

3.2.2.3 Karnataka, 2015 80

3.2.2.4 Tamil Nadu, 2015 81

3.2.3 Laboratory diagnosis based on qRT-PCR 82

3.2.3.1 Telangana sporadic field outbreak, 2014 82

3.2.3.2 Tamil Nadu field outbreaks, 2014 83

3.2.3.3 Telangana field outbreaks, 2015 84

3.2.3.4 Andhra Pradesh field outbreaks, 2015 85

3.2.3.5 Karnataka field outbreaks, 2015 85

3.2.3.6 Tamil Nadu field outbreaks, 2015 85

3.2.4 Samples used to infect KC and BHK cells 86

3.3 Discussion 109

3.4 Summary 114

Chapter 4: Isolation and Molecular typing of BTV from Southern India in 2014-2015. 117

4.1 Introduction 117

4.2 Results 119

4.2.1 Isolation of BTV 119

4.2.2 Assigning unique collection number to BTV field strains 120

4.2.3 Virus propagation for typing 122

4.2.4 Typing of BTV isolates 126

4.2.4.1 BTV isolates from vaccinated flock of Tamil Nadu state, 2015 127

4.2.4.2 BTV isolates from vaccinated flock of Karnataka state, 2015 128

4.2.4.3 BTV isolates from unvaccinated flock of Tamil Nadu state, 2014 .. 128

4.2.4.4 BTV isolates from unvaccinated flock of Tamil Nadu state, 2015 .. 128

4.2.4.5 BTV isolates from unvaccinated flock of Telangana state, 2014 129

4.2.4.6 BTV isolates from unvaccinated flock of Telangana state, 2015 129

4.2.4.7 BTV isolates from unvaccinated flock of Andhra Pradesh state, 2015 130

4.2.4.8 Mixed infections 130

4.2.4.9 BTV serotype distribution 137

4.3 Discussion 137

4.4 Summary 144

Chapter 5: Phylogenetic analysis of genome segment 2 from bluetongue virus strains isolated in India during 2014-2015 146

5.1 Introduction 146

5.2 Results 148

5.2.1 Phylogenetic characteristics of Seg-2/VP2 148

5.2.1.1 Seg-2 based phylogenetic analysis for confirmation of serotypes detected by real-time RT-PCR. 154

5.2.2 Phylogenetic analysis of genome segment 2 of BTV-1 within the Indian context 157

5.2.3 Phylogenetic analysis of BTV-1 Seg-2 in a global context 158

5.2.4 Phylogenetic analysis of Seg-2 from Indian BTV-2 isolates 163

5.2.5 Phylogenetic analysis of Seg-2 / VP2 of Indian BTV-2 isolates within a global context 163

5.2.6 Phylogenetic analysis of Seg-2 from Indian BTV-4 isolates 167

5.2.7 Phylogenetic analysis of Seg-2 / VP2 of Indian BTV-4 isolates within a global context 167

5.2.8 Phylogenetic analysis of Seg-2 from Indian BTV-5 isolates 171

5.2.9 Phylogenetic analysis of Seg-2 / VP2 of Indian BTV-5 isolates within a global context 171

5.2.10 Phylogenetic analysis of Seg-2 from Indian BTV-9 isolates..... 174

5.2.11 Phylogenetic analysis of Seg-2 / VP2 of Indian BTV-9 isolates within a global context 175

5.2.12 Phylogenetic analysis of Seg-2 from Indian BTV-10 isolates..... 178

5.2.13 Phylogenetic analysis of Seg-2 / VP2 of Indian BTV-10 isolates within a global context 178

5.2.14 Phylogenetic analysis of Seg-2 from Indian BTV-12 isolates..... 181

5.2.15 Phylogenetic analysis of Seg-2 / VP2 of Indian BTV-12 isolates within a global context 182

5.3 Discussion 184

5.4 Summary 194

Chapter 6: General discussion and future work 196

6.1 Bluetongue in India 196

6.2 Attending BT field outbreaks from Southern India 197

6.3 Clinical signs in BT suspected cases 200

6.4 Serogroup specific qRT-PCR to diagnose BT 201

6.5 Well characterised and documented BTV strains 206

6.6 Typing of BTV isolates 207

6.7 Seg-2 based phylogenetic analysis of 46 Indian BTV strains from Southern India, 2014-15 211

6.8 Future work 215

Appendix 1 List of primers and probes for serotype-specific qRT-PCR (Maan et al., 2016) 218

Appendix 2 Details of BTV isolates from Southern India, 2014-15. 221

Appendix 3 Detail of Seg-2 sequences of BTV reference strains available from GenBank.. 227

Appendix 4 Percentage of nucleotide identity in Seg-2 between BTV strains sequenced in this study and global reference strains of BTV. 239

List of tables

Table 1.1 The families of dsRNA viruses. 2

Table 1.2 The genera within *Reoviridae* family 4

Table 2.1: Primer and probe sequences to detect BTV RNA by BTV specific qRT-PCR targeting BTV Seg- 10 52

Table 2.2: Primer and probe sequences to detect BTV RNA by BTV specific qRT-PCR targeting BTV Seg- 9. 52

Table 2.3: Primer sequences for full-length amplification of cDNA (FLAC) technique (Maan et al., 2007b)..... 53

Table 2.4 Samples collected from four Southern states during 2014-2015 BT outbreaks in India. 56

Table 2.5: Cell culture adapted Indian BTV isolates obtained from collaborators under the Indo-UK project. 57

Table 2.6 Composition of serogroup-specific quantitative RT-PCR assay targeting Seg-10 60

Table 2.7: Composition of serogroup-specific quantitative RT-PCR assay targeting Seg-9 60

Table 2.8: Composition of type-specific TaqMan based qRT-PCR assay with two sets of primers and probes..... 63

Table 2.9: Composition of type-specific TaqMan based qRT-PCR assay with one set of primers and probe 64

Table 2.10 Details of the BTV field strains for Illumina sequencing in this study 67

Table 3.1 Main clinical signs and their frequency in sheep and goats affected by BTV. 80

Table 3.2 Seg-9 based qRT-PCR results from Telangana state, 2014..... 82

Table 3.3 Seg-9 based qRT-PCR results from Tamil Nadu state, 2014. 83

Table 3.4 Seg-10 based qRT-PCR results of samples from Telangana state, 2015 84

Table 3.5 Serogroup-specific real-time RT-PCR results to diagnose BT 86

Table 3.6 Serogroup-specific real-time RT-PCR results in vaccinated flocks. 86

Table 3.7 Samples with Ct value <30 by BTV group specific qRT-PCR 87

Table 3.8 Detail of clinical samples positive for BTV and used for virus isolations..... 102

Table 4.1 Isolation of BTV from qRT-PCR positive clinical samples (n=141) 122

Table 4.2: Indian field strains of BTV (n=63) serotyped by Seg-2 based qRT-PCR. 124

Table 4.3 Details of Indian field strains of BTV (n=10) obtained from collaborators for typing by Seg-2 based qRT-PCR 126

Table 4.4 ®Serotyping of Indian field isolates of BTV (n=63) from 2014-15 BT outbreaks in Southern India. 130

Table 4.5: Serotyping of BTV isolates provided by various collaborators. 133

Table 4.6 BTV isolates (n=36*) with single serotype infections during BT outbreaks in Southern India, 2014-15. 134

Table 4.7 Serotype detected in BTV isolates (n=36) involving single serotype during BT outbreaks in Southern India, 2014-15. 135

Table 4.8 BTV isolates with mixed infections(n=26) during BT outbreaks in Southern India, 2014-15. 135

Table 4.9 Dominant serotype detected in mixed co-infection BTV isolates (n=26). 136

Table 4.10 Distribution of BTVserotypes in BTV isolates (n=62) during 2014-15 BT outbreaks in Southern India. 136

Table 5.1 Details of the BTV field strains sequenced in this study 150

Table 5.2 Genetic characterization of Seg-2 from Indian BTV isolates sequenced. 161

List of figures

Figure 1.1 Diagram of the BTV virus particle..... 6

Figure 1.2 Schematic representation of the BTV replication cycle..... 16

Figure 1.3 Map of India showing all the states of India. 36

Figure 1.4 BTV serotypes reported from different States of India since 2001. 45

Figure 2.1 The author attending suspected BT outbreaks in mixed flocks of sheep and goats in various states of Southern India during 2014-2015. 56

Figure 2.2 TruSeq index plate fixture setup for 24 libraries..... 73

Figure 3.1 Hyperaemic oral mucosa and swollen muzzle in sheep suspected for bluetongue disease, Karnataka 2015. 88

Figure 3.2 Oral erosions and ulcers particularly on the dental pad in BT suspected sheep, Karnataka 2015..... 89

Figure 3.3 Erosion, crusting of muzzle and nasal discharge speckled with blood in BT suspected sheep, Karnataka 2015. 90

Figure 3.4 Vulvar lips haemorrhages & pyrexia in sheep suspected for bluetongue disease, Karnataka 2015. 90

Figure 3.5 Excessive salivation, frothing at the mouth and swollen cyanotic tongue in BT suspected sheep, Karnataka 2015. 91

Figure 3.6 Submandibular and head oedema in BT suspected sheep, Andhra Pradesh 2015. 92

Figure 3.7 Coronitis resulting in lameness and break in wool in BT suspected sheep,..... 93

Figure 3.8 BT suspected cases from Tamil Nadu, 2015. 94

Figure 3.9 Map of India showing the geographical location of the study, 2014-15..... 95

Figure 3.10 Locations of bluetongue suspected outbreaks in this study from Southern India, 2015 96

Figure 3.11 Locations of bluetongue suspected outbreaks in this study from Southern India, 2014 97

Figure 3.12 Locations of bluetongue suspected outbreaks in Karnataka recorded by ICAR-NIVEDI, 2014-15..... 98

Figure 3.13 Locations of bluetongue suspected outbreaks in Karnataka recorded by ICAR-NIVEDI, 2007-11..... 99

Figure 3.14 Different serotypes isolated from Telangana, 2002-2013. 100

Figure 3.15 Different serotypes isolated from Andhra Pradesh, 2006-2011.101

Figure 4.1 Cytopathic changes induced by the BTV isolate from sheep in BHK-21 121

Figure 4.2 Electrophoretic analysis of BTV dsRNA in a 1 % agarose gel... 123

Figure 5.1 Unrooted Maximum Likelihood (ML) phylogenetic tree of the nucleotide sequences of Seg-2 coding region of Indian BTV isolates (n=46) and BTV reference strains of 27 serotypes isolated worldwide. 155

Figure 5.2 ML unrooted phylogenetic tree of Seg-2 coding sequences of Indian BTV isolates (n=46) during 2014-2015 (Table 5.1) and other Indian BTV field strains (n=46) available in GenBank (n=46). 156

Figure 5.3 ML unrooted phylogenetic tree of coding region Seg-2/VP2 gene of Indian BTV-1 isolates (n=3) during 2015 (Table 5.1) with reference and field strains of BTV-1 from around the world. 159

Figure 5.4 ML unrooted phylogenetic tree of Seg-2 coding region of Indian BTV-2 isolates (n=4) during 2015 (Table 5.1), reference strains of BTV-2 from around the world represented as circular (top) and rectangular (bottom) tree. 165

Figure 5.5 ML unrooted phylogenetic tree of coding region Seg-2/VP2 gene of Indian BTV-4 isolates (n=2) during 2014-2015 (Table 5.1) with reference strains of BTV-4 around the world represented as circular (top) and rectangular (bottom) tree. 169

Figure 5.6 ML unrooted phylogenetic tree of coding region Seg-2/VP2 gene of Indian BTV-5 isolates (n=17) during 2014-2015 (Table 5.1) with reference and field strains of BTV-5 around the world represented as circular (top) and rectangular (bottom) tree. 173

Figure 5.7 ML unrooted phylogenetic tree of coding region of Seg-2/VP2 gene of Indian BTV-9 isolate (n=1) during 2015 from Tamil Nadu, India

(Table 5.1) with reference strains of BTV-9 around the world represented as circular (top) and rectangular (bottom) tree. 176

Figure 5.8 ML unrooted phylogenetic tree of coding region Seg-2/VP2 gene of Indian BTV-10 isolates (n=2) during 2014-2015 (Table 5.1) with reference strains of BTV-10 all over the world 180

Figure 5.9 ML unrooted phylogenetic tree of coding region Seg-2/VP2 gene of Indian BTV-12 isolates (n=17) during 2014-2015 (Table 5.1) with reference strains of BTV-12 from all over the world. 183

Acknowledgements

I express my gratitude towards my supervisor Professor Peter Mertens, Chair in Virology, Faculty of Medicine and Health Sciences, University of Nottingham and former head of Arbovirus Molecular Research Group and head of Pirbright Institute's Vector-borne Viral Diseases (VVD) Programme, for his valuable guidance, suggestion, diligent supervision and constant encouragement throughout the research work.

I wish to record my sincere gratitude to Professor Massimo Palmarini, Director, Centre for Virus Research, MRC-University of Glasgow for his scientific, patient counseling, talented direction, constant encouragement and untiring help amidst of his busy scientific schedule.

I wish, if I could find words to express my feelings love and regards for Dr.Lynda Moore, Head of academic affairs and training at The Pirbright Institute, Pirbright, Surrey who left no stone unturned for giving me the best of support. Without her support, I could not have come to this level. My sincere regards to Yvonne for valuable help during my work.

I am thankful to my assessors Professor Alain Kohl and Pablo Murcia for their constant encouragement and suggestion during my research work. I express my gratitude to Maude Jacquot, at the Institute of Biodiversity, Animal Health and Comparative Medicine, (College of Medical, Veterinary & Life Sciences, University of Glasgow) for her having taught me phylogenetic analysis.

I am thankful to ICAR for providing the necessary financial support in the form of "ICAR international fellowship".

I sincerely acknowledge the help of Dr. Sushila Maan and Dr. Narender Maan, Department of Biotechnology, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana, India for their support during the course of this project.

I am greatly thankful to my parents, sister and husband for their encouragement and support till completion of my thesis. Echo of sweet little voice of my son Arjun will always be a reason for me to smile.

I express my gratitude towards Dr.Madhusudan Hosamani for his constant encouragement, support and also for helping me in field work.

I would also like to acknowledge the help of farmers and field veterinarians for their kind cooperation in the field work.

I thank Dr. Inderjeet Singh, Director, Central Institute for Research on Buffaloes, Haryana, India for his constant encouragement to accomplish this endeavor.

My feelings of indebtedness and a deep sense of gratitude for Dr. Kyriaki Nomikou at the Medical Research Council, Centre for Virus Research (MRC-CVR), University of Glasgow, without whose devoted support and encouragement, it would have never ever been possible for me to complete PhD.

Authors Declaration

I declare that, except where explicit reference is made to the contribution of others, that this research is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Sarita Yadav

University of Glasgow, 2018

Chapter 1: General Introduction

1.1 Introduction

Bluetongue (BT) is an arthropod-borne, non-contagious and economically important haemorrhagic viral disease of domestic and wild ruminants (Verwoerd and Erasmus, 2004, MacLachlan, 1994), causing direct and indirect losses to livestock industries worldwide, estimated at 3 billion US$ per year (Tabachnick et al., 1996). BT disease is endemic in most tropical and subtropical countries of the world, causing mortality up to 70% in naïve populations of sheep (Elbers et al., 2008b). *Bluetongue virus* is the type species, of 22 virus species classified within the genus *Orbivirus*, family *Reoviridae*, although several additional species have recently been proposed (Mertens et al., 2005).

Most bluetongue virus (BTV) strains are transmitted by 'vector competent' species of biting midge, infecting ruminants, including most domestic and wild species and camelids. BTV can also infect several carnivorous feline and canine species via an oral route, although these are usually regarded as dead-end hosts (Jauniaux et al., 2008). Severe BT disease is usually only seen in naïve sheep populations, and may be more severe in certain breeds, primarily the European fine wool and mutton breeds, or some species of wild ruminants such as white tailed deer, or brocket deer (Erasmus, 1975, Taylor, 1986, Vosdingh et al., 1968). BT disease is characterized by various clinical signs, that range from acute to subacute, mild or inapparent depending on the virus strain as well as the species, breed, age and immune status of the infected host (Verwoerd and Erasmus, 2004, Elbers et al., 2008b). In sheep, BTV can infect the vascular endothelium and is characterized by haemorrhages, fever, nasal discharge, oral erosions and ulcers, lameness, coronitis, weakness, depression, torticollis, facial and pulmonary oedema (Spreull, 1905, Verwoerd and Erasmus, 2004, Maclachlan et al., 2009).

The disease is transmitted primarily by adult females of certain species of biting midges belonging to the genus *Culicoides*, family *Ceratopogonida*e which blood-feed to obtain protein for the production of eggs (Mellor, 2000, Tabachnick, 2004). Other BTV transmission modes can include venereal transmission (e.g. through infected bull semen - (Bowen and Howard, 1984), oral transmission (Batten et al., 2014), transplacental infection (De Clercq et al., 2008, Menzies et al., 2008) and by mechanical vectors (Wilson and Mellor, 2008, Luedke et al., 1965, Bouwknegt et al., 2010). Due to its economic impact on international livestock trade, BT is a notifiable disease, formerly classified among the list

A diseases by the Office International des Epizooties (OIE) (OIE, 2017). Twenty four distinct serotypes of BTV have been known and identified for many years. However BTV-25 was recently identified in asymptomatic goats in Switzerland (Hofmann et al., 2008b, Chaignat et al., 2009), BTV-26 was identified in clinical samples from sheep in Kuwait (Maan et al., 2011b) and BTV 27 from asymptomatic goats on Corsica, France (Zientara et al., 2014, Schulz et al., 2016, Jenckel et al., 2015, Savini et al., 2017b). Several further putative types, including: BTV-28 from the Middle East, detected in a Sheep pox vaccine preparation (Bumbarov et al., 2016), BTV-29, isolated from an Alpaca in South Africa have also been identified (Wright, 2014), and BTV-30, 31 and 32 from ruminants in Mongolia (EPIZONE, 2017).

1.2 Taxonomy

1.2.1 dsRNA viruses

There are eight families of dsRNA viruses which are recognised by the International Committee for the Taxonomy of Viruses (ICTV), including a diverse group of pathogens affecting a large variety of vertebrates, invertebrates, plants, fungi and bacteria, and are of medical, veterinary and agricultural significance (Mertens, 2004). Important examples include: rotavirus, causing neonate mortality; bluetongue virus, causing bluetongue disease in ruminants; plant viruses of wheat and rice; birnavirus in poultry; infectious pancreatic necrosis virus affecting fish; and cypoviruses causing losses in silk industry (Table 1.1). The *Reoviridae* is the largest of the eight dsRNA virus families (Mertens, 2000).

Table 1.1 The families of dsRNA viruses. Compiled from (ICTV, 2011)

Family	Host
Birnaviridae	Vertebrate and invertebrate
Chrysoviridae	Fungi
Cystoviridae	Bacteria
Endornaviridae	Algae, fungi and plants
Partitiviridae	Fungi, protozoa and plants
Picobirnaviridae	Vertebrate
Totiviridae	Fungi and protozoa
Reoviridae	Fungi, protozoa, vertebrate, invertebrate and plants

1.2.2 The family *Reoviridae*

The family *Reoviridae* comprises two subfamilies, the *Spinareovirinae* containing nine genera, and the *Sedoreovirinae* containing six genera, which are found in a broad range of econiches, collectively infecting a diverse group of hosts including vertebrates, invertebrates, plants and fungi (Mertens, 2004, Mertens et al., 2005, Attoui, 2012). Viruses of the subfamily *Spinareovirinae* have large surface projections or turrets present at the 12 vertices of the icosahedral virus or core particle, whereas viruses in the subfamily *Sedoreovirinae* are non-turreted or smooth. The family *Reoviridae* (the reoviruses) includes 15 distinct genera that are currently recognized by ICTV, as well as several unassigned viruses that may include representatives of additional genera (Table 1.2). The reoviruses are usually considered to be non-enveloped, although some viruses (including BTV) can exit infected cells by budding and may acquire a temporary external membrane in the process. The intact reovirus particles are 60-80nm in diameter and have icosahedral capsid consisted of one, two or three concentric proteins layers, surrounding a single copy of 9, 10, 11 or 12 linear dsRNA segments that compose the viral genome (Mertens, 2004, Attoui, 2012). The reoviruses show similarities in their replication strategies and structure. The phylogenetic relationships of their more conserved genome segments, suggest a common ancestry and a process or divergence involving coevolution and co-speciation with their respective hosts (Attoui et al., 2002).

1.3 The genus *Orbivirus*

According to the 9th report of the International Committee on Taxonomy of Viruses (ICTV), twenty-two species of *Orbivirus* have been recognized and 10 tentative species are waiting to be incorporated in the genus (Mertens et al., 2005, Attoui, 2012). A number of additional putative species have recently been identified by whole genome sequencing of different orbivirus isolates (Belaganahalli et al., 2012, Belaganahalli et al., 2011, Cowled et al., 2009).

The *Orbiviruses* are arthropod-borne viruses that are transmitted by arthropod vectors including mosquitoes, biting midges, sandflies and ticks (Belaganahalli et al., 2015). Closely related segmented RNA viruses that infect a single host cell can exchange genome segments during co-infection generating hybrid progeny virus strains by a process called as reassortment. This is considered as the primary determinant for different virus strains belonging to the same virus species (Calisher and Mertens, 1998, Batten et al., 2008). The

polythetic parameters recognized by ICTV (van Regenmortel and Mahy, 2004) that can be used (singly or in combination) to identify members of an *Orbivirus* virus species include: reassortment (primary parameter), serological cross-reaction at high level, conserved genome segments showing high level similarity in RNA sequence and cross-hybridization, reverse transcription polymerase chain reaction (RT-PCR) assays against conserved genome segments, identifying the virus serotype, agarose gel electrophoresis (AGE) to analyze the viral RNA electropherotype, identification of conserved terminal regions on the genome segments, common vector or host species identification (Mertens et al., 2005, Mertens et al., 2009, Mertens, 2000, Attoui et al., 2001). African horse sickness virus (AHSV), Epizootic hemorrhagic disease virus (EHDV), Equine encephalosis virus (EEV) and Bluetongue virus (BTV) are considered to be the most economically important *Orbiviruses* affecting ruminants and equines.

Table 1.2 The genera within *Reoviridae* family (Mertens et al., 2005)

Genus	Genome segments(numbers)	Host	Vector
Aquareovirus	11	Molluscs,fish, Crustacea	-
Cardoreovirus	12	Crustacea	-
Coltivirus	12	Mammals (including humans)	Ticks
Cypovirus	10	Insects	-
Fijivirus	10	Plants	Planthooper
Dinovernavirus	9	Mosquitoes	-
Idnoreovirus	10	Insects	-
Mimoreovirus	11	Phytoplankton	-
Mycoreovirus	11 or 12	Fungi	-
Orbivirus	10	Mammals (including humans), birds	*Culicoides* (midges), mosquito, sandfly, ticks
Orthoreovirus	10	Mammals, birds and reptiles	-
Oryzavirus	10	Plants	Planthooper
Phytoreovirus	12	Plants	Leafhopper
Rotavirus	11	Mammals (including humans) and birds	-
Seadornavirus	12	Mammals (including humans)	Mosquitoes
Unassigned reoviruses	10,11or 12	-	-

1.4 The structure of the Bluetongue virus particle

A combination of cryoelectron microscopy, X-ray crystallography and radiolabelling techniques have been used for high resolution structural studies of the bluetongue virus particle (Grimes et al., 1998, Nason et al., 2004). Bluetongue virus is usually regarded as a nonenveloped virus, it is icosahedral, 80 nm in diameter (Verwoerd et al., 1972, Mertens, 1999) with three concentric protein layers, consisting of seven structural proteins, VP1 to VP7 (Grimes et al., 1998, Mertens, 2000), which encapsidate a 10-segmented double-stranded (ds) RNA genome (Mertens et al., 1984, Roy et al., 1990b) (Figure 1.1). The outer-most capsid-layer consists of two major structural proteins, 60 trimers of VP2 (110 kDa) and 120 trimers of VP5 (60 kDa) (Mertens et al., 1987, Mertens et al., 1984, Grimes et al., 1998, Roy, 1989). The larger of these two outer-capsid proteins, 'VP2' is the most variable of the BTV proteins and is responsible for cell attachment during initiation of infection (Hassan and Roy, 1999). VP2 also represents a target for the neutralising antibodies that are generated during infection of the vertebrate host. As a consequence variation in the amino acid sequence of VP2 determines the serotype of BTV as detected in serum neutralisation assays (Cowley and Gorman, 1989, Mertens et al., 1989b). The smaller of the two outer-capsid proteins, 'VP5', which is the second most variable of the BTV proteins, is responsible for membrane penetration during initiation of infection and is thought to play a secondary role in determining the specificity of interactions with neutralising antibodies, and therefore virus serotype (Mertens et al., 1989b, Cowley and Gorman, 1989, DeMaula et al., 2000).

The inner transcriptionally-active BTV 'core' particle is composed of the remaining five highly conserved, structural proteins and the viral genome segments. The core consists of two concentric protein layers, the outermost of which (the 'core surface' layer) is composed of 780 copies of the 38kDa VP7 protein, arranged as 260 trimers. The innermost, 'sub-core' capsid layer is composed of 120 copies of the 100 kDa VP3 'scaffolding' protein, arranged as 12 dish shaped decamers (Grimes et al., 1998). The BTV sub-core layer, which determines the overall size and organisation of the BTV particle, surrounds the dsRNA genome segments and the transcriptase complexes (TCs), composed of the three minor structural proteins, namely: VP1(Pol), 149 kDa (RNA-dependent RNA polymerase): VP4(CaP), 76 kDa (RNA capping enzyme): VP6(Hel), 36 kDa (dsRNA helicase). These TCs are situated on the inner-surface of the sub-core, at the

twelve fivefold axes of icosahedral symmetry of the particle (Verwoerd et al., 1972, Mertens, 1999, Mertens et al., 1987).

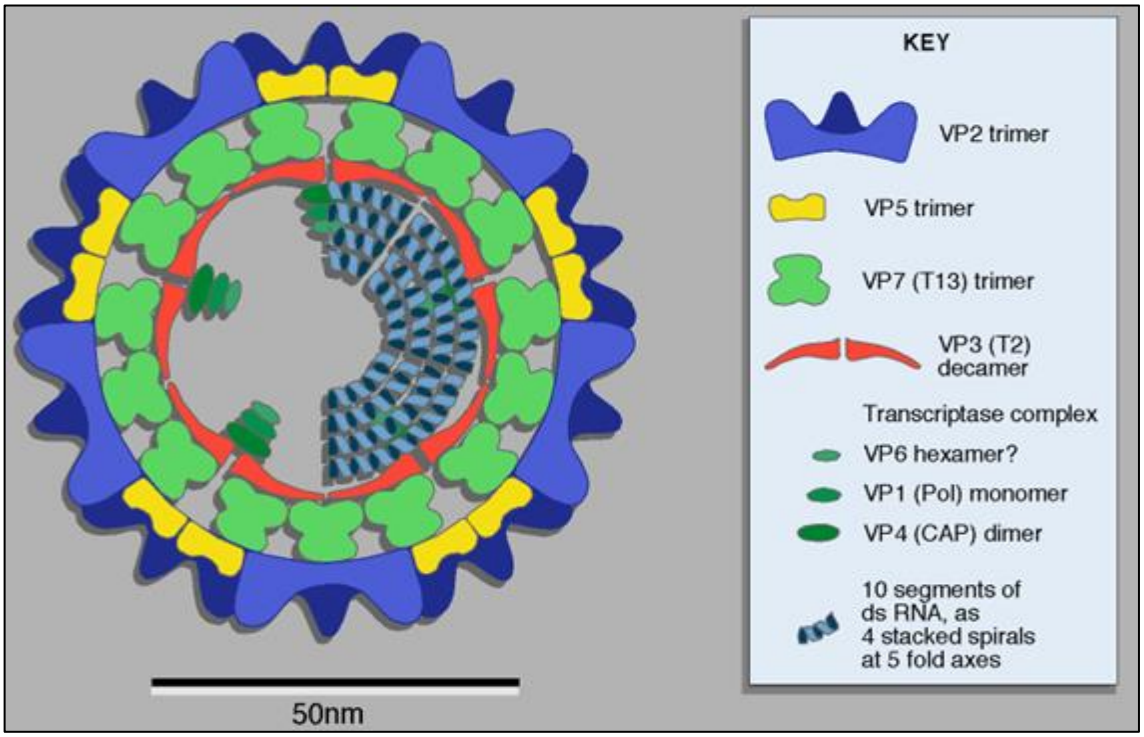


Figure 1.1 Diagram of the BTV virus particle (Mertens et al., 2003)

During cell-entry, the outer-capsid of the virion is lost, with removal of VP2 and VP5 prior to the release of the core-particle from the early endosome into the host-cell cytoplasm (Martin and Zweerink, 1972, Forzan et al., 2007). Large electron-dense structures, called viral inclusion bodies (VIBs) are considered to be the primary sites for BTV genome replication and the assembly of progeny virions (Brookes et al., 1993). Four BTV non-structural proteins (NS1, NS2, NS3/NS3a and NS4) are synthesized in virus-infected cells, but are not detected in purified virus particles. These include: the tubule protein NS1(TuP); the viral inclusion body matrix protein, NS2(ViP), the virus release protein, NS3/NS3a (Mertens, 2004) and the recently discovered NS4 protein which has a role in virus replication and in modulating the host-cell response to infection (Belhouchet et al., 2011, Ratinier et al., 2011). A putative fifth non-structural protein, 50-59 residues length, encoded by an overlapping open reading frame (S10-ORF2) in segment 10 has recently been recognised, although further evidence for its expression in infected cells is required (Stewart et al., 2015). Two of the non-structural proteins, NS1and NS2, are highly conserved (Huismans et al., 1987, Huismans and Els, 1979, Owens et al., 2004) while the

smaller NS3 protein is more variable and in the closely related African horse sickness virus (AHSV), is the second most variable protein after VP2 (Huismans et al., 2004).

1.5 The BTV genome

The genome of BTV is 19.2 Kbp in size (Roy et al., 1990b) with genome segments ranging from 3954 to 822 bp for BTV-10 (Mertens, 2004). The BTV genome encodes seven structural proteins (VP1-VP7) and four (possibly five) non-structural proteins (NS1, NS2, NS3/NS3a, NS4 and NS5), which are synthesized during virus replication in infected cells (Mertens et al., 1984, Ratinier et al., 2011, Belhouchet et al., 2011). It was originally thought that the BTV genome was monocistronic, with a single viral protein encoded by each dsRNA segment (Mertens et al., 1984), until the identification and characterization of NS4, encoded by a second out of phase and overlapping open reading frame of genome-segment 9 (Belhouchet et al., 2011, Ratinier et al., 2011, Firth, 2008). A putative fifth non-structural protein, 50-59 residues length, has also recently been identified, encoded by an overlapping open reading frame on segment 10 (S10-ORF2), (Stewart et al., 2015).

The BTV genome segments are identified as segment 1 (Seg-1) to segment 10 (Seg-10) in order of decreasing size, which matches their characteristic pattern of migration during agarose gel electrophoresis (AGE) (Mertens et al., 2009). The genome-segment migration pattern by polyacrylamide gel electrophoresis (PAGE) can vary with both polyacrylamide concentration, and virus strain (Pedley et al., 1988). The migration pattern of the genome segments, or viral RNA electropherotype, during polyacrylamide gel electrophoresis (PAGE) depends on the primary sequences of the RNAs (point mutations, insertions or deletions) and thus shows significant differences between different strains and serotypes of BTV, whereas the migration pattern of the viral dsRNA genome segments by agarose gel electrophoresis (AGE) reflects the difference in their molecular weights and thus show a more consistent electropherotype pattern, characteristic of BTV isolates, regardless of virus strain (Maan et al., 2007a, Mertens et al., 1989a, Mertens, 2000).

Conserved terminal sequences are a common feature of the genome segments of members of the family *Reoviridae* (Mertens and Sangar, 1985, Mertens, 2004) and may provide important recognition signals for initiation of transcription and RNA packaging. The positive strand of the BTV genome segments has conserved hexanucleotides at the noncoding termini of both 5’ and 3’ ends , 5’-GUUAAA…….. ACUUAC-3’ (Mertens and Sangar, 1985).

Comparisons of Seg-2 sequences show that the terminal hexanucleotides are the longest regions that are fully conserved across reference strains of the 24 types (Maan et al., 2007a). The noncoding region (NCR) at 5' varies from 8-34 bp in length whereas at 3'end, NCR ranges from 24-116 bp in length for BTV (Batten et al., 2008, Mertens et al., 2005).

Recently conserved hexanucleotide regions have been identified on the +ve strands of at least some of the BTV genome segments, that are essential for successful rescue of ssRNA transcripts from cDNA copies, into viable virus particles. These regions are therefore thought to play an essential role in the identification and packaging of the individual genome-segments during BTV genome replication and packaging (Boyce et al., 2016).

1.6 BTV structural proteins

1.6.1 Outer capsid proteins (VP2 and VP5)

Both core proteins and the non-structural proteins (NS) are highly conserved among BTV serotypes with the exception of the outer capsid proteins VP2 and VP5 which are the most variable (least conserved) proteins encoded by Seg-2 and Seg-6 respectively (Roy, 1989, Mertens, 2004). Outer capsid proteins, VP2 and VP5 are primarily involved in attachment to the cellular receptor and membrane penetration respectively during the initiation of infection (Mertens et al., 1984, Roy, 1992, Hassan and Roy, 1999, Hassan et al., 2001). Variations in VP2 and VP5 proteins are probably regarded to reflect adaptive responses by the virus to the immunological defences by the host and vector and the virus's need to attach host cells from different species (Huismans and Erasmus, 1981, Roy et al., 1994). Among all of the BTV genome segments, Seg-2 (encoding outer capsid protein VP2) has the highest rate of sequence variation, showing 29% to 59% nucleotide variation between different BTV serotypes (Maan et al., 2004, Maan et al., 2007a, Batten et al., 2008). VP2 is the major serotype-determining antigen, based on the specificity of interactions between VP2 and the neutralizing antibodies generated during infection of the host (Mertens et al., 1989b, Inumaru and Roy, 1987, Gould and Eaton, 1990, Cowley and Gorman, 1989).

Phylogenetic analyses of Seg-2 from multiple BTV isolates have established that Seg-2 divides into 27 distinct groups, precisely reflecting virus serotype, with <33% variation in nucleotide sequence within each serotype, and 29–59% nucleotide sequence variation between types (Maan et al., 2007a, Maan et al., 2010, Schulz et al., 2016, Mertens et al., 2007b). As the least conserved of the BTV genome segments, sequence variations in Seg-2 represents a basis for molecular evolutionary and phylogenetic studies and can be used for

identification of virus serotype, the geographical origins of the virus lineage and the design of primers and probes for typing specific RT-PCR assays to identify serotype and topotypes of BTV (Mertens et al., 2007a).

The second most variable of the BTV-genome segments is Seg-6, which encodes the smaller outer capsid protein VP5, which plays a minor role in determining the specificity of the neutralising antibody response, contributing to serotype determination (Maan et al., 2008, Singh et al., 2004, Cowley and Gorman, 1989).

1.6.2 Major core proteins (VP3 and VP7)

The atomic structure of VP7 revealed by x-ray crystallography, presents two distinct domains within each VP7 molecule. The upper domain of VP7 interacts with the VP2 and VP5, while the lower domain interacts with VP3 (Basak et al., 1996, Basak et al., 1992, Grimes et al., 1998). The outer/upper domain of VP7 includes an exposed arginine-glycine-aspartic acid (RGD) tripeptide motif (amino acids 168 to 170) which may mediate cell attachment and penetration of insect cells by BTV core particles (Tan et al., 2001, Basak et al., 1996, Xu et al., 1997, Darpel et al., 2009).

BTV core particles are poorly infectious for BHK cells, but show high levels of infectivity (comparable to intact virus particles) for adult *Culicoides* midges or a *Culicoides sonorensis* cell line (KC cells)(Mertens et al., 1987). Infectious subviral particles (ISVPs) of BTV are generated by treatment of virus particles with a protease (e.g. chymotrypsin), resulting in modification of the outer-capsid layer, which includes cleavage products of the VP2 protein (Marchi et al., 1995, Mertens et al., 1987, Mellor, 1990, Mertens et al., 1996). ISVP show an enhanced infectivity for adult *Culicoides* midges or a KC cells, suggesting that VP2 cleavage may be an important early step during transmission of the virus and infection of the vector insect (Mertens et al., 1987).

VP7 is the third least conserved of the BTV proteins and the most immuno-dominant of the serogroup specific BTV antigens, exhibiting serological cross-reactions at high level between any BTV strains but meagre or no cross-reaction with viruses belonging to other *Orbivirus* species (Maan et al., 2008, Huismans and Erasmus, 1981, Wilson et al., 2000, Anthony et al., 2007, Mecham et al., 2003).

Sequence analyses of BTV Seg-3 encoding VP3 have demonstrated that nucleotide sequence variations reflect correlation with the geographical grouping of BTV isolates into

topotypes with distinct eastern or western geographic variants (Nomikou et al., 2009, Maan et al., 2011b, Gould and Pritchard, 1991, Gould, 1987, Pritchard et al., 1995, Sailleau et al., 2017).

The nucleotide sequences of Seg-7 branches into multiple distinct clades that give evidence for only a partial correlation with the geographical origins of the virus lineage (Wilson et al., 2000, Maan et al., 2008). These distinct clades suggest that VP7 functions in infection of insect cells and insect vectors from various geographic regions, have resulted in additional variations that reflect adaptations to different vector populations/species (Mertens et al., 1996). However, as a result of its immune-dominant and antigenically conserved properties, VP7 provides as an appropriate target for serogroup-specific serological assays (Afshar et al., 1992a, Lunt et al., 1988, Anderson, 1984) and RT PCR assays (for designing primers and probes) to identify BTV serogroup (Anthony et al., 2007, Mertens, 2000).

1.6.3 Minor enzymatic core proteins (VP1, VP4 and VP6)

The three minor core proteins VP1 (Pol) (a monomer), VP4 (CaP) (a dimer) and VP6 (Hel) (a hexamer) of transcriptionally active BTV core particles, together form the Transcriptase or replicase complexes (TCs) which are located at each of the 5 fold axes of VP3 sub-core layer (Gouet et al., 1999). These minor proteins play a vital role in viral genome transcription and replication (Roy, 2008b) during viral infection.

VP1: VP1 is the viral polymerase VP1 (149.5 kDa, encoded by Seg-1) is the largest and most highly conserved BTV protein, (Huang et al., 1995, Roy et al., 1988). VP1 is located within the sub-viral capsid layer as 12 copies per core particle (Stuart et al., 1998) where it uses the BTV genome segments as templates for the transcription of the ten BTV mRNAs, protected from host cell cytoplasm and antiviral surveillance mechanisms (Grimes et al., 1998, Mertens and Diprose, 2004, Urakawa et al., 1989). VP1(Pol) also has replicase activity that is responsible for the synthesis of -ve sense RNA strands using the viral mRNAs as templates, reforming the dsRNA genome segments during the assembly of progeny virus particles (Boyce et al., 2004, Matsuo and Roy, 2011).

Seg-1 of BTV, which encodes VP1(Pol), is not only highly conserved across the BTV virus species but also shows significant conservation across all members of family *Reoviridae* (Huang et al., 1995). Seg-1 represents a potential target for serogroup-specific

RT-PCR assays (for design of primers and probes) to detect any BTV strains, and potentially to identify different geographic variant or topotypes of BTV (Shaw et al., 2007). Distantly related reoviruses can also be compared and their species/genus identified by analysis of the conserved nucleotide sequence of their genome segment that encode the viral polymerase (Pol) (Attoui et al., 2000a, Mertens et al., 2004, Attoui et al., 2005, Attoui et al., 2001, Belaganahalli et al., 2012, Belaganahalli et al., 2011, Belhouchet et al., 2010, Maan et al., 2011b).

VP4: VP4 is also a highly conserved BTV protein encoded by genome segment 4 (Seg-4). BTV mRNAs (+ve sense transcripts) that are synthesised at an early stage during infection of the host-cell, are modified by VP4 (Hel) within the parental core particles, which has four distinct enzyme functions: an RNA triphosphatase; guanyltransferase and two distinct methyltransferase activities (Le Blois et al., 1992, Martinez-Costas et al., 1998, Ramadevi and Roy, 1998, Roy, 2008a). These mRNAs are subsequently translated into the viral proteins by the host cell ribosomes. The 'Cap-structure' is to stabilises the viral mRNA and promotes efficient translation of most eukaryotic mRNAs (Sutton et al., 2007). However BTV mRNAs do not have a poly 'A' tail.

VP6: VP6 is the smallest enzymatic core protein (35.7 kDa) and is encoded by the conserved genome segment 9 of BTV, along with the NS4 protein. VP6 displays RNA-dependent ATPase, an RNA binding site and helicase functions (Roy et al., 1990a, Stauber et al., 1997). It is thought to unwind the dsRNA BTV genome segments, facilitating transcription by VP1, helping in separation of the newly synthesised positive sense RNA (mRNA transcript) after transcription. There are two related forms of VP6 (VP6 and VP6a) that are translated from the separate in-frame initiation codon of same ORF on Seg-9 of BTV (Wade-Evans et al., 1992).

Seg-1, Seg-4 and Seg-9 (encoding minor core protein) from different BTV strains can be separated into eastern and western geographically distinct variants / topotypes of BTV isolates by phylogenetic sequence comparison (Maan et al., 2011b, Maan et al., 2008, Maan et al., 2010).

1.7 BTV non-structural proteins (NS1, NS2, NS3, NS4 and NS5)

NS1 (64kDa viral protein, encoded by Seg-5)**:** forms tubular structures within the cell cytoplasm, that are the characteristic morphological features of BTV infection (Hewat et

al., 1992, Urakawa and Roy, 1988). NS1 'tubules' are formed by multimers of NS1 as helically coiled ribbons, 68 nm diameter and 1000 nm long (Huismans and Els, 1979). Tubules are also present in viral inclusion bodies (VIB) during early stage in infection (Brookes et al., 1993, Eaton et al., 1988). NS1 is also involved in regulating the egress of virus from the mammalian and *Culicoides* cells (Owens et al., 2004). Owens and colleagues demonstrated that high NS1: NS3 ratio correlates with virion egress by cell lysis in mammalian cells, whereas a lower NS1:NS3 ratio correlated with BTV egress via a budding (Owens et al., 2004). NS1 has been shown to increase the translation of specific viral proteins (Boyce et al., 2012).

NS2: NS2 is expressed abundantly and is a major component of the viral inclusion bodies (VIBs) that form as perinuclear structures in infected cells (Brookes et al., 1993, Thomas et al., 1990, Butan and Tucker, 2010, Kar et al., 2007, Lymperopoulos et al., 2006). VIBs play a major role in BTV replication, viral protein synthesis and the assembly of core proteins, and viral RNAs to form progeny core particles. NS2 binds to viral ssRNA and interacts with VP3 to start BTV core formation (Kar et al., 2007).

Phylogenetic sequence comparison of the highly conserved Seg-5 (encoding NS1) and Seg-8 (encoding NS2) from different BTV strains gives a clear split into geographically distinct eastern and western variants (topotypes) of BTV in a manner similar to the proteins of virus subcore (Maan et al., 2008).

NS3: early in infection, *Bluetongue virus* is released from infected mammalian cells by budding through the plasma membrane. Later in infection, larger numbers of progeny virus particles are released by cell lysis, while in insect cells, virus egress occurs without significant damage to the host cell or cytopathic effects, through the action of NS3 as a viroporin (Celma and Roy, 2009, Guirakhoo et al., 1995, Hyatt et al., 1989, Hyatt et al., 1993). There are two related forms of NS3, a 229-amino acid NS3 protein and its truncated form NS3a which is a 216-amino acid protein. These proteins are translated from the two in-frame initiation codon of same ORF on the positive strand of Seg-10 of BTV. Seg-10 also encodes the NS5 protein. NS3 helps in trafficking and release of viral particles from infected cells (Mertens et al., 2004, Beaton et al., 2002, Celma and Roy, 2009, Doceul et al., 2014, Vitour et al., 2014). It has also been proposed that NS3 hinders the host innate immune response and down regulates IFN-β promoter activation in reporter assays (Chauveau et al., 2013).

NS3 and its truncated form NS3a are the membrane glycoprotein (Wu et al., 1992) which acts as a viroporin, hydrophobic viral transmembrane proteins that enhance membrane permeability by forming pores (Han and Harty, 2004) thereby assisting in virus release by promoting membrane permeabilization. NS3/NS3a are synthesised in only very small amounts in mammalian cells but represent major translation products of the viral genome in insect cells, mediating non-lytic release of progeny virus (Noad and Roy, 2009).

Seg-10 is the fourth least conserved of the BTV genome segments and phylogenetic sequence comparison give evidence for separation into multiple distinct clades, with some geographic bias (Billinis et al., 2001, Balasuriya et al., 2008, Bonneau et al., 1999, Nikolakaki et al., 2005, Nikolakaki et al., 2004, Maan et al., 2008). It has been suggested that (like Seg-7/VP7) in view of the significance of NS3 for release of BTV virions from infected insect cells that these clades reflect adaptation to different insect vector populations / species in different geographic regions.

NS4: Bioinformatics analysis of Seg-9 encoding VP6 showed the presence of an overlapping +2 open reading frame (ORF), positioned at +1 position downstream to the VP6 ORF (Firth, 2008, Belhouchet et al., 2011, Ratinier et al., 2011). NS4, is a 77-79 amino acid protein which localises in the nucleolus and is highly conserved among different BTV serotypes as well as in most other *orbiviruses* (Ratinier et al., 2011, Belhouchet et al., 2011, Firth, 2008). Although the role of NS4 has not been full determined, its association with cell membrane is thought to indicate a role in virus cell-exit (Belhouchet et al., 2011). Ratinier and colleagues found that BTV-8 NS4 counteracted antiviral responses of the host, and it may therefore associated with evasion of the host immune system (Ratinier et al., 2011).

NS5: A putative fifth non-structural protein, 50-59 residues in length, encoded by an overlapping open reading frame (S10-ORF2) in segment 10, has recently been identified, although further evidence for its expression in infected cells is still required (Stewart et al., 2015).

1.8 The BTV replication cycle

1.8.1 Attachment and entry

Following attachment of virions to specific receptors on the host cell surface, BTV enters the cell by receptor mediated endocytosis into clathrin-coated vesicles (Forzan et al., 2007)

or alternatively by micropinocytosis (Gold et al., 2010). Outer capsid protein, VP2 is primarily involved in attachment to the cellular receptor, while VP5 is involved in cell-membrane penetration during the initiation of infection (Mertens et al., 1984, Roy, 1992, Hassan and Roy, 1999, Hassan et al., 2001).

Cell attachment and penetration of insect cells by BTV core particles is mediated by VP7, and can be neutralised by VP7 specific antibodies, suggesting more than one mechanisms of BTV entry. This interaction may be mediated by the arginine-glycine-aspartate (RGD) motifs (integrin binding domains) that are displayed on the outer/head domain of VP7 (Mertens et al., 1987, Mertens et al., 1996, Hynes, 1992, Tan et al., 2001, Grimes et al., 1998, Forzan et al., 2007).

1.8.2 Uncoating

The low pH of early endosome favours dissociation of VP2 from the core layer (Forzan et al., 2004, Zhang et al., 2010) and conformational changes in VP5 that can induce membrane fusion and release of the transcriptionally active core particle through the endosome membrane into the host cell cytoplasm (Forzan et al., 2004, Hassan et al., 2001, Hassan and Roy, 1999, Noad and Roy, 2009).

1.8.3 Transcription

For the family *Reoviridae*, transcription and replication of the viral genome take place within the cytoplasm of host cell. However, in order to protect the viral dsRNA from antiviral surveillance mechanisms of host cell cytoplasm, the viral genome stays contained within the core particle (Mertens and Diprose, 2004). The three minor core proteins VP1 (Pol), VP4 (CaP) and VP6 (Hel) form the transcriptase / replicase complexes, located at each of the 5 fold axes of VP3 sub-core layer of the transcriptionally active BTV core particles. Together these proteins mediate viral genome transcription and replication during infection (Roy, 2008b, Gouet et al., 1999, Mertens and Diprose, 2004).

The RNA-dependent RNA polymerase (transcriptase) activity of VP1 transcribes +ve strand ssRNAs (mRNA) from negative strand of each of ds RNA genome segment within the core particle (Boyce et al., 2004, Urakawa et al., 1989). The helicase function of VP6 (Hel) unwinds the dsRNA genome segments to allow transcription by VP1 (Pol) and may help in separation of the newly synthesised mRNA after transcription (Roy et al., 1990a,

Stauber et al., 1997). The four distinct enzyme functions of VP4 (CaP) which include: an RNA triphosphatase; guanyltransferase and two methyltransferase activities, generate methylated cap structures (^{7}mGpppGm) at the 5' termini of viral mRNAs synthesised early during infection (Le Blois et al., 1992, Martinez-Costas et al., 1998, Ramadevi et al., 1998, Roy, 2008b). The cap structures stabilise the viral mRNAs and promote their efficient translation (Sutton et al., 2007). The capped transcripts leave the core structure through channels at the 5 fold axes (Diprose et al., 2001, Gouet et al., 1999, Grimes et al., 1998, Mertens and Diprose, 2004, Verwoerd and Huismans, 1972). BTV mRNAs don't possess a poly (A) tail at their 3' termini. Translation of BTV proteins utilizes the translational machinery of the host cell, competing with host cell mRNAs (Huismans, 1979). BTV infection results in shut-off of host-cell protein synthesis in mammalian cells, but has little overall effect on host-protein synthesis in insect-vector cells. NS1 facilitates up-regulation of specific BTV genes expression for the synthesis of viral proteins (Boyce et al., 2012). Viral inclusion bodies (VIBs) play a major role and represent a site for virus genome-replication and assembly of progeny core particles, from viral proteins that are synthesised within the cytoplasm of infected cells (Brookes et al., 1993).

1.8.4 Replication

VP1 with its replicase activity synthesizes the negative strand RNA from positive strand RNA or early mRNAs within the nascent progeny subviral particles (Boyce et al., 2004).

1.8.5 Assembly and release

Viral transcripts are directed to the viral inclusion bodies (VIB), possibly by interactions with the phosphorylated form of NS2, where negative strand synthesis, nascent progeny core particle assembly and genome encapsidation take place. NS2 binds to viral single stranded RNA and interacts with VP3 to start BTV core formation (Kar et al., 2007). The progeny virions are surrounded by outer-capsid proteins at the periphery of VIBs, when virions exit the VIBs to enter the host cell cytoplasm (Kar et al., 2007, Brookes et al., 1993). VP3 proteins can self-assemble to form the subcore-shell and act as a scaffold for attachment of the internal replicase complexes (Grimes et al., 1998) as well as the trimmers of outer-core protein VP7. Bluetongue virus is released from infected cells by lytic cycle (Schwartz-Cornil et al., 2008), cell membrane permeabilization (Fu, 1995), or through the role of NS3 in trafficking and release of BTV particles by its viroporin activity in insect cells, predominantly without inducing cytopathic effect (Noad and Roy, 2009, Celma and

Roy, 2009, Guirakhoo et al., 1995, Hyatt et al., 1989, Hyatt et al., 1993, Owens et al., 2004). There is also evidence that orbiviruses can leave infected cells by incorporation of multiple particles into intracellular vacuoles, that can subsequently fuse with the host cell-membrane releasing their contents (Maree and Huismans, 1997).

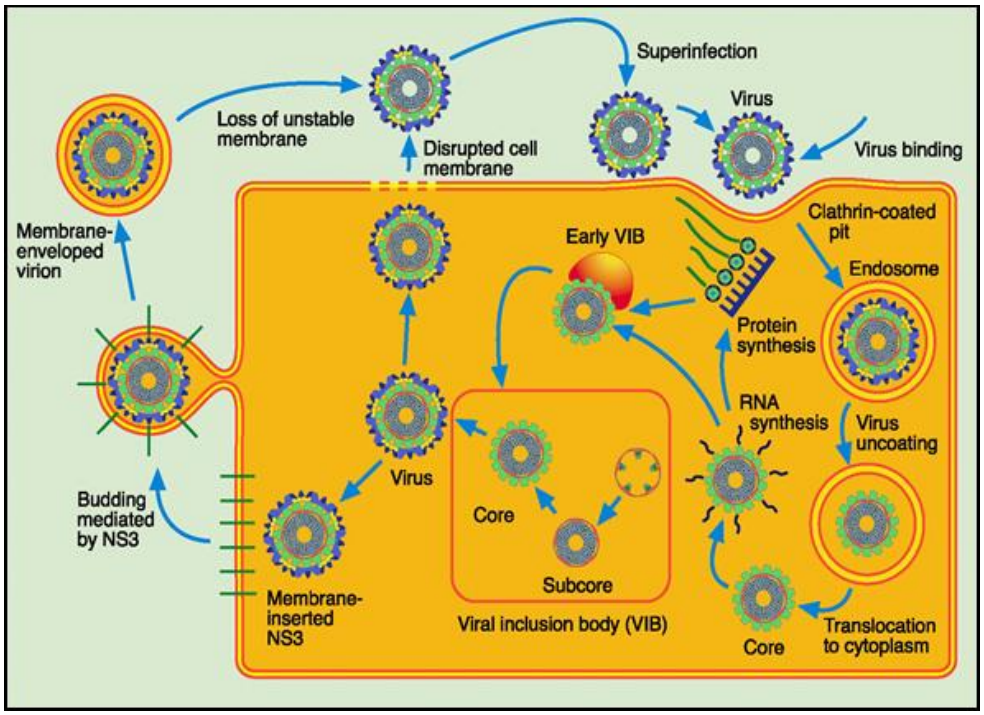


Figure 1.2 Schematic representation of the BTV replication cycle

(Adapted from Mertens and Diprose, 2004 – with permission).

1.9 BTV pathogenesis

1.9.1 Clinical signs

Although BTV can infect most domestic and wild ungulates, camelids and even certain carnivores (via an oral route) severe disease is usually seen primarily in certain breeds of sheep particularly in naïve populations of European fine wool and mutton breeds as well as some species of wild ruminants such as white tailed or brocket deer (Erasmus, 1975, Taylor, 1986, Vosdingh et al., 1968, Fernandez-Pacheco et al., 2008, Meyer et al., 2009). In adult cattle and goats, BT disease is often asymptomatic or inapparent (Parsonson, 1990, Barratt-Boyes and MacLachlan, 1995, Verwoerd and Erasmus, 2004), although infection can cause reproductive failure, abortion and loss of milk yield. The symptoms of bluetongue in sheep have been well documented, and include fever, serous or blood stained nasal discharge, ocular discharge, excessive salivation, oral erosions and ulcers, wool

break, lameness, coronitis, weakness, depression, torticollis/ wryneck, respiratory distress, facial and pulmonary oedema (Spreull, 1905, Verwoerd and Erasmus, 2004, Moulton, 1961, Maclachlan, 2011, MacLachlan et al., 2008, Maclachlan et al., 2009). The clinical course of disease is variable with symptoms ranging from acute to subacute, mild or inapparent depending on the virus strain as well as species, breed, age and immune status of infected host (Verwoerd and Erasmus, 2004, Elbers et al., 2008b, MacLachlan, 1994, Parsonson, 1990, Barratt-Boyes and MacLachlan, 1995). The clinical manifestations of BT in adult sheep are at least in part the result of vascular injury mediated by virus that causes increased vascular permeability, oedema, hyperaemic congestion and haemorrhagic infarction (Verwoerd and Erasmus, 2004, Moulton, 1961, Chiang et al., 2006, MacLachlan, 1994). Mortality generally varies from 2 to 30% but may be more in naïve and susceptible flocks (Cox, 1954, Conraths et al., 2009, Erasmus, 1975). Viraemia occurs in sheep around three to five days post infection, followed by congestion of oral mucosa as an early sign of disease (Foster et al., 1991). In contrast, goats and cattle usually remain asymptomatic and rarely show symptoms other than anorexia and viremia, typical in enzootic areas (Erasmus, 1975, Luedke and Anakwenze, 1972, Maclachlan et al., 2009, Caporale et al., 2014). The BTV-8 strain outbreak in northern Europe during 2006-2009 did cause some clinical signs in naïve cattle, although their mortality rate was <1%, compared to ~25% in sheep (Backx et al., 2007, Dal Pozzo et al., 2009a, Dal Pozzo et al., 2009b, Elbers et al., 2008a, Barratt-Boyes and MacLachlan, 1995, MacLachlan et al., 1990, Elbers et al., 2008b). It has been reported that cattle have inapparent BTV infection due to less susceptibility to BTV-induced lung microvascular injury (DeMaula et al., 2001, DeMaula et al., 2002a, McLaughlin et al., 2003, Russell et al., 1996).

Clinical manifestation of BTV infections in cattle represent a mild form of disease, showing viremia, anorexia, oral and nasal mucosae congestion, serous or mucopurulant nasal discharge, excessive frothy salivation, tongue oedema, erosions, coronitis, dermatitis (Bekker et al., 1934), udder lesions and decrease in milk production as an additional clinical sign (Bekker et al., 1934, Dal Pozzo et al., 2009b, Thiry et al., 2006, Williamson et al., 2008). Recent studies have confirmed that BTV can cross the placenta in both pregnant cattle and sheep, resulting in teratogenic effects in the foetus (dummy calf syndrome). There is also evidence for reduced reproductive success (and productivity) during BTV outbreaks that may result from failed implantation of the embryo, again causing loss of milk yield.

BTV antibodies have been detected by serological assays, in both large and small carnivorous species from Africa suggesting of BTV infection (Alexander et al., 1994, Coetzee et al., 2012a). There is a recorded sero-prevalence of BTV specific antibodies of ~60% of elephants in India, indicating infection (K.P. Singh personal communication), suggesting that BT may be a factor in the survival of endangered mammalian wildlife species. In addition, there is evidence of BTV infection in dogs through the use of contaminated canine vaccine or in lynx by feeding on infected meat (Akita et al., 1994, Alexander et al., 1994, Fernandez-Pacheco et al., 2008, Jauniaux et al., 2008, Levings et al., 1996, Wilbur et al., 1994, Evermann, 2013, Brown et al., 1996).

1.9.2 Pathogenesis

Adult *Culicoides* can transmit BTV by intradermal inoculation during feeding on susceptible hosts. The protease activity of saliva in competent *Culicoides* can cleave BTV outer capsid protein, VP2 generating infectious subviral particles [ISVP] thereby promoting infectivity of BTV particles for the vector insects (Darpel et al., 2011). Saliva of *Culicoides* can also enhance BTV infectivity by modulating the host's immune reaction, recruiting leucocytes (inflammatory cells), which can become infected, facilitating the initial replication of BTV and spread from the initial bite-site (Darpel et al., 2011, Pages et al., 2014). After primary replication of BTV in regional lymph nodes involving dendritic cells, lymphocytes, mononuclear cells, and macrophages, virus dissemination occurs, allowing virus replication in endothelial cells and mononuclear phagocytes of secondary sites like the lungs, spleen, heart, intestine and other tissues (Barratt-Boyes and MacLachlan, 1995, Barratt-Boyes and MacLachlan, 1994, Barratt-Boyes et al., 1995, MacLachlan et al., 1990, MacLachlan, 2004).

BTV replication at primary sites occurs for 2–3 days, whereas secondary replication takes between 4 and 20 days, depending on the course of the disease (MacLachlan, 2004). BTV can spread through both the lymph (Barratt-Boyes and MacLachlan, 1994) and blood (Brodie et al., 1998) including mononuclear cells, erythrocytes, and thrombocytes.

Diagnosis of BTV by detection of viral RNA using RT-PCR assays, does not by itself confirm that an animal is still infectious. Blood of infected animal had been found positive for BTV RNA by RT PCR for > 220 days post-infection, but virus could not be isolated in cell culture or inoculation of susceptible sheep from RT-PCR positive samples (MacLachlan, 1994, Bonneau et al., 2002). Ruminant blood detected positive by RT- PCR

many months post-infection was also found non-infectious to *Culicoides* by either intra thoracic inoculation or oral feeding (Bonneau et al., 2002, Tabachnick, 1996).

BTV causes cell associated and prolonged viraemia in infected animals but there is little evidence of a persistent infection (Barratt-Boyes and MacLachlan, 1995, Bonneau et al., 2002, MacLachlan et al., 1994, Singer et al., 2001). In sheep, viraemia can persist in the blood for 14 to 54 days (Koumbati et al., 1999) and in cattle up to 60 or even 100 days (Sellers and Taylor, 1980). However, BTV RNA can be detected by RT-PCR for 3-100 days in sheep and up to 220 days in cattle post-infection (Singer et al., 2001, Bonneau et al., 2002, MacLachlan et al., 1994). Virus is found in invaginations of the cell membrane of erythrocytes, thereby promoting longer viraemia, protection from host immune clearance, potentially maintaining infectivity for *Culicoides* that feed on viraemic ruminants (Brewer and MacLachlan, 1992, Brewer and MacLachlan, 1994). BTV can cause persistent infections of gamma-delta T cells (Takamatsu et al., 2003) and infectious virus can persist for long in immune-privileged sites, such as the testes, potentially leading to venereal transmission.

Marked difference in the clinical expression between sheep and cattle after BTV infection could be related to inherent species-specific differences in the sensitivity of endothelial cells to BT infection and differential production of inflammatory cytokines (Coen et al., 1991). Endothelial cells and mononuclear phagocytic cells of the lungs and lymphoid tissues are the main site for BTV replication in both sheep and cattle (Barratt-Boyes and MacLachlan, 1994, Barratt-Boyes and MacLachlan, 1995, MacLachlan et al., 1990, Pini, 1976). After infection activated endothelial cells produce many inflammatory mediators, including PGF2α thromboxane, prostacyclin, IL-1, IL- 6, IL-8, cyclo-oxygenase-2, and nitric oxide synthase (DeMaula et al., 2002a, Schwartz-Cornil et al., 2008). Proinflammatory cytokines produced by ovine endothelial cells are substantially greater in quantity than bovine endothelial cell (DeMaula et al., 2001, Russell et al., 1996, DeMaula et al., 2002b). In addition, markedly increased ratios of prostacyclin to thromboxane are detected in cattle (Schwartz-Cornil et al., 2008). Prostacyclin is a vasodilation and platelet aggregation inhibitor, while thromboxane is a vasoconstrictor and promotes platelet aggregation. Significantly increased ratio of thromboxane to prostacyclin could explain enhanced clinical manifestations of microvascular injury, thrombosis and oedema in ovine species, in contrast to cattle (DeMaula et al., 2001, DeMaula et al., 2002b).

1.10 Evolution and molecular epidemiology of BTV

Viral quasi-species is a continuously changing swarm or cloud of closely related variants centered around a consensus sequence but continuously able to emerge or disappear in different directions as a new mutant within a single population (Andino and Domingo, 2015). Bluetongue virus evolution occurs through the mechanism of quasispecies that is driven by accumulation of mutations or nucleotide substitution (genetic drift), reassortment (genetic shift), intra-segment recombination and concatemerisation (gene duplication), although the latter two mechanisms are thought to be rare but significant outcomes (Carpi et al., 2010, Maan et al., 2012c, He et al., 2010, Anthony et al., 2011, Coetzee et al., 2012b, Mohd Jaafar et al., 2014, Nomikou et al., 2015, Shafiq et al., 2013, Bonneau et al., 2001, Oberst et al., 1987, Stott et al., 1987, Shaw et al., 2013, Batten et al., 2008). Quasispecies evolution, together with founder effect and strong purifying selective pressures have over time accumulated genetic heterogeneity and variations in BTV strains, that can influence replication or transmission efficiency of virion (Niedbalski, 2013, Balasuriya et al., 2008).

Segmented RNA viruses of the same species, that co-infect a single host cell, can exchange genome segments during replication generating hybrid progeny virus strains by a process known as 'reassortment'. The compatibility of viruses for reassortment is considered as the primary determinant showing that different strains belonging to the same virus species (Simon-Loriere and Holmes, 2011). Although several studies have confirmed that BTV reassortment is frequent and widespread most of these studies are based on limited, sub-genomic data sets, impeding specific observations into the frequency and patterns of reassortment between all ten segments (Nomikou et al., 2015). Samal and colleagues demonstrated reassortment in 5% of the progeny clones from a sheep that was infected with BTV 10 and BTV 17 (Samal et al., 1987) whereas 90% of progeny virions were reassortants within a calf infected with BTV-11 and BTV-17 (Stott et al., 1987). Batten *et al* (2008) provided evidence of a European BTV field strain, derived by reassortment of two parental live attenuated-vaccine strains of BTV-2 and BTV-16, supporting concerns about the use and transmission of live attenuated BTV vaccines in the area. The co-circulation of multiple BTV serotypes and strains in BTV endemic areas and their concurrent infection in the same host and cells provide multiple opportunities for reassortment and suggest circulation of a complex quasi-species (McColl and Gould, 1994).

Although BTV outbreak strains are usually identified by serotype, which is determined by the sequence of Seg-2, this does not reveal the extensive genotypic and phenotypic heterogeneity that exists in BTV field strains and populations, even within the same BTV serotype (Nomikou et al., 2015).

1.10.1 BTV serogroup, serotypes and topotype

Orbivirus serogroup reflects antigens that are both conserved and cross-reactive between members of the individual virus species which exists as a complex spectrum of serotypes, topotypes and strains. The BT serogroup specific antigens include most of the non-structural and core proteins. In contrast, BTV serotype is determined by the BTV outer capsid proteins VP2 and VP5, particularly VP2, which primarily controls the specificity of interactions with neutralising antibodies in serum neutralisation assays.

BTV serotypes can be detected and differentiated by RT-PCR and phylogenetic analyses targeting Seg-2 of the virus genome, which codes for outer capsid protein VP2 (Maan et al., 2007a, Mertens et al., 2007a) .There are 24 distinct serotypes of BTV that have been known for several decades, as well as the recently identified Toggenburg virus which was detected during routine surveillance of goats in Switzerland during 2008 and identified as as BTV-25 (Hofmann et al., 2008b, Chaignat et al., 2009), BTV-26 was identified in clinical samples from infected sheep in Kuwait (Maan et al., 2011b) and BTV-27 was recently identified in asymptomatic goats from Corsica, France (Zientara et al., 2014, Schulz et al., 2016). Two further putative types, include BTV-28 from the Middle East detected in a Sheeppox vaccine preparation (Bumbarov et al., 2016) and BTV-29 in an Alpaca from South Africa (Wright, 2014). Studies of BT outbreaks in Mongolia have also identified three additional putative novel serotypes (Hoffman personal communication 2017).

Sequencing studies indicate that BTV strains in separated geographic regions have evolved independently over an extensive period of time. This geographical separation has allowed viruses to gain unique point mutations, some of which may make them more fitted to transmission and survival in their respective local ecosystems. Genetic heterogeneity of BTV strains as a result of genetic drift, genetic shift and selection, have gradually led to the appearance of distinctive regional variants that can be divided into eastern and western topotypes for all of the genome segments of BTV. The eastern BTV group generally constitutes viruses from South Asia, South-East Asia, China and Australia, while viruses

from Africa ,the Americas, Europe and the Middle East are mostly included in the western BTV group (Maan et al., 2007a, Maan et al., 2008, Mertens et al., 2007a).

The most conserved structural and non-structural proteins of the BTV are VP1, VP3, VP4, VP6, NS1, NS2 and NS4 encoded by the conserved Seg-1, Seg-3, Seg-4, Seg-9, Seg-5, Seg-8 and Seg-9 respectively (Maan et al., 2008). Seg-7 and Seg-10 are the third and fourth most variable of the all the BTV genome-segments, while Seg-2 and Seg-6 are the first and second most variable, respectively (Maan et al., 2008, Huismans and Erasmus, 1981, Wilson et al., 2000, Balasuriya et al., 2008, Bonneau et al., 1999, Nikolakaki et al., 2005, Nikolakaki et al., 2004, Anthony et al., 2007, Billinis et al., 2001, Mecham et al., 2003).

Sequencing and phylogenetic studies of Indian BTV isolates, have used full length or partial analyses of selected individual genome segments (particularly Seg-2) of different BTV isolates, rather than full genome analyses (Sreenivasulu et al., 1999, Biswas et al., 2015, Tembhurne et al., 2010, Desai et al., 2009, Shafiq et al., 2013). Although these studies have contributed significantly to our understanding concerning the serotypes, origins and evolutionary relationships of the BTV strains in India, a comprehensive approach to the sequencing and analysis of full genome of Indian BTV isolates is still missing.

Recently, full-length whole-genome sequence data have been generated for multiple Indian BTV isolates, contributing additional knowledge about reassortment of field strains and topotype of each genome segment (Maan et al., 2012a, Maan et al., 2012b, Maan et al., 2012c, Maan et al., 2012d, Rao et al., 2012b, Chand et al., 2016, Kumar et al., 2016, Rao et al., 2015, Maan et al., 2015c, Minakshi et al., 2012).

Sequencing and phylogenetic analyses of 26 Indian BTV isolates by Maan *et al* (2015c) confirmed that most of the genome-segments are derived from eastern lineages, grouping within the major eastern topotype. However, Seg-5 encoding NS1, from multiple Indian BTV isolates post 1982, originate from the major western topotype (Maan et al., 2012b, Maan et al., 2015c, Maan et al., 2012e, Maan et al., 2012d). Maan and colleagues showed that all 10 segments of BTV-2 isolates (IND2003/01, IND2003/02 and IND2003/03) belong to the western topotype and share >99% nucleotide identity with a BTV-2 vaccine-strain from South Africa, indicating an earlier introduction of this vaccine-strain into India (Maan et al., 2015c, Maan et al., 2012b). In addition, all of the segments of BTV-10

isolates (IND2003/06; IND2005/04, IND2004/01) are also derived from western lineages, sharing >99% nucleotide identity to a BTV-10 vaccine-strain from United States (Maan et al., 2012e, Maan et al., 2015c, Rao et al., 2012a). These data raise concerns over the movement of western field and/or vaccine-strains of BTV into India, possibly associated with livestock trade, importation of infected animals, vector movements, and/or the unauthorized importation and use of live attenuated vaccine strains from South Africa and America into the country (Rao et al., 2012a). Sequencing databases can be used as a basis for tracing the origins, spread, and reassortment events during the evolution of individual outbreak strains, as well as improving diagnostics and vaccine development/matching to field strains.

1.11 DNA sequencing

DNA sequencing is the process to determine the exact order of four nucleotide bases- adenine, guanine, cytosine and thymine in a DNA molecule.

1.11.1 The Maxam and Gilbert method

Maxam-Gilbert, or chemical sequencing is based on specific chemical fragmentation of terminally labelled DNA at Adenine, Guanine, Cytosine or Thymine nucleotides site (Maxam and Gilbert, 1977). This was the earliest sequencing technique used to determine the genome sequences of *Orbivirus* isolates (Ghiasi et al., 1985, Purdy et al., 1985, Roy et al., 1985)

1.11.2 Sanger's dideoxy sequencing technology

Sanger or dideoxy chain termination sequencing relies on the enzymatic synthesis of the complementary strand of DNA, coupled with chain termination. Synthesis of complementary strands is terminated by the incorporation of radiolabelled or fluorescently labeled dideoxynucleotides (ddNTP), which block progression of a DNA polymerase, at a specific base, thereby causing chain termination (Sanger et al., 1977). The exact order of the four nucleotide namely adenine, guanine, thymine and cytosine in a particular strand of DNA was identified by separating the fragments of DNA using electrophoresis and exposure of X-ray film to the radiolabeled fragments (at 5' end with P32 or S35), or using fluorescently labeled dideoxynucleotides and automated fluorescence sequencing equipment, in the Sanger sequencing approach. Dideoxy chain termination sequencing

became the principal sequencing method for analysis of the BTV genome segments, using conserved terminal sequence primers (Kowalik and Li, 1989, Gould, 1987, Cooke et al., 1991).

1.11.3 Next generation sequencing

Broadly, there are two sequencing approaches in next generation sequencing: short-read and long-read. Short-read sequencing is further categorized into sequencing by ligation (SBL) and sequencing by synthesis (SBS). Sequencing by synthesis includes cyclic reversible termination and single-nucleotide addition approaches whereas SOLiD and Complete Genomics platforms use sequencing by ligation approach of sequencing. The long read sequencing approach uses a single-molecule real-time (SMRT) and synthetic long-read sequencing approach (Goodwin et al., 2016, Metzker, 2010, Mardis, 2008). Long-read sequencing helps to reveal structural features of highly complex genomes, in terms of repetitive regions, structural variations (larger than single nucleotide polymorphism), genome assembly, large structural features of the genome and copy number alterations.

There are many elemental steps that are common to all high throughout sequencing approaches. These steps are: the library preparation from the genomic DNA or complementary DNA (cDNA); amplification of molecules within the library; immobilization of DNA fragments onto a solid support; massively parallel sequencing; and detection by imaging (Shendure and Lieberman Aiden, 2012, Shendure and Ji, 2008)

1.11.3.1 Illumina (MiSeq, NextSeq 500, and HiSeq)

This is based on cyclic reversible termination approach that is based on the Sanger sequencing principle, involving hybridization of fluorophore-labelled 3´-blocked deoxynucleotides to complementary bases, imaging and finally cleavage of fluorophores, followed by regeneration of 3' OH group. Briefly, in a four color cyclic reversible dye terminator sequencing approach, each adapter ligated DNA molecule of the library is captured on the glass flow cell and undergoes clonal amplification by bridge PCR, to generate a fluorescent cluster. The fluorescence is read for millions of clusters simultaneously and dye terminators are removed to regenerate 3' OH and process is repeated (Goodwin et al., 2016)

1.11.3.2 Ion Torrent (Life Technologies)

This is based on single nucleotide addition approach which uses incorporation of a single specific nucleotide by a DNA polymerase during each cycle, to produce a detectable signal for that nucleotide. Ion Torrent (Life Technologies) use clonal amplification of adaptor ligated DNA, by emulsion PCR and change in pH induced by removal of hydrogen ion during base incorporation is measured (Mardis, 2008).

1.11.3.3 Roche pyrosequencing platforms

Roche (GS FLX sequencing system) is also based on single nucleotide addition approach which uses incorporation of a single specific nucleotide by a DNA polymerase during each cycle, to produce a detectable signal for that nucleotide. However, unlike Ion Torrent, which measures change in pH is induced by removal of hydrogen ion during base incorporation, Roche (GS FLX sequencing system) couples base addition with release of a pyrophosphate, which in turn is converted into light by luciferase enzyme (Mardis, 2008, Goodwin et al., 2016, Metzker, 2010, Niedringhaus et al., 2011, Zhang et al., 2011).

1.11.3.4 SOLiD sequencing

This is based on sequencing by ligation approach of sequencing in which a fluorophore-labelled degenerate probe, with one or two known bases is hybridized to complementary bases in the target DNA fragments, followed by ligation of the fluorescent probe to an anchor (a known sequence) which hybridizes to an immobilized adaptor sequence followed by imaging to identify the template base complementary to a known base within probe (Goodwin et al., 2016).

1.11.3.5 Third generation sequencing

Single molecule real-time sequencing (SMRT): The DNA polymerase enzyme is immobilized to the bottom of zero-mode waveguides (picolitre wells) in contrast to Illumina approach where the polymerase is free to travel along the DNA template. The template fragments are converted into circular molecules by ligated hairpin adapters at each end of the template, to allow multiple repeated sequencing by the same polymerase molecule, for efficient signal production in SMRT approach. This approach involves short-read technology using barcodes (during library preparation) to generate longer long reads

by computational assembly *in silico*. Pacific Biosciences (PacBio) platform is based on SMRT sequencing approach (Goodwin et al., 2016).

1.11.4 Sequencing approaches for dsRNA viruses

The development of sequence-independent approaches, namely single primer amplification technique (SPAT) and full-length amplification of cDNA (FLAC) for the amplification of full-length cDNA copies of segmented dsRNA viruses, permitted full length segment sequencing without prior sequence knowledge (Attoui et al., 2000b, Potgieter et al., 2009, Lambden et al., 1992, Maan et al., 2007b). Sequencing of amplified cDNA is accomplished by sequencing either cloned individual segments in SPAT (Attoui et al., 2000b, Lambden et al., 1992, Potgieter et al., 2002, Vreede et al., 1998) or by using purified PCR amplicons of individual genome segments (without cloning) in FLAC (Maan et al., 2007b). Potgieter *et al*, 2009 showed a more efficient method of genome amplification combined with high throughput approach to obtain the full-length sequence of viral dsRNA genomes from clinical samples without prior virus cultivation, sequence determination, cloning or separation of PCR amplicons (Potgieter et al., 2009). These approaches to sequencing the BTV genome have been further strengthened during the era of 'high-throughput' or 'next generation' sequencing technologies, enhancing our understanding of the molecular epidemiology of BTV (Lorusso et al., 2014, Chand et al., 2016, Yang et al., 2012, Jenckel et al., 2015, van den Bergh et al., 2016, Savini et al., 2017b, Rao et al., 2012c, Boyle et al., 2012, Caporale et al., 2014, Breard et al., 2016, Nomikou et al., 2015, Minakshi et al., 2012). Although extensive BTV sequences are now available in public databases, there is still only a limited number of full-length, whole-genome sequences, for isolates with fully documented metadata concerning their original date and place of isolation, passage history, host species, clinical significance and vector species.

1.12 BTV control

BT was formerly classified as a 'class A disease', by the Office International des Epizooties (OIE) and remains a 'notifiable' disease due to its economic impact on the international livestock trade (OIE, 2017). Taking into account that BTV is non-contagious and virus is transmitted primarily through the bites of infected adults of competent *Culicoides* spp., control measures primarily involve prevention of virus spread and include: (a) movement restrictions of susceptible and infected animals, (b) control of *Culicoides*

vectors, (c) removal of infected animals to interrupt BTV transmission cycle (d) modifications to husbandry practices, which include housing of susceptible animals, removal of manure and drainage of wet areas (e) introduction of surveillance and protection zones and (f) vaccination (Alpar et al., 2009).

Though, many types of BTV vaccines have been prepared including inactivated, attenuated, subunit and recombinant vaccines, only attenuated and inactivated BT vaccines have been widely commercially available to vaccinate livestock (Noad and Roy, 2009, Savini et al., 2008, Di Emidio et al., 2004, Osburn et al., 1996, Niedbalski, 2011). Vaccination approaches in various countries are developed according to national policies and the local epidemiology of BTV.

1.12.1 Attenuated vaccines

Modified live virus (MLV) vaccines can be produced by serial passage of BTV in cell cultures or embryonated chicken eggs in order to attenuate the virus. Although, MLV BT vaccines still infect the vaccinated ruminant host, they produce a strong antibody response, helping to prevent infection with field strains and severe clinical BT disease (Hunter and Modumo, 2001, Lacetera and Ronchi, 2004, Savini et al., 2008). They are also cost effective and require only single inoculation annually.

However, live vaccines can also present many disadvantages. MLV vaccinated animals may show varying degree of clinical signs, including abortions, teratogenic effects, decreased milk yield and poor semen quality. In addition, the attenuated vaccine virus strain may revert to virulent and can reassort with both field strains or other vaccine strains to produce more infectious and more virulent novel strains (Stott et al., 1987, Maclachlan, 2010, Osburn et al., 1971, Young and Cordy, 1964, Batten et al., 2008, Dungu et al., 2004a, Monaco et al., 2004, Savini et al., 2010, Patta et al., 2004, Zientara et al., 2010, Ferrari et al., 2005). Animals vaccinated with BTV MLV strains can also show high levels of viraemia after vaccination, which facilitates transmission of vaccine strain in *Culicoides* vector (Savini et al., 2010, Veronesi et al., 2010, Veronesi et al., 2005, Ferrari et al., 2005). Finally, MLV vaccines are not DIVA compliant, thus it is not possible to differentiate vaccinated animals from naturally infected animals (Savini et al., 2008, Zientara et al., 2010).

BT vaccination in South Africa (an endemic area for multiple BTV serotypes) is based on multivalent live attenuated BT vaccines, administered as three sequential injections containing different BTV strains and a total of sixteen different serotypes, produced by Onderstepoort Biological Products , Pretoria, South Africa (Dungu et al., 2004b, Savini et al., 2010, Veronesi et al., 2010, Savini et al., 2014). Monovalent MLV formulations have also been used since 2000 in Spain, Italy, Portugal and France according to local epidemiology of BT in a given region (Savini et al., 2008, Patta et al., 2004). Due to the disadvantages associated with attenuated vaccines, these vaccines have been replaced with inactivated BT vaccines in Europe after 2005 (Zientara et al., 2010). However, MLV are still in current use in North America (Scott and James, 2015).

1.12.2 Inactivated vaccines

Chemically inactivated, tissue culture grown, BTV vaccines are safe, efficacious and protective against the homologous BTV serotype, but are expensive and may require a booster in comparison to MLV vaccine (Zientara et al., 2010, Savini et al., 2007, Savini et al., 2008, Wackerlin et al., 2010, Hamers et al., 2009). After the BTV-8 incursion into northern Europe in 2006, millions of animals were vaccinated with the BTV-8 inactivated vaccine. Commercial inactivated BTV vaccines are not DIVA compliant using serological assays, but it is possible to detect and identify animals that have been infected with field strains using RT-PCR assays to detect the viral RNA (Calvo-Pinilla et al., 2014).

1.12.3 Virus-like particles

Virus-like particles (VLP) (empty viral capsids) are structurally similar to native BTV particles but do not contain the viral RNA genome and are non-infectious. VLP are formed by the expression and assembly of the four major BTV structural proteins, VP2, VP3, VP5 and VP7 using recombinant Baculoviruses expression system (French and Roy, 1990, French et al., 1990), or by expression of these proteins in plants (Thuenemann et al., 2013). VLP contain functional viral proteins required for penetration and entry into the cell, for eliciting immune response (Perez de Diego et al., 2011). VLP can elicit good protection with homologous virus and partial protection even with heterologous BTV serotypes (Stewart et al., 2012, Roy et al., 1994, Perez de Diego et al., 2011, Stewart et al., 2013, Noad and Roy, 2009). VLP have potential for development of multiserotype and DIVA compatible vaccines. However, for use as commercial vaccines they need to be purifiable from cellular components and be sufficiently stable to give an adequate shelf

life. Other important factors include the efficiency, yield and cost of production (Schwartz-Cornil et al., 2008, Anderson et al., 2014).

1.13 BTV diagnosis

Historically, clinical diagnosis was the standard method for detection and monitoring of BT and outbreaks of BT-8 in Europe in 2006 and in France during 2015, were initially recognised in this way. However, differential clinical diagnosis of the disease in sheep requires a well experienced veterinary service (Bexiga et al., 2007). Sub-clinical infections, particularly in cattle, goats and resistant breeds of sheep may be unrecognized or go unnoticed, particularly in situations where outbreaks are infrequent or previously unknown. Consequently, rapid, efficient and reliable laboratory diagnostic tests are essential to confirm clinical suspicion of BT and to provide veterinary health authorities adequate time to develop and deploy control measures especially during an epidemic. Laboratory diagnosis of bluetongue has traditionally included virus isolation, group-specific and type-specific serological assays, although more recently molecular assays (RT-PCR assays) can be reliably performed (even in the face of inactivated vaccination campaigns) directly on clinical samples (e.g. blood samples) (Alpar et al., 2009).

1.13.1 Virus isolation

Traditionally, initial isolation of BTV required the inoculation of 14 days old embryonated hen's egg, either by yolk sac route (Mason et al., 1940) or more effectively through an intravenous route (Goldsmit and Barzilai, 1985) with blood, semen and tissue samples including liver, spleen, lymph nodes or mucosal epithelium from a viremic animal. This is followed by (blind) subpassaging of material from infected embryos in cell culture including using mammalian cell lines (BHK or Vero cells), or in an insect cell line (*Culicoides sonorensis* (KC) or *Aedes albopictus* (C6-36) cells) for adaptation of virus (Wechsler and Luedke, 1991, OIE, 2017).

Although cell culture based virus isolation remains as a gold standard for diagnosis and provides sufficient material for further characterisation of the virus, it is laborious, time consuming, requires specialized skills and cannot be automated. Consequently, alternative methods of virus detection include RT-PCR assays (to detect viral RNA), immunoperoxidase test (Afshar et al., 1991), fluorescent antibody staining tests (Gould et

al., 1989), immunoelectron microscopy (Brookes et al., 1994) and sandwich ELISAs for group-specific antigen detection of bluetongue (Hawkes et al., 2000).

1.13.2 Serological assays

1.13.2.1 Group-specific antibody detection assays

Serological assays for detection of group-specific antibodies against BTV, include: agar gel immuno-diffusion (AGID) tests (Pearson et al., 1985, Jochim and Chow, 1969, Batten et al., 2009), complement fixation (CF) tests (Boulanger et al., 1967) and ELISA, both competition and blocking (Afshar et al., 1987, Anderson, 1984, Martyn et al., 1991, Thevasagayam et al., 1996, Chand et al., 2009, Reddington et al., 1991).

The AGID and CF tests lack sensitivity and can show cross-reactivity with related *orbiviruses* (Della-Porta et al., 1985), or against host cell proteins present in antigen preparations. Therefore, these tests have largely been replaced by the ELISA. During the 1980s, ELISA lacked specificity due to the use of semi-purified antigens. Anderson, (1984) developed a group-specific monoclonal antibody based ELISA directed against an epitope on the highly conserved outer core protein VP7 of BTV. This assay showed no cross-reactions with related *Orbiviruses*, or against cellular proteins present in the antigen preparation. VP7 is a serogroup-specific antigen (Gumm and Newman, 1982) encoded by genome segment 7 and is highly immuno-dominant. It is therefore particularly suitable for development of group-specific diagnostic assays (Afshar et al., 1992b) to identify members of the *Bluetongue virus* species/serogroup (Mertens, 2000).

The use of ELISA to detect BTV- specific antibodies in natural infection is compromised by non-DIVA vaccination programmes using either live attenuated or partially purified inactivated vaccines (Barros et al., 2009). However, Anderson and colleagues also described a monoclonal antibody based competitive ELISA for the detection of antibodies against BTV serogroup specific non-structural protein 1 [NS1] (Anderson et al., 1993). This ELISA could differentiate between BTV-infected and BTV-vaccinated animals with an inactivated vaccine. However, this assay is no longer in use, as serum from animals vaccinated with live attenuated vaccines gave a positive reaction.

1.13.2.2 Serotype-specific antibody and antigen detection assays

Rapid and reliable BTV serotype differentiation is essential particularly at the start of an outbreak, to allow early selection of an appropriate vaccine to help control the spread of the virus.

Traditionally, laboratory conformation of the BTV serotype includes isolation of virus and subsequent serotyping by serum neutralisation tests / virus neutralization tests (SNT/VNT) via: microtitre neutralization, plaque reduction, plaque inhibition and fluorescence inhibition tests (Blacksell and Lunt, 1996).

VP2 is the serotype-determining antigen, based on the specificity of its interactions with neutralizing antibodies generated during infection of the host (Mertens et al., 1989a). The less variable VP5 protein also plays a minor role in eliciting the antibody response and can also contribute to serotype determination (Mertens et al., 1989a, DeMaula et al., 2000).

SNT is based on interaction of a serial dilution series of the test serum with a fixed amount of a reference strain of each BTV serotype, to determine the concentration at which the test serum neutralises the virus (Parsonson and Snowdon, 1985). VNT requires an initial isolation of the test virus strain in culture cells. It is based on interaction of a serial dilution of the test virus with a fixed amount of reference antisera for each of the BTV serotypes (Afshar, 1994, Clavijo et al., 2000). However, confirmation of virus serotype by this route requires days to weeks. Such delays can have a significant adverse effect on the extent and duration of an epizootic, by causing a delay in selection of an appropriate vaccine strain/serotype.

Serological typing methods require BTV serotype-specific antisera or reference strains for all BTV serotypes (Hamblin, 2004). However reference antisera are expensive to produce in experimental animals and are in short supply. Also, these serological assays can be unreliable particularly if infection and the resulting samples include more than one BTV serotype. Low-level cross-reactions can occur with related orbiviruses thereby producing false positives (low sensitivity and specificity). Since serological diagnosis requires time and often lacks the necessary specificity and sensitivity, molecular assays are currently the method of choice for typing of BTV. The amplified cDNA products from RT-PCR can also be sequenced, not only to confirm virus serotype but also to identify different virus lineages, identification of vaccine/field strains or differentiation of topotypes within each

serotype, which is impossible by conventional serological typing methods (Anthony et al., 2007).

1.13.3 Molecular assays

Molecular diagnostic assays for BTV broadly include conventional and real time RT-PCR, sequencing analysis and polyacrylamide or agarose gel electrophoresis. Nucleic acid-based techniques or 'molecular assays' especially RT-PCR, sequence analyses and phylogenetic comparisons are used for the detection and typing of BTV (Alpar et al., 2009, Mertens et al., 2007b).

RT-PCR can be used to detect RNA viruses, involving initial synthesis of a complementary DNA (cDNA) copy of the RNA using reverse transcriptase (RT), followed by amplification and analysis of the cDNA. RT- PCR assay exists in a number of formats including conventional or gel based RT-PCR where the targeted viral RNA sequence is detected by electrophoretic analyses of the amplified cDNA products.

A quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) can be used to amplify and simultaneously quantify a targeted RNA sequence. Real-time RT-PCR assays based on TaqMan technology detect fluorescence signals from an oligonucleotide probe that is activated during target amplification. A fluorogenic probe, labeled with both a fluorescent reporter and a quencher dye is used in the TaqMan-PCR. The technology exploits the 5' exonuclease activity of *Taq* DNA polymerase to cleave an oligonucleotide probe during PCR, thereby generating a detectable signal. After hybridization of the probe to the target sequence, the *Taq* DNA polymerase cleaves the TaqMan probe with its 5'–3' exonuclease activity. The reporter and quencher dyes are separated upon cleavage, as the target is amplified, resulting in increased fluorescence of the reporter.

The benefits of real-time PCR assays over conventional approach include high sensitivity and specificity (making then suitable for direct use on clinical samples avoiding the need for virus isolation), the ability to quantify viral RNA templates (copy number) and the detection of short amplicons. The reactions also take place in a closed tube, reducing risks of cross-contamination and false positive (Bustin et al., 2005). These assays can be scaled up for high throughput applications, automation and the potential for accurate target quantification. However, the insensitivity of a cell line for BTV isolation or low quality RNA in clinical sample or some technical error may also lead to false negative results by

PCR (Billinis et al., 2001). The most conserved genome segments or regions, that are also unique to the target virus species, should therefore be selected for the design of real-time RT-PCR assays and a wide selection of diverse samples should be used for assay validation.

1.13.3.1 Group-specific RT-PCR assays

Group-specific RT-PCR assays use genome segments that are most conserved across the whole BTV species or serogroup. The most conserved segments of the BTV genome are Seg-1 (VP1), Seg-3 (VP3), Seg-4 (VP4), Seg-5 (NS1), Seg-7 (VP7), Seg-8 (NS2) and Seg-9 (VP6) and represent potential targets for the development of BTV species or group-specific RT-PCR assays (Alpar et al., 2009). Several group-specific conventional RT-PCR assays targeting different regions of the viral genome, such as Seg- 3 (Harding et al., 1995, McColl and Gould, 1991, McColl and Gould, 1994, Parsonson and McColl, 1995), Seg-5 (Shad et al., 1997, Aradaib et al., 1998, Aradaib et al., 2003, Wilson and Chase, 1993, OIE, 2017), Seg-7 (Anthony et al., 2004, Wade-Evans et al., 1990) and Seg-10 (Akita et al., 1992, Billinis et al., 2001) are available for detection of BTV. However, even the most conserved of the BTV genome segments do show sequence variations that correlate with the geographic origins of the virus isolate (Maan et al., 2008).

Quantitative real-time RT-PCR (qRT PCR) assays have been reported for the detection of BTV species or serogroup targeting Seg-1 (Shaw et al., 2007, Toussaint et al., 2007), Seg-5 (Jimenez-Clavero et al., 2006, Wilson et al., 2004, Polci et al., 2007, Toussaint et al., 2007), Seg-10 (Orru et al., 2006, Hofmann et al., 2008a) and Seg-9 (Maan et al., 2015a). However, the specificity and sensitivity of RT-PCR assays and detection of all bluetongue serotypes has been hampered by the genomic diversity of BTV which evolves continuously by genetic drift, recombination and gene reassortment (Bonneau et al., 2001). Seg-7 is the third most variable of the BTV genome segments (Wilson et al., 2000, Mecham et al., 2003, Anthony et al., 2007, Maan et al., 2008) followed by Seg- 10 as the fourth most variable region of the BTV genome segments(Maan et al., 2008). Variations between isolates from different regions may require the use of multiplex primer systems. Shaw and colleagues published a duplex assay in which two sets of genotype specific primers and probes were designed to target genome segment 1 of the eastern and western BTV topotypes, respectively (Shaw et al., 2007). Toussaint and colleagues developed two real-time RT-qPCRs amplifying and detecting two different genomic regions/segments of BTV in parallel. This approach would avoid the risk of missing a mutant, a recombinant or a

reassortant strain and detect all serotypes of BTV (Toussaint et al., 2007). The real time probes are very sensitive to probe-target mismatches (Jimenez-Clavero et al., 2006), therefore mutation in the virus genome within a primer or probe 'foot-print' can result in a mismatch in the fluorescent probe target region and presents a significant challenge.

1.13.3.2 Serotype-specific RT-PCR assays

RT-PCR assays using serotype-specific primers have the potential to reduce the need for BTV serotype-specific antisera for serological typing methods. Full-length sequence data for genome segment 2 from the 24 BTV reference serotypes (Maan et al., 2004) showed that it varies with serotype and represents the least conserved region of the BTV genome. Phylogenetic analyses of Seg-2 show the nucleotide sequence variation of 29% to 59% between reference strains of different BTV serotypes and up to 32% within the serotype from different geographic regions (Maan et al., 2007a, Maan et al., 2010). Nucleotide sequence based typing methods include conventional RT-PCR and real-time RT-PCR assays. Phylogenetic comparisons of Seg-2 are becoming increasingly important for the typing of BTV (Maan et al., 2011b, Maan et al., 2011a, Maan et al., 2010). The conventional and real time RT-PCR assay identifying and differentiating BTV serotypes by amplifying serotype-specific genome segment 2 are available (Maan et al., 2016, Attoui, 2012, Mertens et al., 2007b, Mertens et al., 2007a). In developing a molecular assay for serotype-specific detection of BTV, their genetic diversity presents a significant challenge. Real time RT PCR assays function on high-throughput 96-well diagnostic formats to handle the high volumes of samples needing to be tested during a national surveillance program or diagnostic surge in the event of a BTV outbreak.

1.14 Bluetongue epidemiology in India

1.14.1 Geographical distribution of bluetongue disease in India

The Indian subcontinent is a large geographic area, which includes five countries, namely Pakistan, Nepal, Bhutan, Bangladesh and India. India is the world's seventh largest country, with an area of 3.28 million square km (2.4 per cent of the earth's land surface). It is situated in the Northern hemisphere, between 8^{o} 4' N to 37°6'N latitudes and 68°7'E and 97°25'E longitudes. The land boundary of India is about 15,200 km with a coast line of 7,516.6 km. The total population in India is 1210.2 million people, second highest in world after China (Narayanan et al., 2018). The geographical area of India is 3.28 million km^2 with 648 districts of 29 States and 7 Union Territories covering 6.6 lakhs villages and

64639 urban wards, according to office of the Registrar General and Census Commissioner (ORGI), Ministry of Home Affair, Government of India (ORGI, 2011).

Bluetongue disease is known to be endemic and has been reported from many states of India, including northern, central, western and southern states (Prasad et al., 1992, Sreenivasulu et al., 2004, Ranjan et al., 2015, Prasad et al., 2009). However, overt clinical outbreaks have mostly been reported from rural native mixed flocks of sheep and goats across the four states of Telangana, Andhra Pradesh, Karnataka and Tamil Nadu in the southern region of India (Rao et al., 2016b).

Bluetongue was first reported with overt clinical signs of BT from Maharashtra state in Western India (Sapre, 1964). Then in 1970's, frank clinical outbreaks of BT were recorded in Merino sheep at Kothipura farm of Himachal Pradesh, Northern India (Uppal and Vasudevan, 1980) and in a Corriedale sheep at the Central Sheep Breeding Farm of Haryana, Northern India (Vasudevan, 1982). During 1980's, severe BT outbreaks were reported: in rural sheep flocks from Maharashtra, Western India (Singh et al., 1982, Harbola et al., 1982) and Karnataka, Southern India (Srinivas et al., 1982); in native Chokla and exotic Rambouillet sheep breeds at the Central Sheep and Wool Research Institute of Rajasthan, Northern India (Lonkar et al., 1983, Sharma et al., 1985); and in exotic Rambouillet sheep at the Central Sheep Breeding Farm of Haryana, Northern India (Mahajan et al., 1991).

Clinical BT in India was originally reported in exotic breeds of sheep from 1970-1980, but thereafter, BT outbreaks were reported annually in native breeds of sheep, particularly in Southern states (Mehrotra et al., 1991, Kulkarni et al., 1992, Saravanabava, 1992). Although, BT is endemic in India, classical BT signs have seldom been reported in the native sheep breeds of Northern India during the last two decades. In Tamil Nadu, BT was first reported in 1982 with regular incidence thereafter. In 1989-1990, 1997-1998 and 2005-2006, large epidemics of BT, with severe clinical manifestations, were reported from Tamil Nadu resulting in high mortality of sheep (Reddy et al., 2016a).

1.14.2 Influence of climate of India on bluetongue disease

India can be divided into six physiographic regions based on physical features, including: the Northern and North-eastern Mountains (the Himalayas); the Northern Plain; the Peninsular Plateau; the Indian Desert; the Coastal Plains and the Islands. The Deccan

plateau is part of the peninsular plateau and is a triangular landmass. Telangana, Andhra Pradesh, Karnataka, and Tamil Nadu are the southern states of India and form a part of Peninsular Deccan plateau.

India has a monsoon type of climate, but there are regional and seasonal variations in climate in terms of wind pattern, temperature and rainfall. This explains the unity and diversity of the climate of India. For example, temperature in summer is up to 55^0C in the Indian Desert and -45^0C in Leh (northern mountains) in winter. There are four seasons in India namely the cold weather season, the hot weather season, the southwest monsoon season and the retreating monsoon season (NCERT, 2014).

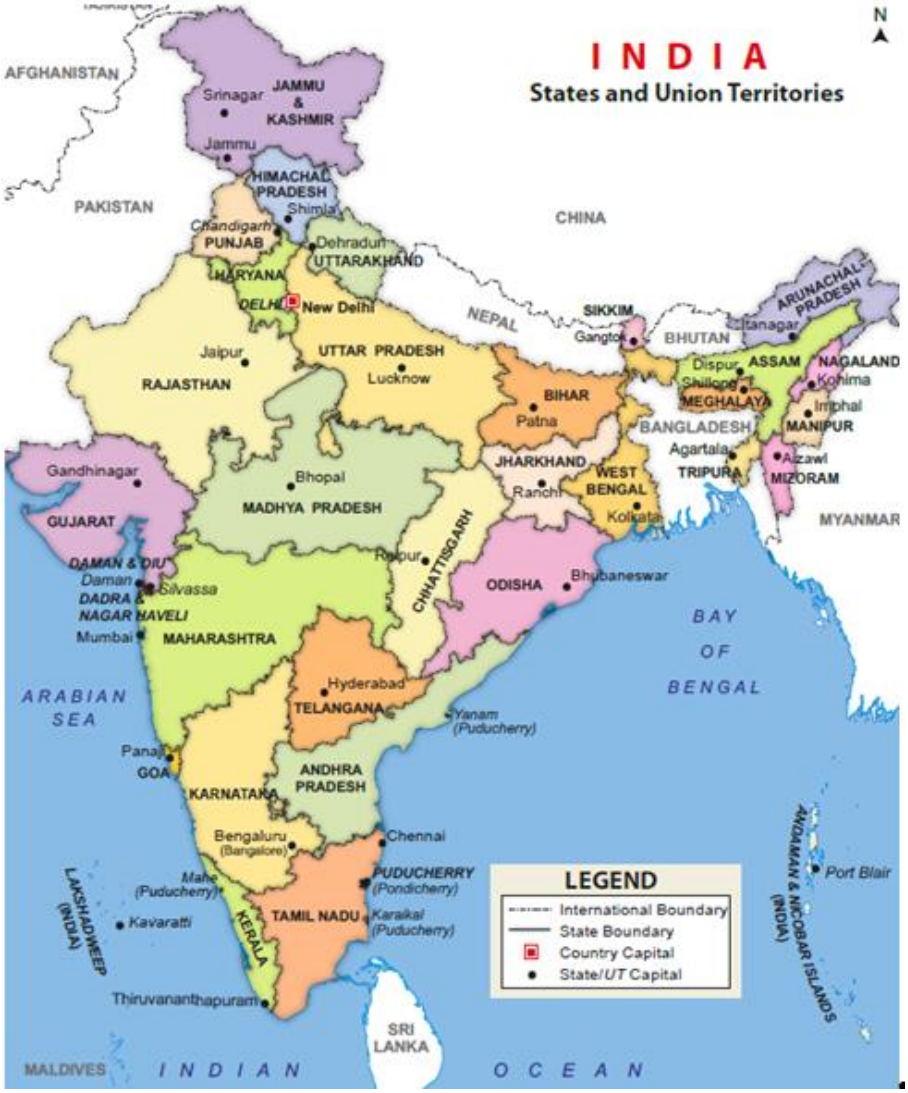


Figure 1.3 Map of India showing all the states of India.

The cold weather season: starts from mid-November in Northern India, with December and January being the coldest months. The mean daily temperature remains less than 21°C in northern India, with night temperature sometimes below freezing point in some areas. The main reasons for the intense cold in north India during winter season are: the Northern landmass away from sea's moderating influence, altitude, cold winds from the snowfall in the Himalayan region and the cold winds coming from the Caspian Sea. The Peninsular part of India does not experience an intense cold weather season because of lower altitude and the sea's moderating influence (www.nios.ac.in/media/documents/316courseE/ch17).

The summer season: is from April to June in North India with temperatures rising to 48^0C in May. Southern India experiences a less intense summer than Northern India, with temperatures between 26^0C to 32^0C, because of moderating influence of the ocean.

The southwest monsoon: During April and May, as the sun is exactly vertical to the Tropic of Cancer, an intense low pressure is created in the North western landmass of the Indian subcontinent. This attracts the southeast trade winds, which get deflected towards the Indian subcontinent (South West) while crossing the Equator, dropping large amounts of rain. Therefore, broadly, the southwest monsoon is a westerly continuation of the southeast trade wind covering the entire subcontinent by mid-July (India, 2017).

North east monsoon and tropical cyclones: Tropical cyclones and depression bring rain in the month of October and November across the Southern Peninsular Coast. These can be very destructive potentially causing disasters each year. The retreating monsoon is the southward movement of north easterly winds in October and November when the weather is dry in North India. However, these winds collect moisture while crossing the Bay of Bengal, making October and November the rainiest months of the year for the Eastern Peninsula (NCERT, 2014).

The Tamil Nadu coast, the southern part of Andhra Pradesh, and southeast Karnataka, receive 30% of their annual rainfall from the North East monsoon, during October and November (Attri and Tyagi, 2010). However, for Tamil Nadu alone, it constitutes nearly 48% of annual rainfall. As a whole, India gets more than 75% of its annual rainfall from June to September. The rest is mainly received in the months of October, November and December in the Southern parts of India (Rao et al., 2016b).

The occurrence of BT outbreaks in India is influenced by meteorological factors which include temperature, relative humidity, and precipitation. The susceptibility of individual sheep breeds to BT and the size of the sheep population as a whole determine the appearance of overt clinical disease in southern India (Rao et al., 2016b). The poopulation of sheep is highest in Andhra Pradesh, followed by Rajasthan State, with more than 70% of the country's sheep population resident in five states, namely Andhra Pradesh, Telangana, Karnataka, Tamil Nadu and Rajasthan (Livestock census, 2014).

An appropriate climate and a high sheep population density appear to be required to maintain BT outbreaks, as indicated by the negligible incidence of BT in Kerala, a Southern State where climatic condition are similar to those of the other four Southern States but the sheep population is low (Arun et al., 2014, Ravishankar et al., 2005). In the Great Indian Desert (Rajasthan), where the incidence of BT is also low, the sheep population is high, (the second largest in the country) but the climate is harsh and not conducive for *Culicoides*.

There are significant differences in the disease pattern and clinical manifestation of BT between Northern and Southern states of India. Frank clinical BT disease has been documented mainly from the Southern states (Prasad et al., 1992, Prasad et al., 2009). In northern parts of Andhra Pradesh and Karnataka, BT outbreaks occur during the south west monsoon, with the highest incidence during August and September. In contrast, in the Southern interior parts of Andhra Pradesh, Karnataka and Tamil Nadu, the disease is observed from October to December, during the North East monsoon, (Sreenivasulu et al., 2004). High ambient temperatures, rainfall and high relative humidity during these two rainy seasons are considered likely to contribute to conducive breeding grounds for *Culicoides* population, potentially helping in the spread of BT in southern India (Rao et al., 2016b). In 2005-2006, a widespread BT outbreak was reported, covering all of the sheep rearing regions in Tamil Nadu, but severe clinical disease, associated with high mortality, was reported mainly in Southern districts during a very heavy north east monsoon in October and December (Reddy et al., 2016a). During the same year, mild to moderate clinical BT was reported from Salem, Dhamapuri, and Erode districts of Tamil Nadu during July (south-west monsoon) but with no mortality of sheep.

Bluetongue has not been reported from the Nilgiris district, part of Western Ghats (a chain of large mountains) situated at an altitude of 2892 m above sea level (Reddy et al., 2016a). However, mild BT was reported in two Nilgiri breed ewes from Ooty, Nilgiri district

during 2009, and BTV-23 serotype was confirmed by serum neutralization test (Venkataramanan et al., 2010).

In contrast, frank outbreaks of BT are not observed in North Western and Northern India due to the short rainy season (only the south west monsoon), semi-arid to arid agro climatic zones , extreme weather conditions with heat waves in summer, and the harsh winters of Western India, all of which are not congenial for *Culicoides* breeding (Rao et al., 2016b).

A recent report of concurrent infection of BTV-12 with *Peste des Petite Ruminant virus* (PPRV) in small ruminants from Haryana State of Northern India, showed early non-specific clinical signs of high fever, malaise and anorexia, followed by predominant PPR symptoms including hyperaemia of the oral mucosa, diarrhoea and mortality (Maan et al., 2017). Biswas and colleagues also reported concurrent infection of PPRV and BTV-1 from a PPR suspected outbreak in goats in Uttar Pradesh State (Biswas et al., 2010, Mondal et al., 2009) during of January, 2008. BT is usually asymptomatic or inapparent in goats and the clinical symptoms in this outbreak included typical PPR signs of pyrexia, erosive stomatitis, diarrhoea, pneumonia and coughing. Mondal and colleagues isolated BTV-1 from asymptomatic goats during a BT seroprevalence study in the Pithoragarh hills of Uttarakhand during January, 2009 (Mondal et al., 2013). Chauhan and colleague isolated BTV-1 from the spleen of an aborted goat foetus in Gujarat, 2007 (Chauhan et al., 2014).

1.14.3 Landless, small and marginal farmers in India

The livestock sector makes a significant contribution in the socio-economic fabric of rural India, helping to reduce rural poverty and improving nutritional security. Millions of small and marginal farmers (the ‘landless’) depend on small ruminants for their livelihood and to augment their income. According to the classification used in the Agricultural Census, 2011 marginal farmers hold less than 1 hectare of land, while ‘small’ farmers have a land holding of 1 to 2 hectares. Small and marginal farmers account for 85% (117.6 million in number) of the total operational holding in the country, but hold only 44.6% of the operated area (71.15 million hectares) showing a huge disparity in terms of land holding by farmers in India (DACFW, 2011).

Small ruminants represent crucial livelihood wealth and can be immediately liquidated in times of need, distress and emergencies. Therefore, small ruminant husbandry plays a significant role in risk management strategies and improving the livelihood of rural India

(DADF, 2016). In recent years, the livestock sector in India has grown faster than the crop sector and contributes nearly 28% of the total agricultural gross value added. A Sustained rise in per capita income and rapid urbanization in the recent past, have led to increased growth in demand for animal food products. Although resource poor families have more opportunities in demand-driven small-ruminants production, they face formidable challenges in terms of fodder scarcity, grazing land shrinkage, ill equipped veterinary services, marketing of produce and a high prevalence of various animal diseases around the country. Furthermore, it is difficult for state animal husbandry departments to continuously monitor migratory flocks for disease, making the situation even more challenging (DADF, 2016). Small and medium farmers, including landless labourers, rear sheep and goats mainly for meat and skin production in Tamil Nadu, Andhra Pradesh and Karnataka, but in some parts, especially in Karnataka state, also for wool production.

In 1997‑1998, 0.3 million small ruminants died due to BT during the north east monsoon in Tamil Nadu (Ilango, 2006). BT causes significant economic losses to the Indian sheep industry annual, particularly in Southern India (Ranjan et al., 2015). During 1991‑2005, BT has caused huge economic losses, amounting to 60.8% of all infectious diseases in Indian sheep, particularly to landless marginal and small farmers (Ranjan et al., 2015).

1.14.4 Livestock population in India

India has the world's largest livestock population, with a total of 512.05 million livestock identified by the 19th livestock census. The livestock census has been conducted in India every five years since 1919, by the Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, Government of India. There are about 299.9 million bovines (cattle, buffalo, mithun and yak), 65.06 million sheep and 135.17 million goats in the country (DAHDF, 2014). According to 19th Livestock Census, distribution of livestock amounted to 37.28% cattle with largest share followed by 26.40% goats, 21.23% buffaloes, 12.71% sheep and 2.01% pigs and 0.37% others (horse, ponies, mule, donkey, camel, mithun and yak) of the total livestock population.

State-wise the percentage share of cattle population is 5.03% for Andhra Pradesh, 4.98% Karnataka, 4.62% Tamil Nadu with a maximum share in Madhya Pradesh and Uttar Pradesh states amounting to 10.27% each in the country. For the sheep population, the state-wise percentage share is 40.57% for Andhra Pradesh with largest share in country, 14.73% Karnataka, and 7.36 % in Tamil Nadu. More than 70% of the country's sheep

population is found in four states namely Andhra Pradesh, Karnataka, Tamil Nadu and Rajasthan.

State-wise the percentage share of the goat population is 6.71% for Andhra Pradesh, 3.55% Karnataka, 6.02% in Tamil Nadu, with largest share in Rajasthan state, amounting to 16.03%. The number of cattle, sheep and goats per thousand households are: 477, 1311, 451 respectively for Andhra Pradesh; 680 cattle, 685 sheep and 343 goats per thousand households in Karnataka state; and 508 cattle, 276 sheep and 469 goats per 1000 households in Tamil Nadu,. These figures show that livestock are reared in very small units per family (DAHDF, 2014).

1.14.5 Contribution in the national economy

Agriculture contributes 17.4%, to the national gross value added (GVA) of India, with ~55% of the work force dependent on agriculture and allied activities (DACFW, 2011). According to the Central Statistics Office (CSO), the livestock sector contributed 28.5% at basic prices to the value output of agricultural and allied sectors, during 2015-16, with ~5 million small and marginal farmers, including landless labourers, are involved in the rearing of sheep and goats in the country, but a total workforce of nearly 70 million are involved in the livestock sector (DAHDF, 2014).

Nearly 0.3 million sheep and goats died due to BT during the north west monsoon season in 1997-1998 (Ilango, 2006) causing significant economic losses to the Indian sheep industry as a whole. From 1991-2005, BT disease caused the highest reported economic losses amongst all leading infectious diseases (Ranjan et al., 2015).

1.14.6 Susceptibility to BT of sheep and goat breeds in Southern India

Breeds of sheep found in the Andhra Pradesh and Telangana, are Nellore (the tallest breed of sheep resembling a goat in appearance), Deccani, Bellari and Macherla .There are no recognized goat breeds from Andhra Pradesh, Telangana and Karnataka states. Sheep breeds of Karnatak include Deccani, Bellary, Bannur/Bandur/Mandya, Hassan and Kenguri. The sheep breeds from Tamil Nadu include meat-purpose breeds: Madras/Chennai Red, Melcher/Mecheri Ramanadhapuram White, Vembur, as well as wool-purpose breeds: Coimbatore, Tricky Black, Kilakarisal/Keezhakaraisal**,** Nilgiri and Chevvadu. The goat breeds from Tami Nadu are: Kanniadu in the Thirunelveli district:

Ramanadhapuram, Kodiadu in Sivagangai; Ramanadhapuram, Tuticorin and Salem Black from the Salem district (Acharya, 1982, ICAR, 1945, Bhatia and Arora, 2005).

Semi-pastoral and semi-intensive management systems are adopted for rearing sheep in these states. While the semi-intensive system, involves both stall feeding and pasture grazing; the semi-pastoral system involves seasonal migration of flocks during periods of fodder scarcity (Kantwa et al., 2017, Rao et al., 2016b).

There are no definite reports on breed predispositions to BT in India, and this may even vary with different virus strains. In India, BT outbreaks were earlier detected in exotic or mixed wool breeds under crossbreeding programmes for breed improvement. However, outbreaks are currently reported mostly in native/local breeds (Prasad et al., 2009, Prasad et al., 1992, Rao et al., 2016b, Susmitha et al., 2012, Sreenivasulu et al., 1999, Bommineni et al., 2008). It has been suggested that this change correlates with the introduction of exotic / western topotypes strains into the Indian sub-continent. Trichy black and Ramnad white breeds in Tamil Nadu are found to be more susceptible to BT than Vambur and Mecheri sheep breeds (Prasad et al., 2009). Similarly in Andhra Pradesh, the short-haired mutton Nellore-breed of sheep is more susceptible to BT than the thick coarse carpet-wool Deccani breed. In the rest of the country, mostly medium quality wool breeds are found which do not show frank clinical BT disease symptoms (Rao et al., 2016b).

1.15 Serotypes of BTV in India

Serological evidence suggests that 21 different BTV serotypes exist in India, with at least 15 BTV-serotypes (BTV- 1, 2, 3, 4, 5, 8, 9, 10, 12, 16, 17, 18, 21, 23 and 24) isolated over the last five decades, including BTV-24 & BTV-5 the most recent additions to list in 2016. Over the last two decades, 12 serotypes have been reported namely BTV-1, BTV-2, BTV-3, BTV-4, BTV-5, BTV-9, BTV-10, BTV-12, BTV-16, BTV-21, BTV-23 and BTV-24 mainly from Southern India (Figure 1.3) (Rao et al., 2016b, Hemadri et al., 2016, Krishnajyothi et al., 2016, Bommineni et al., 2008, Chand et al., 2016, Kumar et al., 2016, Rao et al., 2015, Sreenivasulu et al., 1999, Reddy et al., 2016b, Reddy et al., 2016a, Rao et al., 2012c, Rao et al., 2016a, Pudupakam et al., 2017, Prasad et al., 1992, Prasad et al., 1994, Mondal et al., 2013, Maan et al., 2012e, Maan et al., 2012d, Maan et al., 2015c, Maan et al., 2015b, Maan et al., 2017, Maan et al., 2012c, Maan et al., 2012b, Maan et al., 2012a, Joardar et al., 2009, Desai et al., 2009, Susmitha et al., 2012).

During the last decade, BTV-1 and BTV-12 have been reported from Northern India (Maan et al., 2017, Mondal et al., 2013, Chand et al., 2016, Biswas et al., 2015, Biswas et al., 2010, Mondal et al., 2009). BTV-1 has been isolated from Gujarat, in Western India (Chauhan et al., 2014) and there is one report of the isolation of BTV 3 from west Bengal, North Eastern India (Joardar et al., 2009).

BTV isolates from Andhra Pradesh and West Bengal in 2003 were incorrectly typed as BTV-15 but later confirmed as BTV-10 from Andhra Pradesh and BTV-3 from West Bengal (Joardar et al., 2009, Bommineni et al., 2008, Gollapalli et al., 2012, AINPBT, 2010). Therefore, the only evidence for BTV-15 in India is serological, with detection of BTV-15 specific antibodies (Prasad et al., 2009).

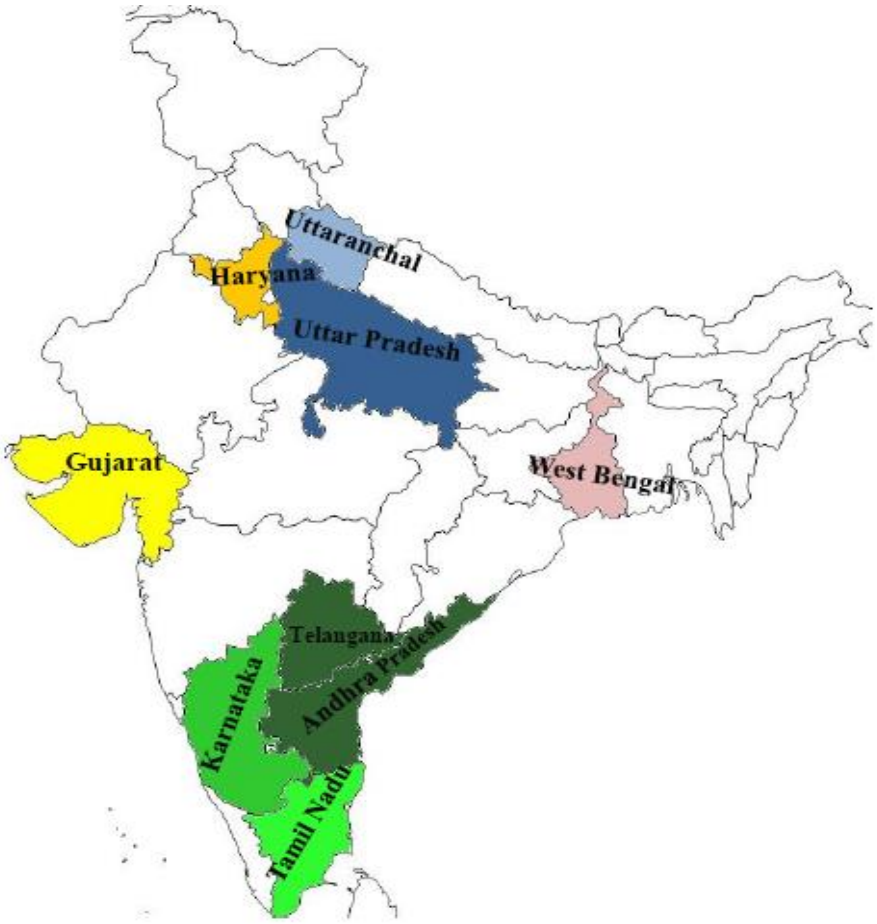
1.16 BTV vaccination in India

Vaccination with a pentavalent inactivated BTV vaccine (containing BTV-1, BTV-2, BTV-10, BTV-16 and BTV-23 serotypes) is currently being carried out in the four Southern states of India: Telangana, Andhra Pradesh, Tamil Nadu and Karnataka as well as Maharashtra, which is a western state. However, inspite of hyperendemic BT transmission in these region, vaccination is not done on a 'mass' scale. The pentavalent inactivated BTV vaccine was developed by Tamil Nadu Veterinary and Animal Science University (TANUVAS) under the All India Network Project on Bluetongue, an initiative of Indian Council of Agricultural Research (ICAR), Government of India, and subsequently technology transfer to Indian Immunological Limited (IIL), Hyderabad was done on February, 2011 (www.icar.org.in/node/3516). Since 2010, vaccine was used in Maharashtra, Telangana, Andhra Pradesh, Tamil Nadu and Karnataka at farmer's doorstep before its recent commercialization in 2015. The Raksha Blu vaccine (commercial name of pentavalent inactivated BTV vaccine, containing serotypes BTV-1, BTV-2, BTV-10, BTV-16 and BTV-23) was launched on 8 January 2015 by Indian Immunologicals Limited in Hyderabad, Telangana (Maan et al., 2015c, Rao et al., 2016b). The vaccine is available to the farmers at the rate of 5 rupees per dose. The five currently circulating and most prevalent serotypes in the country BTV-1, -2, -10, -16 and -23 are inactivated with formaldehyde in addition to binary ethylenimine, adjuvanted with Montanide and then mixed as a pentavalent BT vaccine formulation (Reddy et al., 2010). The Animal Husbandry and Veterinary Science (AH & VS) department of Karnataka State runs a mandatory vaccination programme for four economically important infectious diseases in sheep and goats, namely: Haemorrhagic Septicaemia (HS), Enterotoxaemia (ET), Anthrax

and *Peste des Petits Ruminants* (PPR) but this does not include BT. PPR vaccination is carried out in all districts of Karnataka State, twice in a year (May and November). A similar situation exists in Telangana, Andhra Pradesh and Tamil Nadu.

Twelve BTV serotypes including: BTV-1, BTV-2, BTV-3, BTV-4, BTV-5, BTV-9, BTV-10, BTV-12, BTV-16, BTV-21, BTV-23 and BTV-24 have been isolated from Southern India since 2001, with BTV-5 and BTV-24 as new inclusion to the list in 2016. There are also reports of the co-circulation of multiple BTV serotypes with mixed serotype infections within a single animal in outbreaks from Southern India (Reddy et al., 2010, Reddy et al., 2016b). Therefore a regular, rapid and robust virological surveillance is needed to identify currently circulating serotypes, ensuring regular re-evaluation of the vaccine composition. Indeed, one or more BTV serotypes have been observed as the dominant outbreak strain for one or more years, but these are then succeeded and replaced by new dominant serotypes (Rao et al., 2016b). Different BTV serotypes can therefore be dominant during different years and in different States.

These changes may be explained by the fact that infection with one BTV serotype provides long-lasting herd immunity against that homotypic serotype during its dominance in the region and may provide some protection against severe clinical disease, there is little cross protection against infection by another serotype, which can therefore emerge as dominant in subsequent years. Detection of multiple co-circulating BTV serotypes and co-infection of different BTV serotypes within individual animals emphasizes the need for regular virological surveillance in India, particularly in light of the emergence of and reassortant with exotic (western topotype) BTV strains, and the potential for the generation of novel genotypes with modified phenotypes that display different transmission or virulence characteristics. It may be particularly valuable to compare isolates from before, during and after implementation of the current pentavalent inactivated-vaccine campaign, to see if it has influenced the prevalence of different BTV serotypes.



	Andhra Pradesh & Telangana	BTV-1, BTV-2, BTV-3, BTV-4, BTV-9, BTV-10, BTV-12, BTV-16, BTV-21, BTV-24
	Karnataka	BTV-16, BTV-5
	Tamil Nadu	BTV-1, BTV-2, BTV-3, BTV-16, BTV-23
	Gujarat	BTV-1
	Haryana	BTV-12
	Uttar Pradesh	BTV-1
	Uttaranchal	BTV-1
	West Bengal	BTV-3

Figure 1.4 BTV serotypes reported from different States of India since 2001.

1.17 Aims and objectives of Project

BTV is endemic in India, with regular hyper-endemic outbreaks occurring in the South. Serological evidence suggests that 21 BTV serotypes exist in India. However, only twelve BTV-serotypes (BTV-1, 2, 3, 4, 5, 9, 10, 12, 16, 21, 23 and 24) have been isolated there since 2001. A pentavalent inactivated BTV vaccine, containing dominant serotypes from India, (BTV-1, BTV-2, BTV-10, BTV-16 and BTV-23), is currently being used.

Detection of multiple co-circulating BTV serotypes in India emphasizes the need for regular virological surveillance, particularly in light of the ongoing pentavalent inactivated vaccination programme. It will be particularly valuable to compare isolates from before, during and after implementation of the current pentavalent inactivated-vaccine campaign, to see if it has influenced the prevalence of different BTV serotypes.

Molecular epidemiology studies of BTV depend on the development of sequence databases for the RNA segments of individual virus isolates from defined locations, with well documented isolation dates and passage histories. Generation of whole-genome sequence database for BTV strains from diverse geographical areas of India is needed to support molecular epidemiological studies to determine the origins, movements of different virus lineages and to determine the genetic variation and phylogenetic relationship with other isolates reported from different parts of the India and world. Phylogenetic analyses can help to trace evolutionary relationship between BTV strains and determine the origins and movement of each virus lineage and even each individual genome segment within an outbreak strain. It also helps in the characterization of any novel BTV isolates, as a new BTV lineage, identification of its topotype or cross-topotype reassortant that may be generated, thus contributes to better understanding of BTV molecular epidemiology and supporting the implementation of appropriate control strategies for BT.

The investigation of field outbreaks of BTV from Southern India in this project will help us understand the epidemiology of the disease in endemic regions and in the country as a whole. The Southern states of India have a higher incidence of bluetongue disease. High sheep population density, congenial monsoon climate for multiplication of *Culicoides* and a susceptible host population, may all be the contributing factors to the disease incidence and prevalence.

The project is intended to isolate and 'type' BTV strains in India, then generate full length sequence data, primarily for BTV genome Seg-2, and potentially for the entire genome of selected bluetongue virus isolates from India. These sequencing data support the selection of conventional type-specific primers for Seg-2 that facilitates cDNA synthesis, amplification and sequencing. Full length sequence data for BTV genome Seg-2 provides molecular epidemiology information concerning the strains circulating in the region. An initial step in this process is the typing of viruses (by real-time RT-PCR assays) confirming the circulation of multiple BTV serotypes in the subcontinent.

Aims of the project include:

- The investigation of suspected field outbreaks of BTV from Southern India during 2014-2015. This includes collection of EDTA blood and necropsy (spleen and lymph node) samples from outbreaks in mixed flocks of sheep and goats showing clinical signs in various districts of Telangana, Andhra Pradesh, Karnataka and Tamil Nadu during 2014- 2015. BTV RNA will be detected by BTV specific TaqMan based quantitative RT-PCR targeting genome Seg-10 (NS3) (Hofmann et al., 2008a) and Seg-9 (Maan et al., 2015a). Samples (EDTA blood and tissue) that are positive (Ct<30) by BTV serogroup-specific qRT-PCR targeting Seg-10 or Seg-9, will be mainly used to infect KC and BHK cells for virus isolation (Chapter 3).
- Identification of serotype by serotype-specific TaqMan based qRT-PCR assay targeting BTV Seg-2 in Indian BTV field isolates from four Southern states of India during 2014 and 2015 BTV outbreaks. It will be particularly valuable to compare samples / isolates from before, during and after implementation of the current pentavalent inactivated-vaccine campaign, to see if it has influenced the prevalence of different BTV serotypes (Chapter 4).
- To generate full-length sequence data for Genome segment 2 of Indian BTV strains isolated during 2014-2015 BT outbreaks from India, on a next generation sequencing platform (Chapter 5).
- To identify the serotype of these field isolates from 2014-2015 outbreaks of BTV in India by comparing ORF of Seg-2 sequences with those of reference and field strains of 27 serotypes (Chapter 5).
- Phylogenetic analyses based on Seg-2 of these BTV strains to determine the geographic origins, movements of virus lineage and evolutionary relationship with other isolates reported from different parts of the India and world (Chapter 5).

Chapter 2: Materials and Methods

The chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich (now Merck) company unless otherwise stated. All plastics used for the experiments were purchased from Tarsons Products Ltd, (Kolkata, India) and Nunc, Thermo Fisher Scientific (Mumbai, India) unless indicated otherwise. Cell culture flasks with growth area of 25 cm^2 and 175 cm^2 were purchased from Greiner Bio-One (Noida, India, catalogue number 660160). qPCR optical 96 well reaction plates with barcode, 0.1 mL were purchased from Applied Biosystems, Thermo Fisher Scientific (MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL, catalogue number 4346906).

2.1 Cell culture

2.1.1 Cell culture media

- Dulbecco's modified Eagle's medium, DMEM (Sigma-Aldrich, catalogue number D5280) for culture of BHK cell line.
- Schneider’s insect cell culture medium (Sigma-Aldrich, catalogue number S0146) for culture of KC cell line.
- Trypsin-EDTA solution (10 X) with 5.0 g porcine trypsin and 2 g EDTA • 4Na per liter of 0.9% sodium chloride (Sigma-Aldrich, catalogue number T4174).
- Dulbecco’s phosphate buffered saline without Calcium and Magnesium (Sigma-Aldrich, catalogue number 56064C).
- L-glutamine solution, 200 mM (Sigma-Aldrich, catalogue number G7513)
- Penicillin-Streptomycin (100X) with 10,000 units penicillin and 10 mg streptomycin/mL (Sigma-Aldrich, catalogue number P4333) for cell culture.
- Fetal bovine serum for cell culture (Biological Industries, catalogue number 04-007-1A).
- Sodium bicarbonate solution, 7.5% (Sigma-Aldrich, catalogue number S8761)
- Freezing media: 70% maintenance medium, 20% foetal bovine serum, 10% dimethyl sulphoxide (DMSO) passed through 0.2 µm sterile syringe filter.

2.1.2 Cell lines

2.1.2.1 Mammalian cell lines

- Baby Hamster Kidney cells (BHK-21 clone 13), a fibroblast cell line derived from the kidney of one day old hamster originally procured from the American Type Culture Collection (ATCC, USA), was provided by the Veterinary Type Culture Collection, Hisar, Haryana, India for this study.
- BSR cells (a sub-clone of BHK-21 cells) was kindly provided by Professor Peter Mertens, School of Veterinary Medicine and Science, University of Nottingham, UK.

2.1.2.2 Insect cell lines

- KC cells, an insect cell line derived from embryos of *Culicoides sonorensis* (Wechsler and McHolland, 1988) was kindly provided by Professor Peter Mertens, School of Veterinary Medicine and Science, University of Nottingham, UK.

2.1.3 Cell culture technique

2.1.3.1 Mammalian cell culture

Mammalian cell culture work was carried out aseptically in a Class II biosafety cabinet using sterile media, buffer and plastic/glassware. The growth medium consisted of Dulbecco's modified Eagle's medium (Sigma-Aldrich, catalogue number D5280) supplemented with 10% foetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 2mM glutamine and 7.5% sodium bicarbonate was used to grow BHK-21 cell line in 25 cm^2 and 175 cm^2 flasks. Confluent cell sheets were sub-cultured routinely, with a split of 1/3. Monolayer of cells was washed three times with sterile Dulbecco's phosphate buffered saline, 0.2mL/cm^2 culture flask surface area, to remove traces of serum that would inhibit the action of trypsin. The cell sheet was covered with 0.25% trypsin-EDTA solution, 0.2mL/cm^2 culture flask surface area, and placed for 2 minute in the incubator at 37^0C. The trypsin-EDTA solution was removed from the culture flask by aspiration and flask was again placed in the incubator until the cells detached from the surface of the culture flask. Detached cells were re-suspended in growth medium and split 1 in 3 in culture flask for further cultivation. The BHK-21 cells were incubated at 37^0C in the presence of humidified 5% CO_2.

2.1.3.2 Insect cell culture

KC cells were grown at 26^0C in Schneider's insect medium supplemented with 10% foetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin. KC cells were dispersed by gentle and repeated pipetting up and down over the confluent cell sheets and subcultured with a split of 1/10.

2.1.4 Cryopreservation of cells:

A standard cryopreservation protocol was followed for cell cultures. Near confluent monolayers of BHK cells in log phase were detached from the substrate with 0.25% trypsin-EDTA solution and cells were resuspended in a growth medium. The cell suspension was centrifuged at 1500 rpm for 2 minute to pellet cells. The supernatant was aspirated using a sterile pipette without disturbing the cells pellet and cells were resuspended at a concentration of $1x10^7$ cells per mL in freezing medium, which consisted of 70% Dulbecco's modified Eagle's medium, 20% foetal bovine serum, 10% dimethyl sulphoxide (DMSO). One mL aliquots were transferred to 2 mL cryogenic vials prelabelled with the name of cell line, passage number and date of cryopreservation. Cryopreservation was achieved using a slow freezing approach by placing the vials first at 4°C refrigerator for 2 hour, then at -20^0c for 2 hours , frozen at -80^0C in an insulated box overnight and finally transferring to liquid nitrogen tank at -196^0C.

For KC cells preservation, a standard cryopreservation protocol was followed. However, freezing media consisted of 20% Schneider's insect medium, 70% foetal bovine serum and 10% DMSO was used to dispense cell pellet into 2 mL cryogenic vial.

2.1.5 Revival of cryopreserved cells

Cryogenic vials were taken out from the liquid nitrogen tank and immediately thawed by immersion in a water bath at 37^0C for 2 minute. Swab with 70% (v/v) ethanol was applied to the surface of the cryogenic vial and allowed to air dry for 10 seconds. The thawed cryopreserved suspension was transferred to a 2mL microcentrifuge tube and centrifuged at 1500 rpm for 5 minute at 4^0C, to discard the freezing medium. The cell pellet was resuspended in 1 mL of growth medium and the cell suspension was transferred into a 25 cm^2 culture flask containing 5 mL of growth medium and the flask was incubated at the appropriate temperature. The cell lines were maintained under their respective culture

condition. The cells were observed after 24 hours for cell attachment and medium was then changed with a fresh growth medium to remove traces of DMSO.

2.2 Reagents for RNA extractions

- TRIzol reagent (Ambion, life technologies, Invitrogen, catalogue number 15596026) contains phenol and guanidinium isothiocyanate.
- Chloroform (Sigma-Aldrich, catalogue number 288306)
- Isopropanol (Sigma-Aldrich, catalogue number W292907)
- Ethanol (Sigma-Aldrich, catalogue number E7023)
- Lithium chloride 8 M (Sigma-Aldrich, catalogue number L7026)
- Ammonium acetate 7.5 M (Sigma-Aldrich, catalogue number A2706)

2.3 Blood sample collection

- EDTA Vacutainer tube 9 mL capacity (Greiner bio-one, catalog number 455045).
- Vacutainer needle 20 G, 1inch length, 0.9mm diameter x 25mm length (Greiner bio-one, catalog number 450073)
- Vacutainer needle holder (Greiner bio-one, catalog number 450201)

2.4 Agarose gel electrophoresis

- 10X TAE buffer - 0.4 M Tris, 1.142% (v/v) acetic acid, 0.01 M EDTA, diluted in distilled water. Diluted to 1X in distilled water prior to use.
- Agarose - 1% (w/v) molecular grade agarose (Sigma, catalogue number A9539-100G) diluted in 1×TAE buffer.
- Ethidium bromide - 10mg ethidium bromide per mL of distilled water was made as stock solution. 5ul of stock solution was added to 100 mL of molten agarose gel (Sigma-Aldrich, catalogue number E7637-5G).
- 1 Kb plus DNA Ladder (Invitrogen, catalogue number 10787018).

2.5 Primers for qRT-PCR

2.5.1 Serogroup-specific oligonucleotide primers and probe

Primers and probe sequences for the detection of BTV RNA were based on qRT-PCR assays targeting BTV Seg-10 (NS3), as described by Hofmann et al., 2008a (Table 2.1). The 5' end of TaqMan probes were labelled with 6-Carboxyfluorescein (FAM) and the 3'end with a Black Hole Quencher-1 (BHQ-1). Primer and probe sequences are shown in the 5'-3' orientation. Primers and probes were synthesized by Eurofins (India) and ordered at 100 nM scale with RNAse-free HPLC purification.

BTV RNA was also detected by group specific real-time RT-PCR targeting genome segment 9 (Maan et al., 2015a) – Table 2.2.

Table 2.1: Primer and probe sequences to detect BTV RNA by BTV specific qRT-PCR targeting BTV Seg- 10 . (Hofmann et al., 2008a)

Oligonucleotide	Primer name	Primer sequence (5'-3')
Forward primer	BTV_IVI_F	TGG-AYA-AAG-CRA-TGT-CAA-A
Reverse primer	BTV_IVI_R	ACR-TCA-TCA-CGA-AAC-GCT-TC
Probe	BTV_IVI_P	FAM-ARG-CTG-CAT-TCG-CAT-CGT-ACG-C- BHQ1

Table 2.2: Primer and probe sequences to detect BTV RNA by BTV specific qRT-PCR targeting BTV Seg- 9. (Maan et al., 2015a)

Oligonucleotide	Primer sequence (5'-3')
Forward primer1	GYRCGGGNGGDGAYRYDAARAYG
Reverse primer 1	RWRBRAAATCGCMCTACGTCAAG
Forward primer 2	GTACAGGCGGAGATGTGAAAACG
Reverse primer 2	GTGTAAAACCGCTATATGCCGTG
Probe	CACCTCTAAAGGGTCCAGGGTACC

2.5.2 Serotype-specific oligonucleotide primers and probe

Primers and probes for typing of BTV were based on qRT-PCR assay as described by Maan et al. (2016) (Appendix 1). The assay is based on VP2 nucleotide sequences of the BTV reference strains, available in the *Orbivirus* Reference Collection (ORC) at The Pirbright Institute, as well as nucleotide sequences in the public domain (GenBank). The 5' end of TaqMan probes were labelled with 6-Carboxyfluorescein (FAM) and the 3'end with

a Black Hole Quencher-1 (BHQ-1). Primers and probes were synthesized by Eurofins (India).

2.6 Primers for full-length amplification of cDNA (FLAC)

2.6.1 Anchor-primers for ligation

BTV dsRNA was ligated at its 3' ends to an anchor-primer, as described by Maan et al., (2007b). The iSp9 is a C9 spacer (phosphoramidite) that forms a self-priming hairpin structure, to initiate synthesis of a cDNA (Table 2.3). The 5' terminus of the primer is phosphorylated. Primers were synthesized by Integrated DNA Technologies, UK and ordered at 100 nM scale with dual PAGE & HPLC purification.

2.6.2 Primer for amplification of cDNA

The cDNA that was generated from the anchor-primer ligated RNA, in a reverse transcription reaction, was further amplified by PCR using primer 5-15-1 that is complementary to the anchor-primer, as described by Maan et al., 2007b (Table 2.3). The 5-15-1 primer was synthesized by Integrated DNA Technologies, USA and ordered at 100 nM scale with RNAse-free HPLC purification.

Table 2.3: Primer sequences for full-length amplification of cDNA (FLAC) technique (Maan et al., 2007b).

Primer name	Primer sequence (5'-3')
Anchor-primer	p-ACCTCTGAGGATTCTAAAC/iSp9/TCCAGTTTAGAATCC-OH
5-15-1	GAGGGATCCAGTTTAGAATCCTCAGAGGTC

2.7 Enzymes and kits

2.7.1 Ligation, Reverse transcription, cDNA synthesis / purification

- AMV reverse transcription system (Promega, USA, catalogue number A3500) was used for Reverse Transcription (RT) reactions.
- SuperScript III/Platinum Taq One-Step qRT-PCR Kit (Invitrogen, UK, catalogue number 11732020) was used for quantitative RT-PCRs.
- T4 RNA ligase buffer (10 X) (New England Bio Labs, catalogue number B0216L)

- T4 RNA ligase (10 U) (New England Bio Labs, catalogue number M0204L)
- Triple Master PCR system (Eppendorf) was used for cDNA amplification.

2.7.2 Next Generation Sequencing-Illumina sequencing, kits / reagents

- TURBO-DNA free kit (Ambion, Thermo Fisher Scientific, catalogue number AM1907)
- RNA clean & concentrator kit (Zymo research, catalogue number R1015)
- DNA clean & concentrator kit (Zymo research, catalogue number D4029)
- Agencourt AMPure XP-PCR purification (Beckman Coulter Genomics, catalogue number A63881)
- KAPA HiFi Hot Start Ready Mix (Kapa Biosystems, catalogue number 07958927001)
- T4 RNA Ligase 1, ssRNA Ligase (New England Bio Labs, catalogue number M0204L)
- Tapestation D5000 ScreenTape (Agilent, catalogue number 5067-5588)
- Tapestation D5000 Ladder and Sample Buffer (Agilent, catalogue number 5067-5589)
- Qubit dsDNA HS Assay Kit (Life Technologies, catalogue number Q32851)
- Nextera XT Index kit , 24 indexes (Illumina, catalogue number FC-131-1001)
- Nextera XT DNA Library Prep kit, 24 samples (Illumina, catalogue number FC-131-1024)
- PhiX Control v3 (Illumina, catalogue number FC-110-3001)
- MiSeq reagent kit V2 cartridge V2 (Illumina, MS-102-2002).

2.8 Description of the study area

Telangana, Andhra Pradesh, Karnataka, and Tamil Nadu are Southern states of India and form part of the Deccan Plateau Peninsular. Karnataka is located between latitude $11^{0}31'$ to$18^{0}45'$ north and longitude $74^{0}12'$ to 78^{0} $40'$ east. It shares borders with Andhra Pradesh in the east, Maharashtra and Goa in the North, Tamil Nadu in the South and is bounded by the Arabian Sea in the West. Tamil Nadu extends between latitudes $80^{0}2'$ to $13^{0}09'$ north and longitudes $76^{0}50'$ to $80^{0}27'$ east.

Andhra Pradesh was recently reorganized into two states namely Telangana and Andhra Pradesh in 2014. Andhra Pradesh is located between latitudes $12^0$37' to $19^0$54' north and longitudes $76^0$45' and $84^0$46' east, and shares a border with Orissa and Madhya Pradesh in the north, Maharashtra and Karnataka in the west, Tamil Nadu in the south and is bounded by the Bay of Bengal Sea in the east.

2.9 Field outbreaks of BT

Suspected BT outbreaks were attended in mixed flock of sheep and goats, in the Warangal and Mehboobnagar districts of Telangana state during October 2014, and during November 2014 to January 2015 in Karur, Erode, Tirupur, Chengalpattu, Salem and Thoothukudi districts of Tamil Nadu state (Figure 2.1). Suspected BT Cases were also attended in Warangal, Medak and Karimnagar of Telangana state during August 2015, Anantpur and Chittoor districts of Andhra Pradesh state in October 2015, Devanagere, Tumkure, Chickballapur, Kolar and Chikmagalur districts of Karnataka state in November 2015 and Perambalur, Shivagangai, Pudukottai, Tirunelveli, Erode and Thoothukudi districts of Tamil Nadu state in December 2015.

2.10 Collection of field samples

Four hundred and forty nine (449) EDTA blood samples and seven necropsy (spleen and lymph node) samples were collected from outbreaks from mixed flocks of sheep and goat showing clinical signs of bluetongue disease (BT) in various districts of Telangana, Andhra Pradesh, Karnataka and Tamil Nadu states during 2014-15 (Table 2.4).

Of 150 samples collected during the 2015 BT outbreak in Tamil Nadu, eleven EDTA blood samples and two saliva samples were collected from suspected cattle housed close to the mixed flock of sheep and goat. Blood sampling was carried out with the assistance of the owner who restrained the animals. The blood was collected in a vacutainer using a vacutainer needle and holder. Approximately, 5 mL of blood was collected from each animal and transported at 4^0C to the biotechnology laboratory in LUVAS, Hisar, India.

Figure 2.1 The author attending suspected BT outbreaks in mixed flocks of sheep and goats in various states of Southern India during 2014-2015.

A) Telangana, 2015; B) Karnataka, 2015; C) Andhra Pradesh, 2015 and D) Tamil Nadu, 2015.

Table 2.4 Samples collected from four Southern states during 2014-2015 BT outbreaks in India.

State	Year of collection	Blood samples	Necropsy samples
Hyderabad	2014	9	-
Hyderabad	2015	22	1£
Karnataka	2015	178	1*
Chennai	2014	145¥	5$
Chennai	2014	25	-
Andhra Pradesh	2015	60	-

¥ Including two saliva samples; £ spleen, * spleen, $ four spleen and one lymph nodes.

2.11 Viruses

Ten cell culture adapted BTV isolates were obtained under the Indo-UK project titled "Development of diagnostic systems, reference collections and molecular epidemiology studies for important arboviral pathogens of livestock in India" funded jointly by the Department of Biotechnology, Government of India, and the Biotechnology and Biological Sciences Research Council of the UK. The origins (where known) as well as the nomenclature of these viruses are listed in Table 2.5, although their passage history and year of isolation information are not available. These BTV isolates were provided to me by various collaborators namely the Indian Council for Agriculture Research-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Karnataka; ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Uttar Pradesh and Ella Foundation, Hyderabad.

Table 2.5: Cell culture adapted Indian BTV isolates obtained from collaborators under the Indo-UK project.

Name of BTV isolate	Obtained from	Geographic origin
BTV2DH	Dr.Hemadri, ICAR-NIVEDI	Karnataka
BTV1 IVRI	Dr. Singh, ICAR-IVRI	Uttar Pradesh
BTV2 IVRI	Dr. Singh, ICAR-IVRI	Uttar Pradesh
BTV10 IVRI	Dr. Singh, ICAR-IVRI	Uttar Pradesh
BTV 23 IVRI	Dr. Singh, ICAR-IVRI	Uttar Pradesh
BTV M1	Ella Foundation, Hyderabad	Telangana
BTV WG103	Ella Foundation, Hyderabad	Telangana
BTV IPGR/10	Ella Foundation, Hyderabad	Telangana
BTV VJW 764	Ella Foundation, Hyderabad	Telangana
BTV KMN 07/05	Ella Foundation, Hyderabad	Telangana

2.12 Preparation of clinical sample

One mL of clinical blood sample was taken in a 2 mL sterile microcentrifuge tube and pelleted by centrifugation at approximately 1900 g for 1 min in a bench top microcentrifuge, washed (resuspended and pelleted) three times with 1 mL of Dulbecco's phosphate buffered saline. After the final wash, the blood cells were resuspended in Dulbecco's phosphate buffered saline up to a final volume of 1 mL. The washed blood cells were then lysed by adding equal volume sterile water. The sample was then centrifuged at 12,000 rpm at 4^0C to collect the supernatant. The supernatant was passed

through a 0.22 µm sterile syringe filter (Millex, Merck, India, catalogue number SLGV033RS) then used to infect KC cells.

For virus isolation a small piece of tissue sample (0.02gm) was transferred to 1.5 mL microcentrifuge tube and homogenized in 100 µL of PBS supplemented with 1000 units/mL penicillin and 1000 µg streptomycin/mL using a tissue homogenizer. After grinding, 900 µL of PBS, supplemented with 1000 units/mL penicillin and 1000 µg streptomycin/mL was added and the total sample centrifuged at 10,000 g for 5 minute to sediment coarse particles and bacterial contamination. The supernatant was transferred to a fresh microcentrifuge tube and passed through 0.2 µm syringe filter. The processed tissue sample was then used to infect KC cells.

2.13 Total RNA extraction from clinical samples

During RNA extraction care was taken to avoid contamination with RNase and cross-contamination between different BTV samples. Total RNA was extracted from clinical samples (EDTA treated blood or supernatant from homogenized tissues) using a guanidinium isothiocyanate procedure previously described by (Attoui et al., 2000b), with some modifications. Briefly, 400µL of blood sample or supernatant was vigorously vortexed with 600 µL of TRIzol reagent (Invitrogen) in a 1.5 mL eppendorf tube, followed by addition of 200 µL of chloroform. The solution was mixed by vortexing till a cloudy pink solution was obtained, followed by incubation on ice for 10 min. After centrifugation at 10,000 g for 10 min, the clear top aqueous phase was transferred into a 1.5 mL labelled eppendorf tube and mixed with 900 µL of isopropanol. RNA was allowed to precipitate at -20^0C for 2 hours followed by centrifugation at 10,000 g for 10 min to pellet the RNA. The pellet was washed with 1 mL of 70% ethanol and centrifuged at 10,000 g for 10 min to remove supernatant. The pellet was air dried and dissolved in 50 µL of nuclease free water (NFW) to get total RNA and kept at -20^0C until used.

2.14 Laboratory diagnosis by quantitative real-time RT-PCR

A fluorogenic probe labelled with both a fluorescent reporter (e.g. 6-Carboxyfluorescein (FAM) at the 5' end) and a quencher dye (e.g. Black Hole Quencher (BHQ) at the 3' end) is used in the TaqMan-PCR. When the reporter and the quencher are both attached to the probe, energy from the excited reporter is transferred to the quencher by fluorescence resonance energy transfer (FRET) resulting in the absence of a fluorescent signal. After

hybridization of the probe to the specific target template sequence, by complementary base pairing, the *Taq*DNA polymerase extends the upstream primer on the same strand leading to cleavage and displacement and of the TaqMan probe by its 5'–3' exonuclease activity. ,. The reporter and quencher dyes are separated by this cleavage mechanism, as the target is amplified. The energy is no longer transferred by FRET but is released from the reporter as fluorescence, resulting in an increased fluorescence signal. The fluorescence signal can be detected and measured in real time using a real-time thermal cycler.

2.14.1 Serogroup-specific qRT-PCR

The one-step, BTV specific TaqMan based, quantitative RT-PCR assay, targeting BTV Seg-10 (Hofmann et al., 2008a) was performed using the SuperScript III/Platinum Taq One-Step qRT-PCR Kit (Invitrogen). The qRT-PCR were carried out in 96-well 'fast optical PCR plates' (Applied Biosystems) using a real time PCR system apparatus (Applied Biosystem step one plus real time PCR system, catalog number 4376600) and Software, version 2.3 (Applied Biosystems). The composition of the assay reaction mix is presented in Table 2.6. Total RNA extracted from blood or tissue samples were heat denaturated at 99^0C for 5 minute and then chilled on ice prior to addition of the reaction mix. The reaction cycling conditions were: 30 min at 55^0C for cDNA synthesis; inactivation of RT and Taq activation for 2 min at 95^0C; followed by 45 cycles of: 15s at 95^0C, 30s at 56^0C and 30s at 72^0C (Hofmann et al., 2008a). The cycle threshold (Ct) value was defined as the number of amplification cycles required to cross a certain threshold level of fluorescence. The analysis of fluorescence data was conducted using real time PCR system software version 2.3 (Applied Biosystems). Negative results (for assays that did not exceed the threshold level of signal) are reported as 'No Ct'. Negative results were defined as Ct values greater than 40 cycles of amplification (threshold set automatically following the instructions of analysis software). The samples (EDTA blood or homogenised tissue supernatant) that were positive (Ct<30) by BTV serogroup-specific qRT-PCR targeting Seg-10, were mainly used to infect KC cells for virus isolation.

Cycling conditions for group specific real-time RT-PCR targeting genome segment 9 were: 30 min at 55^0C for cDNA synthesis; inactivation of RT and Taq activation for 10 min at 95^0C; followed by 50 cycles of 30 s at 95^0C and 60s at 60^0C (Maan et al., 2015a). The composition of the assay reaction mix is presented in Table 2.7.

Table 2.6 Composition of serogroup-specific quantitative RT-PCR assay targeting Seg-10

Reagents	Amount (µl)
2X reaction Mix (SuperScript III/Platinum Taq One-Step qRT-PCR Kit (Invitrogen, UK, catalogue number 11732020)	12.5
Forward primer (10 mM)	1.0
Reverse primer (10 mM)	1.0
Probe (5 mM)	0.5
ROX (1:10)	0.5
Nuclease free water	5.6
SuperScript III RT/Platinum Taq Mix	0.5
dsRNA	3.4
Final reaction volume	25

Table 2.7: Composition of serogroup-specific quantitative RT-PCR assay targeting Seg-9

Reagents	Amount (µl)
2X reaction Mix SuperScript III/Platinum Taq One-Step qRT-PCR Kit (Invitrogen, UK, catalogue number 11732020)	12.5
Forward primer 1 (20 mM)	1.0
Reverse primer 1(20 mM)	1.0
Forward primer 2 (20 mM)	1.0
Reverse primer 2 (20 mM)	1.0
Probe (5 mM)	0.5
ROX (1:10)	0.5
Nuclease free water	2.0
SuperScript III RT/Platinum Taq Mix	0.5
dsRNA	4.0
Final reaction volume	25

2.15 Virus propagation

2.15.1 Virus propagation in KC cells

Primary isolation of virus from clinical samples (blood and tissue) was attempted by one passage in KC cells, followed by three passages in BHK-21 cells. Washed confluent cell sheets of KC cells in 25 cm^2 culture flasks were inoculated with 500µL of processed clinical blood sample (see section 2.12.) in 1mL of Schneider's insect cell culture medium. After incubation for 1 h at 26^0C for virus adsorption, 5mL of Schneider's insect medium supplemented with 10% foetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin was added and cells were incubated for 7 days at 26^0C. After 7 days incubation, the KC culture flask was gently shaken and the cell suspension harvested. As

BTV does not produce cytopathic effect in KC cells, BTV RNA was detected by group-specific real time RT-PCR targeting genome Seg- 10 (Hofmann et al., 2008a) or by 1% agarose gel electrophoresis to visualize typical dsRNA migration pattern of BTV RNA, after dsRNA extraction from the cell pellet.

2.15.2 Virus propagation in BHK-21 cells

The growth medium was removed from confluent layers of BHK-21 (a susceptible cell line for attempting BTV isolation) in 25 cm^2 culture flasks which were then washed with Dulbecco's phosphate buffered saline (Sigma-Aldrich, catalogue number 56064C). The monolayer of BHK-21 cells was inoculated with 100 µL of KC cell-suspension that had previously been infected with the sample, and then incubated for 1 h at 37^0C under humidified 5% CO_2 for virus adsorption. During incubation, the flask was rocked gently every 20 min for an even spread of inoculum on the entire cell surface. The monolayer was washed with Dulbecco's phosphate buffered saline to remove unabsorbed virus particles and 5mL of maintenance medium consisting of Dulbecco's modified Eagle's medium supplemented with 100 units/mL of penicillin and 100 µg/mL streptomycin and 10% foetal bovine serum was added. Inoculated and non-inoculated (negative control) 25 cm^2 flask cultures were incubated at 37^0C in 5% CO_2 and monitored daily for visualization of morphological changes, cytopathic effects (CPE) in the infected cells, for up to 7 days.

At least, three blind passages, with an incubation of 7 days each, were attempted in the samples not exhibiting cytopathic effects, before discarding the culture. Following successful isolation, generally after one passage, the presumptive positive isolates in BHK-21 cell culture were confirmed as bluetongue virus (BTV) by group-specific real time RT-PCR targeting genome Seg-10, to detect BTV RNA (Hofmann et al., 2008a), or after dsRNA extraction from cell pellet by 1% agarose gel electrophoresis, to visualize the typical dsRNA migration pattern of BTV RNA. The cells infected with BTV were harvested, transferred to a 15 mL sterile centrifuge tube and stored at 4^0C till further use.

2.16 Storage of BTV field isolates

Field isolates of BTV were assigned unique collection numbers, consisting of the country code for India (IND), the year of collection and the sample number. Each BTV isolate was tested for BTV RNA by group-specific real-time RT-PCR targeting genome segment 10 (NS3) prior to storage at -80^0C. The processed original blood or tissue sample, field BTV

isolations from KC passage 1(KC1), KC 1 and BHK passage 1 (KC1-BHK1), KC 1 and BHK passage 2 (KC1-BHK2) and KC1 and BHK passage 3 (KC1-BHK3) were all stored at -80^{0}C and were given unique collection numbers. For this purpose, the harvested virus in supernatant of the infected cell culture was resuspended in an equal volume of glycerol and then this mixture was aliquoted to one ml per cryogenic vial and stored at -80^{0}C.

2.17 BTV dsRNA extraction from virus-infected cell culture

BTV dsRNA was extracted from virus-infected cell cultures (175 cm^2 confluent layer of BHK-21) using Trizol (Invitrogen, USA). Care was taken to avoid cross-contamination of different BTV isolates during RNA extraction. The total RNA was extracted from virus-infected cell cultures using a guanidinium isothiocyanate procedure as previously described by (Attoui et al., 2000b). Briefly, the contents of one 175 cm^2 culture flask of infected BHK cells showing 100% CPE was decanted into a 50 mL centrifuge tube, followed by centrifugation at 1912 g for 10 minutes. The supernatant was discarded and the pellet was immediately vortexed after addition of 1 mL of Trizol. The mixture was transferred into a 1.5 mL labelled eppendorf tube and vortexed after adding 200 µL of chloroform, until a cloudy pink solution was obtained. After incubation on ice for 10 min, the suspension was centrifuged at 10,000 g for 10 min and the clear top aqueous phase was transferred into a fresh 1.5 mL labelled eppendorf tube. Then 900 µL of isopropanol was added to precipitate the RNA at -20^{0}C for 2 hours. The sample was then centrifuged at 10,000 g for 10 min to pellet the RNA, which was subsequently washed with 1 mL of 70% ethanol, air dried and dissolved in 100 µL of nuclease free water (NFW). Now, 100 µL of 4 M lithium chloride (LiCl) was added to separate cellular ssRNA from the viral dsRNA by incubation at 4^{0}C overnight. After centrifugation at 10,000 g for 5 min to pellet ssRNA, the supernatant was subsequently pooled in a fresh, labelled 1.5 mL eppendorf tube. An equal volume of isopropanol and 0.25 volumes of 7.5 M ammonium acetate were added and dsRNA was allowed to precipitate at -20^{0}C for 2 hours. The dsRNA was pelleted by centrifugation at 10,000 g for 10 min, washed with 70% ethanol and air dried. The dsRNA pellet was finally suspended in 50 µL of nuclease free water and then kept at −20^{0}C until used.

Agarose gel electrophoresis was carried out using 1% agarose in 1X TAE buffer with 0.5 µg/mL ethidium bromide and run at 7 V/cm in 1X TAE running buffer for an hour. The gels were visualized under UV using a transIlluminator, looking for the typical 3-3-3-1 dsRNA migration pattern of BTV isolates.

2.18 Type-specific TaqMan based qRT-PCR

BTV serotyping qRT-PCR assays were performed to detect serotypes, BTV-1 to BTV-27 and BTV-29 (Appendix 1). A significant sequence variation occurs in Seg-2 of BTV strains from different geographic origins but of the same serotype (Maan et al., 2009).Therefore, in this assay, two sets of primers and probes as a duplex format in qRT-PCR were used for BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16, against eastern and western topotypes/strains of same serotype. The assays used the SuperScript III/Platinum Taq One-Step qRT-PCR Kit (Invitrogen, UK, catalogue number 11732020) in 96-well Fast optical PCR plates (Applied Biosystems, UK, catalogue number 4346907) in a real time PCR system apparatus and Software, version 2.3 (Applied Biosystems, UK).

Briefly, the dsRNA test-samples were heat denaturated at 99^0C for 5 minute, then chilled on ice prior to addition of the reaction mix. The composition of the reaction mixes for assays with one set of primers and probe, or two sets of primers and probe respectively are presented in Table 2.8 and 2.9. Cycling conditions were: 30 min at 55^0C for cDNA synthesis; 10 min at 95^0C for inactivation of RT and Taq activation; followed by 45 cycles of 15s at 95^0C, and at 10 min 60^0C. The cycle threshold (Ct) value was defined as the number of amplification cycles required to cross a certain threshold level of fluorescence. The analysis of fluorescence data was conducted using a real time PCR system Software, version 2.3 (Applied Biosystems, UK). Negative results were defined as Ct values greater than 40 cycles of amplification (threshold set automatically following the instructions of analysis software).

Table 2.8: Composition of type-specific TaqMan based qRT-PCR assay with two sets of primers and probes

Reagents	Amount (ul)
2X reaction Mix (SuperScript III/Platinum Taq One-Step qRT-PCR Kit (Invitrogen, UK, catalogue number 11732020)	12.5
Forward primer 1 (10 mM)	1
Reverse primer 1(10 mM)	1
Probe 1(5 mM)	0.5
Forward primer2 (10 mM)	1
Reverse primer2 (10 mM)	1
Probe 2(5 mM)	0.5
MgSO4(50mM)	1
ROX	0.5
Nuclease free water	2.1
SuperScript III RT/Platinum Taq Mix	0.5

dsRNA	3.4
Final reaction volume	25

For BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16, two sets of primer and probes as a duplex format in qRT-PCR were used against eastern and western topotypes/strains of same serotype.

Table 2.9: Composition of type-specific TaqMan based qRT-PCR assay with one set of primers and probe

Reagents	Amount(µL)
2X reaction Mix (SuperScript III/Platinum Taq One-Step qRT-PCR Kit (Invitrogen, UK, catalogue number 11732020)	12.5
Forward primer (10 mM)	1
Reverse primer (10 mM)	1
Probe (5 mM)	0.5
MgSO4(50mM)	1
ROX	0.5
Nuclease free water	4.6
SuperScript III RT/Platinum Taq Mix	0.5
dsRNA	3.4
Final reaction volume	25

2.19 Agarose gel electrophoresis

AGE was carried out using 1% agarose in 1X TAE buffer with 0.5 µg/mL ethidium bromide and run at 7 V/cm in 1X TAE running buffer for an hour at room temperature. The gels were visualized under a UV transIlluminator. Five (5) µL of amplified cDNA PCR product, or BTV genomic dsRNA was mixed with 1µL 6X loading dye and gel electrophoresis was carried out alongside a 1 kb DNA ladder. The image produced was stored as TIFF or JPEG files.

2.20 RT-PCR for full-length cDNA amplification (FLAC)

The viral dsRNA was used for cDNA synthesis as described by (Maan et al., 2007b). Full-length cDNA copies of BTV genome segments were synthesised and amplified in a sequence-independent manner using the “anchor spacer-ligation” method (Maan et al., 2007b).

2.20.1 Anchor-primer ligation

BTV dsRNA was ligated (at its 3' ends) to an anchor-primer, using T4 RNA ligase at 4 °C for12 h. The ligation reactions were carried out in a total volume of 10 µL and consisted of 7 µL dsRNA, 1 µL anchor primer, 1 µL T4 RNA ligase buffer (10 X) (New England Bio Labs) and 1 µL T4 RNA ligase (10 U) (New England Bio Labs). The reaction was incubated at 4^0C overnight.

2.20.2 Heat denaturation of dsRNA

The ligated dsRNA was denatured at 99^0C for 5 minute, separated into single strands of dsRNA molecules and then snap chilled on ice to prevent renaturation.

2.20.3 Reverse transcription (RT)

The ligated RNA was reverse transcribed to generate full length cDNAs using the AMV reverse transcription system (Promega). Nine microliters of RT mix containing 4µL of 25mM MgCl2, 2 µL of 10mM dNTP mix, 0.6 µL (22 U/µL) of AMV reverse transcriptase, 0.5 µL (20 U) of RNAsin and 2 µL of 10X RT buffer (Reverse transcription System, Promega) was added to 11 µL of denatured ligated RNA. Reverse transcription was carried out at 25^0C for 10 min, then 37^0C for 40 min and finally 40^0C for 10 min. The cDNA was then used for PCR amplification.

2.20.4 Amplification of cDNA

The cDNA that was generated in RT reactions was further amplified by PCR using the 'Triple Master PCR system' (Eppendorf) with primer 5-15-1(`5-GAGGGATCCAGTTTAGAATCCTCAGAGGTC-3`) that is complementary to the anchor-primer. The PCR was carried out in a final reaction volume of 12 µL containing 1 µL (~400 ng) of reverse transcribed cDNA product, 1µL of 10x PCR buffer , 0.2 µL dNTPs (10mM each stock), 0.2 µL of 5-15-1 (100 µM) primer, 0.1µL PCR extender polymerase mix (5U/µL) and 9 µL of nuclease free water. Amplification was performed with cycling conditions consisting of an initial denaturation at 94^0C for 2 min, followed by 35 cycles of denaturation for 15s at 95^0C, annealing for 30s at 62^0C, extension for 4 min at 68^0C and a final extension step for 10min at 72^0C. Then 5µL of the PCR reaction was mixed with 1 µLof 6X loading dye and analysed by 1% agarose gel. The PCR products,

corresponding to each of the 10 genome segments, were purified and outsourced for sequencing on a 3730 capillary sequencer using BTV- gene specific primers to generate near-terminal sequences (Maan et al., 2007b).

2.21 Next-generation-Sequencing/Illumina-sequencing of Indian BTV isolates

Next generation sequencing and bioinformatics analysis of the whole genome of selected BTV isolates was performed by Dr. Kyriaki Nomikou, at the Medical Research Council, Centre for Virus Research (MRC-CVR), University of Glasgow, using the Illumina platform. Briefly, dsRNA was extracted from the cell pellets of BSR or BHK cell adapted BTV field isolates (n = 46) (Table 2.10) using TRIzol Reagent (Invitrogen). dsRNA was extracted using 2M lithium chloride and precipitated with isopropanol in the presence of ammonium acetate (7.5M) as described in section 2.17. The viral dsRNA was used for cDNA synthesis, and amplification of BTV genome segments was performed in a sequence-independent manner using the "anchor spacer-ligation" method, as described by (Maan et al., 2007b). After purification of amplified PCR products, libraries were constructed using Nextera XT DNA Library Prep kit (Illumina) according to the manufacturer's instructions. Sequencing was performed on an Illumina MiSeq on a 300-cycle V2 cartridge, as per manufacturer's protocol. Quality assessment and sequencing artefacts were removed, reference sequences were used to map the reads and the consensus sequences were finally extracted.

2.21.1 DNase Treatment of BTV dsRNA samples

BTV dsRNA was subjected to DNase treatment in order to eliminate containing DNA. Briefly, 12 µL of 10X Turbo DNase buffer and 5 µL of 2 Units/ C TURBO DNase (Turbo DNase, Ambion Life Technologies) were added to 50 µL of dsRNA and incubated at 37^0C for 30 minutes. Then, 5 µL of DNase inactivation reagent was added per 50 µL of ds RNA followed by incubation at 25^0C for 5 minute with occasional mixing. The DNase treated supernatant was finally transferred to a fresh labelled tube after centrifugation at 12000 rpm for 5 minute

.

Table 2.10 Details of the BTV field strains for Illumina sequencing in this study

Virus collection number*	Collection date (day/month/year)	Place of origin (District)	State	Latitude	Longitude	Host species	Source	Vaccination status of the animal sampled	Passage history (KC£/BHK$ or BSR&)
[IND2015/15]22	18/12/2015	Thoothukudi	TN	8°56'48.63"N	77°46'25.61"E	S	B	V	KC1/BSR6
[IND2015/18] 40	22/12/2015	Tuticorin	TN	8°56'48.63"N	77°46'25.61"E	G	B	UV	KC1/BHK4
[IND2015/148]491	25/12/2015	Kanchipuram	TN	12°50'3.02"N	79°42'13.10"E	S	B	UV	KC1/BHK5
[IND2015/151]492	25/12/2015	Kanchipuram	TN	12°50'3.02"N	79°42'13.10"E	S	B	UV	KC1/BHK6
[IND2015/157]494	25/12/2015	Kanchipuram	TN	12°50'3.02"N	79°42'13.10"E	S	B	UV	KC1/BHK6
[IND2015/208]404	25/12/2015	Erode	TN	11°20'27.73"N	77°43'1.79"E	S	B	V	KC1/BHK4
[IND2015/211]406	25/12/2015	Erode	TN	11°20'27.73"N	77°43'1.79"E	S	B	UV	KC1/BHK5
[IND2015/214]408	25/12/2015	Erode	TN	11°20'27.73"N	77°43'1.79"E	S	B	UV	KC1/BHK4
[IND2015/169]470	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	S	B	V	KC1/BHK4
[IND2015/172]471	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	C	B	UV	KC1/BHK5
[IND2015/175]472	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	C	B	UV	KC1/BHK4
[IND2015/178]473	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	S	B	V	KC1/BHK4
[IND2015/181]474	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	C	B	UV	KC1/BHK4
[IND2015/184]475	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	S	B	V	KC1/BHK4
[IND2015/187]476	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	S	B	V	KC1/BHK5
[IND2015/190]477	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	S	B	V	KC1/BHK5
[IND2015/193]478	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	G	B	V	KC1/BHK4
[IND2015/196]480	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	S	B	V	KC1/BHK5
[IND2015/199]483	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	S	B	V	KC1/BHK4

[IND2015/202]484	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	G	B	V	KC1/BHK4
[IND2015/205]486	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	S	B	V	KC1/BHK4
[IND2015/160]CS4	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	S	B	V	KC1/BHK4
[IND2015/163]CS6	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	S	B	V	KC1/BHK4
[IND2015/166]CS9	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	S	B	V	KC1/BHK5
[IND2015/76]534	25/12/2015	Thoothukudi	TN	8°45'51.00"N	78° 8'5.41"E	G	B	UV	KC1/BHK4
[IND2015/2017]547 tissue	25/12/2015	Thoothukudi	TN	8°45'51.00"N	78° 8'5.41"E	S	Sp	UV	KC1/BHK4
[IND2015/220]228 tissue	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	S	Sp	UV	KC1/BHK4
[IND2015/223]34 tissue	25/12/2015	Thoothukudi	TN	8°45'51.00"N	78° 8'5.41"E	S	Sp	UV	KC1/BHK4
[IND2015/232]556 saliva	25/12/2015	Thoothukudi	TN	8°45'51.00"N	78° 8'5.41"E	C	Sa	UV	KC1/BHK5
[IND2014/34]BTV-1	06/12/2014	Thanjavur	TN	10°47'13.20"N	79° 8'16.18"E	S	B	UV	KC1/BHK4
[IND2014/35]BTV-2	06/12/2014	Thanjavur	TN	10°47'13.20"N	79° 8'16.18"E	S	B	UV	KC1/BHK4
[IND2015/118]V1	26/11/2015	Chikballapur	K	13°11'35.03"N	78° 1'14.86"E	S	B	V	KC1/BHK5
[IND2015/121]V2	26/11/2015	Chikballapur	K	13°11'35.03"N	78° 1'14.86"E	S	B	V	KC1/BHK5
[IND2015/136]V41	26/11/2015	Chikballapur	K	13°29'43.12"N	77°58'4.05"E	S	B	V	KC1/BHK5
[IND2015/142]V44	26/11/2015	Chikballapur	K	13°29'43.12"N	77°58'4.05"E	S	B	V	KC1/BHK4
[IND2015/145]V44 lamb	26/11/2015	Chikballapur	K	13°29'43.12"N	77°58'4.05"E	S	B	V	KC1/BHK4
[IND2015/133]V Severe	26/11/2015	Chikballapur	K	13° 2'21.52"N	77°23'33.83"E	S	B	V	KC1/BHK4
IND2015/241]K3	10/09/2015	Karimnagar	T	18°19'20.63"N	80°14'29.70"E	S	B	UV	KC1/BHK4
[IND2015/244]K4	10/09/2015	Karimnagar	T	18°26'22.78"N	79° 7'44.26"E	S	B	UV	KC1/BHK4
[IND2015/250]K9	10/09/2015	Karimnagar	T	18°32'54.90"N	78°17'29.98"E	S	B	UV	KC1/BHK6
[IND2015/252]K13	12/09/2015	Karimnagar	T	18°41'50.52"N	79° 4'26.61"E	S	B	UV	KC1/BHK6
[IND2015/256]K14	12/09/2015	Karimnagar	T	18°41'50.52"N	79° 4'26.61"E	S	B	UV	KC1/BHK4
[IND2015/238]6PK	07/09/2015	Warangal	T	18°11'51.79"N	79°42'9.80"E	S	B	UV	KC1/BHK4
[IND2014/37] 10NLG	03/10/2014	Nalgonda	T	16°56'34.67"N	79°12'55.83"E	S	B	UV	KC1/BHK4
[IND2015/271]7A	08/11/2015	Anantpur	AP	14°41'37.97"N	77°38'3.41"E	S	B	UV	KC1/BHK4
[IND2015/]14A	08/11/2015	Anantpur	AP	14°41'37.97"N	77°38'3.41"E	G	B	UV	KC1/BHK4

[*] Virus collection number: [Country code, year / sample number]

[£] Number of passages in KC cell line

[$] Number of passages in BHK cell line.

[&] Number of passages in BSR cell line.

Abbreviations: TN, Tamil Nadu; K, Karnataka; T, Telangana; AP, Andhra Pradesh; B, Blood; Sp, Spleen; Sa, Saliva; V, Vaccinated; UV, Unvaccinated; S, Sheep; G, Goat; C, Cattle.

2.21.2 Anchor-primer ligation and purification of dsRNA

DNase treated dsRNA was ligated to an anchor-primer (as explained in section 2.20.1), then 'cleaned' using the RNA Clean & Concentrator kit (Zymo research, USA) according to the manufacturer's protocol. Briefly, 20 µL of RNA binding buffer was added to 10 µL ligated dsRNA product followed by addition of 30 µL absolute ethanol. The content was transferred to the Zymo-spin column in a collection tube**,** centrifuged at 12,000 g for 30 seconds and flow-through was discarded. Then 400 µL RNA-prep-buffer was added to the column and centrifuged at 12,000 g for 30 seconds to remove flow-through. This was followed by addition of 700 µL RNA-wash-buffer and then 400 µL RNA wash buffer to the column prior to centrifugation at 12,000 g for 30 seconds. The RNA was eluted in 15 µL RNase-free-water by transferring the column to a new tube followed by centrifugation at 12,000 g for 30 seconds and stored at -80^{0}C till used.

2.21.3 Synthesis and amplification of cDNA

The ligated and purified dsRNA was denatured at 99^{0}C for 5 minute to separate the ssRNA strands, then snap chilled on ice to prevent renaturation. The ligated RNA was reverse transcribed to generate full length cDNAs using the AMV reverse transcription system (Promega). Second strand synthesis was done by PCR using the 'Triple Master PCR system' (Eppendorf) with primer 5-15-1 as described in section 2.20.3 and 2.20.4 respectively.

Alternatively, the ligated and purified dsRNA was reverse transcribed to generate full length cDNA using SuperScript III first-strand synthesis system (Thermo Fisher Scientific, catalogue number 18080051). Briefly, 10 µL of ligated RNA, 10 mM dNTPs, 2 µL 10X RT buffer, 1 µL SuperScript III RT (200 U/µL), 1 µL RNase-OUT (40 U/ µL), 2 µL 0.1 M DTT and 4 µL of 25 mM MgCl2 were mixed and incubated at 25^{0}C for 10 min followed by 50^{0}C for 50 min. Finally, the reaction mix was incubated with 1 µL RNase H (2 U) at 37^{0}C for 20 min. Amplification of the resulting cDNA was performed using KAPA HiFi Hot Start Ready Mix (Kapa Biosystems, UK) using the '5-15-1' primer, which is complementary to the anchor primer. Briefly, 12.5 µL of 2 × KAPA HiFi HotStart DNA Polymerase ready mix, consisting of KAPA HiFi HotStart DNA Polymerase (0.5 U/25ul reaction), 0.3 mM of each dNTP and 2.5 mM of MgCl2), was added with 0.2 µL of 100 µM primer 5-15-1 to 2 µL of cDNA and 10.3 µL nuclease free water. Cycling conditions

were 95^{0}C for 3 min followed by 35 cycles of 98^{0}C for 20 s, 65 °C for 15 s, 72 ^{0}C for 4 min with a final cycling step of 72 ^{0}C for 10 min.

2.21.4 Amplified cDNA purification

The dsDNA PCR products were purified using the DNA Clean & Concentrator kit (Zymo research) according to manufacturer's protocol. Briefly, 5 volumes (60 µL) of DNA binding buffer was added to one volume of PCR product (12 µL) and transferred to Zymo-spin column inside a collection tube followed by centrifugation at 12,000 g for 1 minute. Now, 600 µL DNA wash buffer was added to column and centrifuged for 60 seconds to remove flow-through. The DNA was eluted in 20 µL RNase-free water by transferring column to a new tube followed by centrifugation at 12,000 g for 30 seconds.

2.21.5 DNA Quantification

The amount of ds DNA (in ng/ul) was measured prior to library preparation using a Qubit fluorometer and Qubit dsDNA high sensitivity (HS) Assay Kit (Life Technologies), according to manufacturer's instructions. After quantification the concentration of each sample was adjusted to 0.2 ng/µL for library preparation using Nextera XT DNA library preparation kit.

2.21.6 Library preparation

One nanogram of dsDNA in 5 µL (0.2ng/µL) was taken to prepare sequencing libraries using the Nextera XT DNA sample preparation kit (Illumina, USA) according to manufacturer's protocol. All reagents were thawed on ice and mixed properly before use. Nuclease free 0.2 µL PCR tubes were used for all reactions.

2.21.7 'Tagmentation' of input DNA

To each reaction, 10 µL of tagment DNA buffer (TD), 5 µL containing 1 ng of DNA sample and 5 µL amplicon tagment mix (ATM) were added, mixed by pipetting gently and centrifuged briefly at 280 g for 1 minute. Reactions were then incubated on a PCR thermocycler at 55^{0}C for 5 minute and then a 10^{0}C hold for a second followed by a brief spin down at 280 g for 1 minute. Then, 5 µL of neutralize-tagment-buffer (NT) was added, mixed and centrifuged briefly at 280 g for 1 minute to stop the reaction. The mixture was incubated at room temperature for 5 minutes.

2.21.8 cDNA Library Indexing and PCR amplification

Nextera PCR master mix (NPM) was thawed on ice while the index primers (S5XX and N7XX) were thawed at room temperature, for 20 minutes each before making the master mix. The i7 index primers with orange caps were arranged in order horizontally whereas the i5 index primers with white caps were arranged in order vertically (Figure 2.2). Now 15 µL of NPM was added to each sample, followed by 5 µL of the corresponding index primer 1 and index primer 2. The original caps of each primer were replaced with fresh caps to avoid cross-contamination. The mixture was mixed by gentle pipetting and briefly centrifuged at 280 g for 1 minute room temperature, prior putting on a PCR machine using the following programme: 72^0C for 3 min, 95^0C for 30 seconds, 15 cycles of 95^0C for 10 seconds, 55^0C for 30 seconds, 72^0C for 30 seconds, then a final extension step of 72^0C for 5 min, followed by a hold at 10^0C. Barcode tags were incorporated into to each sample through PCR amplification. The reaction could be stored at -20^0C or at 4^0C for next day's work.

2.21.9 Ampure XP dsDNA purification

The amplified PCR reaction products as described in section 2.21.8 were purified using Ampure XP beads (Agencourt, Beckman Coulter). The beads were resuspended by vortexing and then allowed to warm up at room temperature. The samples were also equilibrated at room temperature and 90 µL of Ampure XP beads were added to 50 µL of each sample (at a ratio of 1:1.8) followed by gentle pipetting to mix the components, followed a brief centrifugation at 280 g for 2 minutes, and finally incubation at room temperature for 15 min. The solution was then put on a magnetic rack until the beads and solution were completely separated. The supernatant was removed and the beads were washed twice with 200 µL of freshly prepared 80% ethanol. The beads were allowed to air dry after removing traces of ethanol for 5-10 minute at room temperature. The dried pellet was eluted in 52.5 µL resuspension buffer (RSB) followed by incubation at room temperature for 2 minutes. The samples were then put on a magnetic rack for 5 minutes, until the beads and solution were completely separated. Finally, 50 µL dsDNA supernatant was transferred to fresh tube and stored at -20^0C, or 4^0C for the next day's work

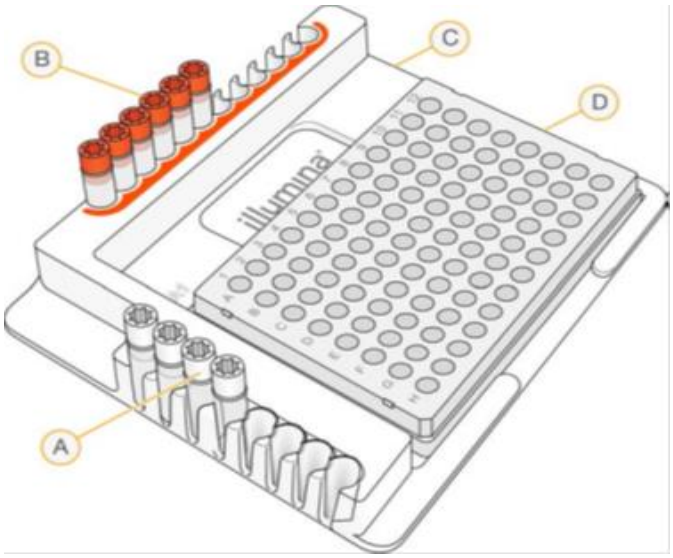


Figure 2.2 TruSeq index plate fixture setup for 24 libraries

A) Index 1 (i7) adapters with orange caps in columns 1–6, B) Index 2 (i5) adapters with white caps in rows A–D, C) TruSeq index plate fixture, D) PCR plate. Image adapted from Nextera XT DNA library preparation kit reference guide (Illumina).

2.21.10 Library Quantification

The concentration of library DNA was measured using Qubit fluorometer and Qubit dsDNA high sensitivity (HS) Assay Kit (Life Technologies), as described in Section 2.21.5. The library sizing (quality and distribution) and quantification were determined using the High Sensitivity D5000 Screen Tape assay (Agilent) in a 4200 Tape Station System (Agilent) according to manufacturer's instructions. Readings on Tape Station should be more than 100 and can range from 100 to 5,000 bp. Another washing with Ampure XP beads was required if primer dimers were detected by Tape Station analysis.

2.21.11 Pooling of sequencing library, denaturation and dilution

Unique barcode indexes attached to the samples during the sequencing library preparation subsequently allowed multiple libraries to be pooled i.e. barcode multiplexing of multiple samples and could be sequenced simultaneously during a single sequencing run. Each library for sequencing was diluted to 4 nM with nuclease-free water, based on their sizing and quantification of DNA fragments on Tape Station. 5 µl from each diluted library were pooled into a single fresh tube and the pooled library quantified again to measure its concentration using a Qubit florometer and the fragment size by Tape station. The pooled libraries were denatured with 0.2 N NaOH for 5 min at room temperature. The pooled

denatured library was further diluted to 12.5 pM with HT1 buffer (Illumina). An internal control library, PhiX control v3 library (Illumina) was added to the concentration of 1%. The diluted library pool was loaded in the MiSeq reagent kit Version (V) 2 cartridge (Illumina) and pair-end reads of 150 bp were produced on a MiSeq platform.

2.22 Processing and assembly of the raw sequencing data

Bioinformatics analysis of the raw sequencing data was carried out by Dr. Kyriaki Nomikou. Briefly, quality check of the raw sequence reads was done by using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). TrimGalore was used for Primer, adapter and low quality read trimming (www.biopinformatics.babraham.ac.uk/projects/trim_galore/). Mapping of quality passed and trimmed sequenced reads to reference genome has been done using Bowtie 2 (www.bowtie-bio.sourceforge.net/bowtie2/index.shtml) and Tanoti (http://bioinformatics.cvr.ac.uk/Tanoti/). Post assembly processing and consensus sequence generation was done using SAMtools (Li et al., 2009).

2.23 Sequence data phylogenetic analysis of Seg-2

Sequence analysis of Seg-2 was done to identify the serotype of Indian field isolates from BT outbreaks in Southern India, 2014-15, by comparing Seg-2 sequences with those of reference strains of the 27 BTV serotypes. The phylogenetic analyses of Seg-2 of these Indian BTV strains will help to determine their geographic origins, movements of different virus lineages and evolutionary relationships with other isolates from different parts of the India and world.

Sequences of Seg-2 used in the phylogenetic analyses were initially aligned in Seaview using Clustal W program (Gouy et al., 2010). Additionally, sequence alignment of Seg-2/VP2 gene sequences of Indian BTV isolates of 2014-2015 generated in this study, together with Seg-2/VP2 gene sequences of the 27 BTV reference serotypes and Seg-2/VP2 sequences of the corresponding serotype sequences publically available retrieved from NCBI (accessed on 01.12.17) , was done using Clustal Omega for phylogenetic analysis (Sievers et al., 2011).

Maximum Likelihood (ML) phylogenetic trees were built using PhyML program (Guindon and Gascuel, 2003) under the best-fit nucleotide substitution model calculated by

JModel test v2.1.4 using Corrected Akaike information criterion (AICc) (Darriba et al., 2012, Posada, 2008). The Maximum Likelihood (ML) method implemented in PhyML was used to attain the best tree topology. The bootstrapping method was used with 100 replicates for maximum-likelihood method. Graphical representation and phylogenetic tree editing was achieved in FigTree (Rambaut, 2009) and further graphic adjustments were done in Inkscape 0.48.4 (Inkscape, 2013).

In a tree, each strain retrieved from the Genbank was annotated by serotype followed by country code, year of sample collection where applicable and Genbank accession number as "serotype/country code/year of sample collection_Genbank accession number". The Indian strains sequenced in this study were indicated as country code, IND followed by year of sample collection, original sample ID and date of sampling.

Chapter 3: Field outbreaks of bluetongue disease in Southern India during 2014-15.

3.1 Introduction

India has the largest livestock population of any country in the world and more than 70% of country's sheep population is found in four states, including: Rajasthan which is a North-western desert state; Telangana, Andhra Pradesh, Tamil Nadu and Karnataka. About 5 million small and marginal farmers including 'landless labourers' are involved in the rearing of sheep and goats in India (Livestock census, 2014), with both 'semi-pastoral' and 'semi-intensive' management systems used for rearing sheep in the Southern states (Kantwa et al., 2017, Rao et al., 2016b).

Clinical BT was first reported from Maharashtra state in Western India (Sapre, 1964), but is now considered both endemic and widespread on the sub-continent, with outbreaks reported in Northern, Central, Western and Southern states (Prasad et al., 1992, Sreenivasulu et al., 2004, Ranjan et al., 2015, Prasad et al., 2009). From 1970-1980, clinical BT disease was reported mostly in exotic breeds of sheep involved in a crossbreeding programme for breed improvement, in Himachal Pradesh (Uppal and Vasudevan, 1980), Haryana, (Vasudevan, 1982) and Rajasthan states of Northern India (Lonkar et al., 1983, Sharma et al., 1985, Mahajan et al., 1991). However there were also a few reports of disease in native sheep breeds in Maharashtra, Western India (Singh et al., 1982, Harbola et al., 1982) and Karnataka, Southern India., (Srinivas et al., 1982). After 1982, BT outbreaks were reported annually in native sheep breeds, particularly in the Southern states (Mehrotra et al., 1991, Kulkarni et al., 1992, Saravanabava, 1992, Prasad et al., 2009, Prasad et al., 1992, Rao et al., 2016b, Susmitha et al., 2012, Sreenivasulu et al., 1999, Bommineni et al., 2008).

There is a clear difference in the pattern of disease and clinical symptoms of BT between the Northern and Southern states of India. While frank clinical BT outbreaks have mostly been reported from sheep and goats in the Southern part of India over the last two decades (Rao et al., 2016b), overt BT signs have only occasionally been reported in local or crossbred sheep breeds in Northern India.

Concurrent infections of small ruminants with BTV and *peste des petite ruminant virus* (PPRV) have been reported in Haryana, Uttar Pradesh and Uttarakhand States of Northern India, showing clinical signs of fever, anorexia, oral mucosa congestion, gastroenteritis and mortality (Maan et al., 2017, Biswas et al., 2010, Mondal et al., 2009, Mondal et al., 2013). In 1989-1990, 1997-1998 and 2005-2006, BT outbreaks with severe clinical manifestations were reported from Tamil Nadu, resulting in high sheep mortality (Reddy et al., 2016a) and nearly 0.3 million small ruminants died in Tamil Nadu in the 1997-98 BT outbreak (Ilango, 2006).

The incidence of BT outbreaks in India is considered to be influenced by temperature, humidity, precipitation and sheep population densities, which collectively provide conducive breeding grounds for the local *Culicoides* population that is involved in BTV transmission, and these are therefore factors in the widespread incidence of overt clinical BT disease in the Southern states (Rao et al., 2016b). In Telangana, northern parts of Andhra Pradesh and Karnataka, BT outbreaks occur during south west monsoon with highest incidence during August and September. In contrast, in the Southern interior parts of Andhra Pradesh, Karnataka and Tamil Nadu, the disease is observed during north east monsoon from October to December (Sreenivasulu et al., 2004).

The clinical signs of BT disease are not pathognomonic and could be confused with diseases such as foot and mouth disease (FMD), peste des petits ruminants (PPR), sheep pox, goat pox and contagious ecthyma which are also endemic in India. Consequently, differential diagnosis of BT requires confirmation by laboratory testing (Prasad et al., 2009).

The four Southern states of India Telangana, Andhra Pradesh, Tamil Nadu, Karnataka including Maharashtra, which is a western state, are being covered by pentavalent inactivated BTV vaccine (containing BTV-1, BTV-2, BTV-10, BTV-16 and BTV-23) but on voluntary basis. The vaccine was tested in Maharashtra, Telangana, Andhra Pradesh, Tamil Nadu and Karnataka at farmer's doorstep from 2010 onwards. The Raksha Blu vaccine (commercial name of pentavalent inactivated BTV vaccine, containing serotypes BTV-1, BTV-2, BTV-10, BTV-16 and BTV-23) was launched on 8 January 2015 by Indian Immunologicals Limited in Hyderabad, Telangana (Maan et al., 2015c, Rao et al., 2016b). However, inspite of hyperendemic status of BTV transmission in these states, vaccination is not done at mass scale. The presence of multiple co-circulating BTV serotypes also shows the importance of routine virological surveillance, especially as it

provides a basis for the current vaccination programme and would help to ensure the use of vaccines of the appropriate BTV serotype (Rao et al., 2016b).

It will be therefore useful to analyse BTV isolates from before, during and after the start of the current pentavalent inactivated-vaccine programme, to see if it has altered the prevalence of different BTV serotypes circulating in the region. An initial step in this process is to attend BT field outbreaks from Southern India, to collect well documented samples for virus isolations, which will subsequently help us understand the epidemiology of the disease in endemic regions and in the country as a whole.

The objectives for the work described in this chapter include:

- To attend suspected field outbreaks of BTV in various districts of Telangana, Andhra Pradesh, Karnataka and Tamil Nadu in India during 2014- 2015.
- To collect blood and necropsy samples from outbreaks in small ruminants and cattle along with well documented information including sample collection date, geographical location of sample collection, geographical coordinates, host species affected, vaccination status against BT and symptoms of disease.
- Process and analyse the collected samples, to detect BTV RNA by TaqMan based quantitative RT-PCR targeting genome Seg-10 (NS3), thereby identifying infected animals.
- To use samples those are positive by BTV serogroup-specific qRT-PCR for BTV isolation, by infecting KC and BHK cells.

3.2 Results

3.2.1 Field outbreaks in 2014

In October 2014, nine EDTA blood samples (n=9) were collected from sheep that were suspected of BTV infection, in flocks that had not been vaccinated against BT in the Warangal (n=5) and Mehboobnagar (n=4) districts of Telangana (Figure 3.11). There was no official BT outbreak reported from Telangana and Andhra Pradesh in 2014 (AICRP, 2014). State Andhra Pradesh was divided into two states, namely Telangana and Andhra Pradesh in 2014. The pentavalent inactivated BTV vaccine is being used in four states of Southern India: Telangana, Andhra Pradesh, Tamil Nadu and Karnataka since 2011 at farmer's doorstep under All India Network Project on Bluetongue (www.icar.org.in/node/3516) to test the efficacy of vaccine and now recently, the vaccine

has been commercialized in 2015 for use by farmers on voluntary basis. These sporadic suspect BT cases showed mostly nonspecific signs, including nasal discharge (89%) and mild congestion of gum mucosa (78%). However other clinical manifestations including head oedema, pyrexia (40 - 41.1^{0}C) and lameness were observed in one case each (Table 3.1 and 3.2).

Suspected BT cases were also attended in the Karur, Erode, Tirupur, Chengalpattu, Salem and Thoothukudi districts of Tamil Nadu, during November 2014 to January 2015, and twenty five (n = 25) EDTA blood samples were collected (Figure 3.11). The most obvious clinical signs included congested oral mucosa (76%), limping (72%), erosions of oral mucosa (56%), fever (32%), excess salivation (16%) and nasal discharge (12%). These flocks had not been vaccinated with pentavalent inactivated BT vaccine (Table 3.1 and 3.3).

3.2.2 BT Field outbreaks in 2015

3.2.2.1 Telangana, 2015

Twenty four (24) animals (suspect BT cases that had not been vaccinated against BTV) were sampled from mixed flocks of sheep and goats (including twenty three EDTA blood samples, twenty one from sheep and two from goats and one necropsy (spleen) sample from PPR suspected goat) from Telangana during September 2015 (Table 3.4, Figure 3.10). These sporadic suspect BT cases were from Warangal, Karimnagar and Medak districts of Telangana. The most common clinical signs were congested oral mucosa (58%), oral lesions (50%), nasal discharge and high fever, 104-106^{0}F (38% each) in these flocks (Table 3.1).

3.2.2.2 Andhra Pradesh, 2015

A severe outbreak of BT occurred in mixed flocks of sheep and goat, which had not been vaccinated against BT, in the Anantpur and Chitoor districts of Andhra Pradesh during early November 2015. The suspected animals (n=60) showed clinical signs that included ulcers and erosions in the mouth, particularly on the dental pad (58%), congested mucosa (48%), high fever, 40 - 41.1^{0}C (43%), salivation (8%), nasal discharge (5%), limping (7%), head oedema (3%) and blood speckled nasal discharge (2%) (Table 3.1 and 3.8, Figure 3.6, 3.10). The goats were apparently healthy and did not show clinical signs, except pyrexia and nasal discharge.

Table 3.1 Main clinical signs and their frequency in sheep and goats affected by BTV.

Symptoms	T 2014	TN 2014	T 2015	AP 2015	K 2015	TN 2015	Overall
Total clinical cases (n)	9	25	24	60	179	145*	442
Pyrexia (n)	1	8	9	26	79	57	180
Percentage (%)	11	32	38	43	44	39	41
Oral lesions (n)	0	14	12	35	120	89	270
Percentage (%)	0	56	50	58	67	61	61
Nasal discharge (n)	8	3	9	3	32	19	74
Percentage (%)	89	12	38	5	18	13	17
Salivation (n)	0	4	1	5	23	25	58
Percentage (%)	0	16	4	8	13	17	13
Limping (n)	1	18	0	4	76	103	202
Percentage (%)	11	72	0	7	42	71	46
Head oedema (n)	1	0	0	2	4	1	6
Percentage (%)	11	0	0	3	2	1	1
Torticollis (n)	0	0	0	0	2	4	6
Percentage (%)	0	0	0	0	1	3	1
Blood stained nasal discharge (n)	0	0	0	1	3	5	4
Percentage (%)	0	0	0	2	2	3	1
Congested oral mucosa (n)	7	19	14	48	80	60	228
Percentage (%)	78	76	58	80	45	43	52

*Mortality cases (five) from Tamil Nadu state are not included.

Abbreviations: AP, Andhra Pradesh; T Telangana, TN; Tamil Nadu and K, Karnataka

3.2.2.3 Karnataka, 2015

One hundred and seventy eight EDTA blood samples (n=178) and one necropsy (spleen) sample were collected, from severe disease outbreaks (suspected BT) during November 2015, that were attended in mixed flocks of sheep (n=176) and goats (n=2) in the Chickballapur, Devanagere, Tumkure, Kolar and Chikmagalur districts of Karnataka State (Figure 3.10). The majority of BT-suspect sheep showed clinical signs that included ulcers on oral mucosa (67%) particularly on the dental pad, congested oral mucosa with swollen muzzle and lips (45%), fever (44%), limping (42%) and excessive salivation and frothing at the mouth (13%). Swollen cyanotic tongue (2%), nasal discharge speckled with blood (2%), submandibular & head oedema (2%), oral mucosa bled on touch (2%) and torticollis (1%) were the rare clinical symptoms (Table 3.1 and 3.8, Figure 3.1-3.5). Wool-break was evident in almost all cases (Figure 3.7).

Although some animals had been vaccinated, only ten blood samples could be collected from three small flocks showing clinical signs of BT. Of these, three blood samples were collected from a vaccinated sheep in village Vemgal, Sidlaghatta, Chikballapur district of Karnataka State using the inactivated bivalent (BTV-1 and 23) vaccine. Three blood samples from inactivated pentavalent (BTV-1, -2, -10, -16 and -23) vaccinated sheep in village Lakkenahalli, Sidlaghatta and four blood samples from inactivated pentavalent vaccinated sheep in village Genjigunte, Sidlaghatta of Chikballapur district of Karnataka State were collected. Primary vaccination with pentavalent BTV vaccine was done in the month of August followed by a booster dose of vaccine 28 days after the primary vaccination. The clinical presentation and disease severity during BT outbreaks, 2015 in this study varied from less severe to more severe, although severity of disease did not differ substantially in vaccinated and non-vaccinated field cases of BT outbreak in this study.

3.2.2.4 Tamil Nadu, 2015

In mid-December 2015, suspected outbreaks of BT were reported from Perambalur, Shivagangai, Pudukottai, Tirunelveli, Erode and Thoothukudi districts of Tamil Nadu. One hundred and fifty samples were collected (Figure 3.10). Of these, one hundred and forty five animals, from mixed populations of sheep (n=106), goat (n=26) and cattle (n=13) were sampled to take EDTA blood and five necropsy (spleen) samples from sheep were also collected. The clinical signs observed in sheep, included limping (71%), erosions and ulcers of the oral mucosa (61%), pyrexia (39%), congested oral mucosa (43%), salivation (17%) and nasal discharge (13%). Torticollis was observed in four cases (3%) whereas blood tinged nasal discharges in five (3%) and head oedema in one (1%) case respectively (Table 3.1, Figure 3.7, 3.8). The goats were apparently healthy and did not show clinical signs of infection, other than pyrexia. However, abortion and still birth cases were observed in four goats with no fever but with a history of limping.

Of one hundred and fifty samples from Tamil Nadu, 2015, thirty seven samples were from sheep (n=33) and goats (n=4) vaccinated with the pentavalent inactivated BT vaccine (Table 3.6). Clinical manifestations of BT were noticed even in the sheep vaccinated with the pentavalent inactivated BTV vaccine and disease severity was similar in vaccinated and non-vaccinated field cases varying from acute to subclinical in both in this study.

Interestingly ten cattle from Thoothukudi, in a mixed population of infected sheep and goat flocks, showed clinical sign of lameness and excessive salivation. These included, two rare cases of nasal discharge speckled with blood, as well as profuse salivation. Mild oral lesions and limping were also evident in three cattle from infected mixed flocks of sheep and goats in Tirunelveli, Tamil Nadu. The cattle had not been vaccinated against BT.

3.2.3 Laboratory diagnosis based on qRT-PCR

3.2.3.1 Telangana sporadic field outbreak, 2014

RNA extracted from clinical samples collected during field outbreaks in 2014, were subjected to serogroup-specific real-time RT-PCR targeting genome segment 9 (Maan et al., 2015a). These were not tested with BTV serogroup-specific qRT-PCR targeting Seg-10 (Hofmann et al., 2008a).

Only one sample, 4WGL/14 from Warangal, Telangana (11%) was positive for the presence of BTV RNA with cycle threshold (Ct) value of 27.5 (Table 3.2). All other eight samples had cycle threshold (Ct) values greater than 40 and thus were considered negative for BTV RNA.

Table 3.2 Seg-9 based qRT-PCR results from Telangana state, 2014

Sample No.	*Ct value	Clinical signs
1MBNR/14	>40	103^0F temperature, depressed, nasal discharge and congestion of gum mucosa.
2MBNR/14	>40	103^0F temperature, nasal discharge and congestion of gum mucosa.
3MBNR/14	>40	103^0F temperature, nasal discharge and congestion of gum mucosa.
4MBNR/14	>40	103^0F temperature, nasal discharge and congestion of gum mucosa, lameness.
1WGL/14	>40	102^0F temperature ,Congestion of gum mucosa
2WGL/14	>40	105^0F temperature, nasal discharge and congestion of gum mucosa.
3WGL/14	>40	103^0F temperature, nasal discharge and congestion of gum mucosa.
4WGL/14	27.5	102^0F temperature, nasal discharge and swelling of head.
5WGL/14	>40	103^0F temperature, nasal discharge.

* Ct values in TaqMan serogroup-specific qRT- PCR targeting Seg-9 of BTV.

3.2.3.2 Tamil Nadu field outbreaks, 2014

Out of twenty five clinical samples (EDTA blood) from Tamil Nadu BT 2014 outbreak, sixteen samples (69.5%) were positive for BTV RNA by BTV serogroup-specific qRT-PCR targeting Seg-9, with cycle threshold (Ct) value ranging from 23 to 38 (Table 3.3). All other samples had cycle threshold (Ct) value more than 40 and thus were considered negative for BTV RNA. These were not tested with BTV serogroup-specific qRT-PCR targeting Seg-10 (Hofmann et al., 2008a).

Table 3.3 Seg-9 based qRT-PCR results from Tamil Nadu state, 2014.

Sample ID	Type of sample	*Ct value	Host spp	Date of sampling	Place of sampling
CHN 1	blood	35	sheep	22/11/2014	Erode, TN
CHN 2	blood	28	sheep	22/11/2014	Erode, TN
CHN 3	blood	34	sheep	22/11/2014	Karur, TN
CHN 4	blood	23	sheep	22/11/2014	Thoothukudi,TN
CHN 5	blood	34	sheep	22/11/2014	Tiruppur, TN
CHN 6	blood	34	sheep	22/11/2014	Tiruppur,TN
CHN 7	blood	40	sheep	22/11/2014	Karur,TN
CHN 8	blood	36	sheep	22/11/2014	Karur, TN
CHN 9	blood	38	sheep	22/11/2014	Karur,TN
CHN 10	blood	32	sheep	22/11/2014	Karur,TN
CHN 11	blood	32	sheep	22/11/2014	Karur,TN
CHN 12	blood	30	sheep	22/11/2014	Karur,TN
CHN 13	blood	30	sheep	22/11/2014	Karur,TN
CHN 14	blood	29	sheep	22/11/2014	Karur,TN
CHN 15	blood	34	sheep	4/12/2014	Kolathur,Salem,TN
CHN 16	blood	40	sheep	4/12/2014	Kolathur,Salem,TN
CHN 17	blood	40	sheep	4/12/2014	Kolathur,Salem,TN
CHN 18	blood	30	sheep	4/12/2014	Kolathur,Salem,TN
CHN 19	blood	40	sheep	2/01/2015	$Chengalpattu,TN
CHN 20	blood	40	sheep	2/01/2015	Chengalpattu,TN
CHN 21	blood	40	sheep	2/01/2015	Chengalpattu,TN
CHN 22	blood	40	sheep	2/01/2015	Chengalpattu,TN
CHN 23	blood	40	sheep	2/01/2015	Chengalpattu,TN
CHN 24	blood	40	sheep	2/01/2015	Chengalpattu,TN
CHN 25	blood	36	sheep	22/11/2014	Thoothukudi,TN

* Ct values in TaqMan serogroup-specific qRT- PCR targeting Seg-9 of BTV

$ Chengalpattu is a town in Kanchipuram district of Tamil Nadu.

3.2.3.3 Telangana field outbreaks, 2015

RNA extracted from blood samples and necropsy samples collected from 2015 field outbreaks in Telanagana were subjected to BTV RNA detection using BTV serogroup-specific qRT-PCR targeting Seg-10 (Hofmann et al., 2008a). Initially, samples were tested by BTV serogroup-specific qRT-PCR targeting Seg-9, but no amplification was detected in any samples by this assay.

Out of twenty three EDTA blood samples and one necropsy sample from Telangana BT 2015 outbreaks, twenty one samples (91%) were positive for BTV RNA by BTV serogroup-specific qRT-PCR targeting Seg-10, with cycle threshold (Ct) value ranging from 20 to 32 (Table 3.4). Three samples had cycle threshold (Ct) value more than 40 and thus were considered negative for BTV RNA.

Table 3.4 Seg-10 based qRT-PCR results of samples from Telangana state, 2015

*Ct value	Place of sampling	Date of sampling	Virus collection number
ND	Dharmaram, Warangal	07/09/2015	1/PKL/15
32	Dharmaram, Warangal	07/09/2015	2/PKL/15
ND	Dharmaram, Warangal	07/09/2015	3/PKL/15
ND	Jukal,Parkal, Warangal	07/09/2015	4/PKL/15
31	Jukal,Parkal, Warangal	07/09/2015	5/PKL/15
24	Jukal,Parkal, Warangal	07/09/2015	[IND2015/236]6PK
30	Medaram, Karimnagar	10/09/2015	K1/15
32	Medaram, Karimnagar	10/09/2015	K2/15
22	Medaram, Karimnagar	10/09/2015	[IND2015/239]K3
27	Mallesham, Karimnagar	10/09/2015	[IND2015/242]K4
32	Oongathurthi, Karimnagar	10/09/2015	K5/15
32	Oongathurthi, Karimnagar	10/09/2015	K6/15
33	Oongathurthi, Karimnagar	10/09/2015	K7/15
28	Oongathurthi, Karimnagar	10/09/2015	[IND2015/245]K8
21	Oongathurthi, Karimnagar	10/09/2015	[IND2015/248]K9
30	Madhurampally Karimnagar	12/09/2015	[IND2015/251]K10
31	Madhurampally, Karimnagar	12/09/2015	K11/15
31	Madhurampally, Karimnagar	12/09/2015	K12/15
31	Madhurampally, Karimnagar	12/09/2015	[IND2015/252]K13
20	Madhurampally, Karimnagar	12/09/2015	[IND2015/254]K14
25	Madhurampally, Karimnagar	12/09/2015	[IND2015/257]K15
31	Nalthur , Medak [$]	14/09/2015	[IND2015/260]PPR2
30	Nalthur , Medak [$]	14/09/2015	[IND2015/224]PPR spleen
31	Rauiapalli, Medak [$]	14/09/2015	[IND2015/263]PPR1

Abbreviations: ND, not detectable by q RT-PCR.

[$] Host spp was goat. All other samples were from sheep.

[*] Ct value by BTV serogroup-specific qRT-PCR targeting Seg-10 (Hofmann et al., 2008a).

3.2.3.4 Andhra Pradesh field outbreaks, 2015

BTV RNA was detected in all sixty EDTA blood samples (100%) using BTV serogroup-specific qRT-PCR targeting Seg-10, with cycle threshold (Ct) value ranging from 18-37 (Table 3.5 and 3.7). Initially, samples were tested by BTV serogroup-specific qRT-PCR targeting Seg-9, but no amplification was obtained in any sample by this assay.

3.2.3.5 Karnataka field outbreaks, 2015

Eighty samples, EDTA blood and one necropsy sample (44.7%, 80/179) from Karnataka were positive for the presence of BTV RNA using BTV serogroup-specific qRT-PCR targeting Seg-10, with cycle threshold (Ct) value ranging from 12-39 (Table 3.5). Ninety nine samples had cycle threshold (Ct) value more than 40 and thus were negative for BTV RNA. Of the 10 samples from BT vaccinated sheep, all samples were positive by serogroup-specific qRT-PCR targeting Seg-10, with cycle threshold (Ct) value ranging from 18-30 (Table 3.6).

3.2.3.6 Tamil Nadu field outbreaks, 2015

Of the one hundred and forty three EDTA blood, two saliva and five necropsy samples from BT suspected 2015 outbreaks in Tamil Nadu, seventy five samples (50%) including saliva and necropsy samples were positive for BTV RNA by BTV serogroup-specific qRT-PCR targeting Seg-10, with cycle threshold (Ct) value ranging from 15 to 39 (Table 3.5). Seventy five samples had cycle threshold (Ct) value more than 40 and thus were considered negative for BTV RNA. Of the thirty seven samples from BT vaccinated sheep and goats, twenty five samples were positive by serogroup-specific qRT-PCR targeting Seg-10, with cycle threshold (Ct) value ranging from 20 to 37 (Table 3.6). Ten cattle from Thoothukudi and three from Tirunelveli were found positive by BTV serogroup-specific qRT-PCR targeting Seg-10 with Ct value ranging from 25 to 34. Interestingly, four vaccinated healthy sheep without clinical signs were positive in qRT-PCR for BT with Ct value ranging from 25 to 32 (Table 3.8).

One possible reason for BTV detection in animals with no clinical signs could be the collection of blood samples during the initial incubation period, or later on, during the recovery period after the animal has recovered from clinical signs. The presence of active

hemagglutinin associated with VP2 on the BTV virus surface, allows the virus to stick to red blood cells, even after it has been inactivated by neutralising antibodies (Hassan and Roy, 1999, Eaton and Crameri, 1989). Some animals are known to remain asymptomatic even though infected with BTV (MacLachlan, 1994).

Table 3.5 Serogroup-specific real-time RT-PCR results to diagnose BT

State	Samples collected	qRT-PCR[$] positive (Ct <40)	qRT-PCR[$] negative (Ct >40)	Prevalence of BTV in samples
Telangana,2014	9	1*	8	11%
Tamil Nadu,2014	25	16*	9	69.5%
Telangana,2015	24	21	3	91%
Andhra Pradesh, 2015	60	60	0	100%
Karnataka, 2015	179	80	99	44.7%
Tamil Nadu, 2015	150	75	75	50%
Overall	447	236	194	53%

* Clinical samples collected from field outbreaks, 2014 were subjected to serogroup-specific real-time RT-PCR targeting genome segment 9 (Maan et al., 2015a).

[$] All samples except 2014 outbreaks were subjected to BTV RNA detection using BTV serogroup-specific qRT-PCR targeting Seg-10 (Hofmann et al., 2008a).

Table 3.6 Serogroup-specific real-time RT-PCR results in vaccinated flocks.

	Samples collected	qRT-PCR positive[$]	Ct value (min - max)	Samples with Ct <30
Karnataka, 2015	10[*]	10	18-30	10
Tami Nadu, 2015	37	25	20-37	15

[*] Seven samples were collected from pentavalent inactivated BT vaccinated sheep and three samples were from bivalent inactivated BT vaccinated animals.

[$] Samples were subjected to BTV RNA detection using BTV serogroup-specific qRT-PCR targeting Seg-10 (Hofmann et al., 2008a).

3.2.4 Samples used to infect KC and BHK cells

Out of four hundred forty seven samples, one hundred and eighty samples were positive with Ct value <30 by BTV serogroup-specific qRT-PCR. Of these one hundred and eighty samples, only one hundred and seventeen samples from 2015 outbreak and six from 2014

outbreaks with Ct value <30 were attempted for virus isolation (Table 3.7). These samples were used to infect KC and BHK cells for virus isolation. Eighteen samples from vaccinated flocks and cattle with Ct value > 30 from 2015 outbreaks were also used to make virus isolation. Overall, one hundred and forty one samples were attempted for virus isolation from 2014-2015 outbreaks. Detail about these 141 samples was given in Table 3.8.

Table 3.7 Samples with Ct value <30 by BTV group specific qRT-PCR

State	Samples collected	qRT-PCR positive (Ct<30)
Telangana,2014	9	1*
Tamil Nadu,2014	25	6*
Telangana, 2015	24	16
Andhra Pradesh, 2015	60	30
Karnataka, 2015	179	57
Tamil Nadu, 2015	150	70
Overall	447	180

* Clinical samples collected from field outbreaks, 2014 were subjected to serogroup-specific real-time RT-PCR targeting genome segment 9 (Maan et al., 2015a)

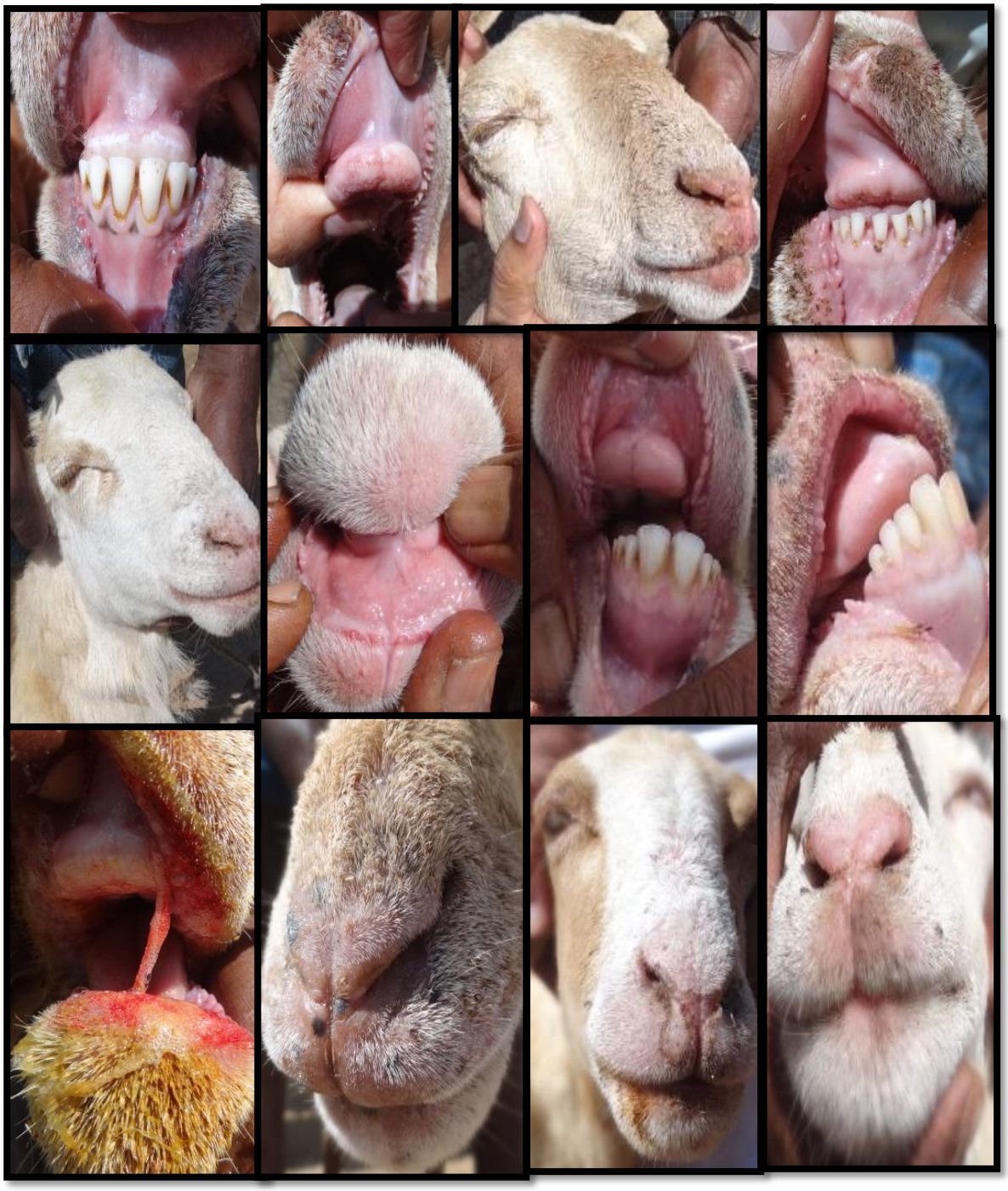


Figure 3.1 Hyperaemic oral mucosa and swollen muzzle in sheep suspected for bluetongue disease, Karnataka 2015.

Muzzle and lips were congested and swollen, hyperaemic and inflamed oral mucosa bled on touch

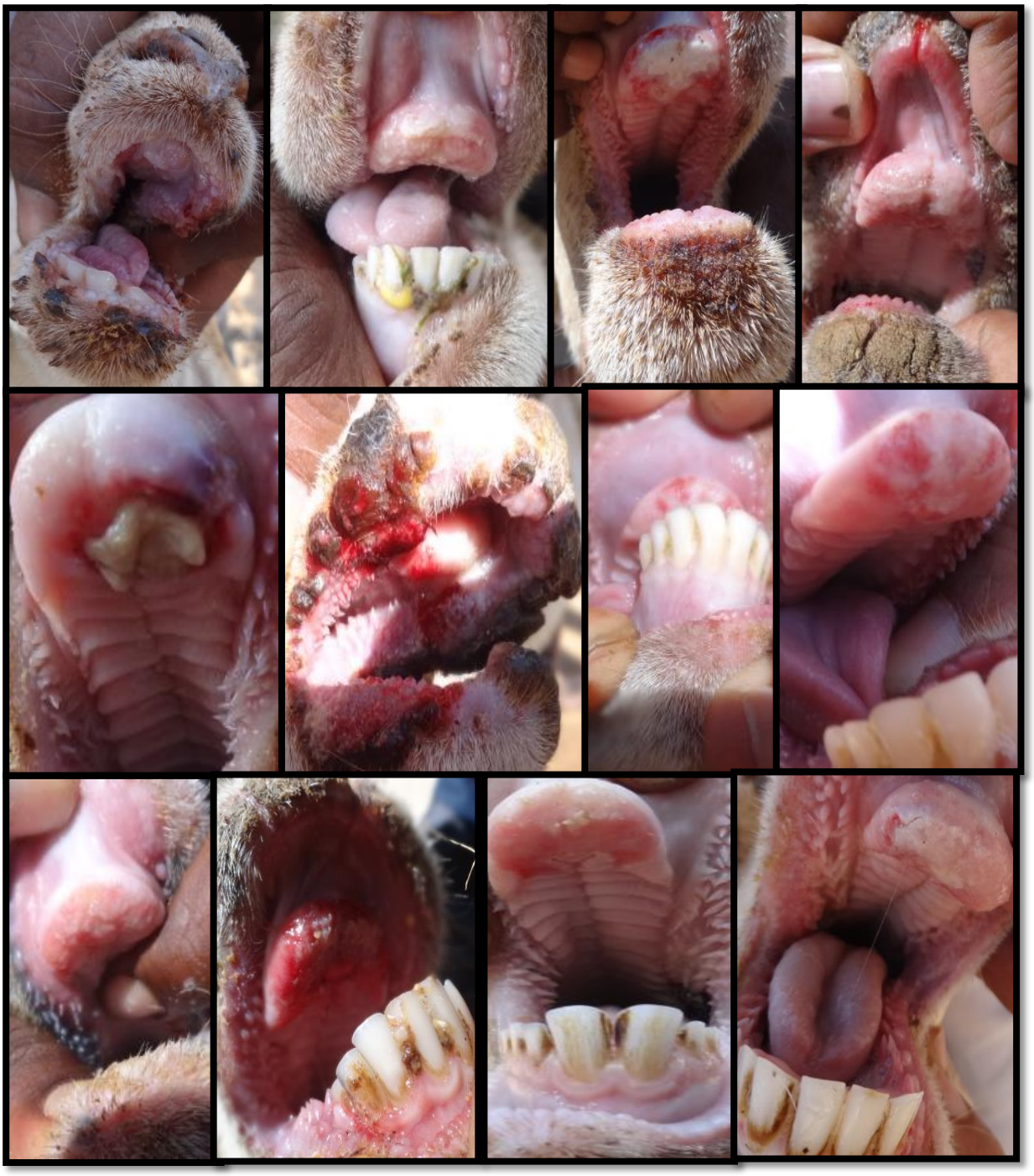


Figure 3.2 Oral erosions and ulcers particularly on the dental pad in BT suspected sheep, Karnataka 2015.

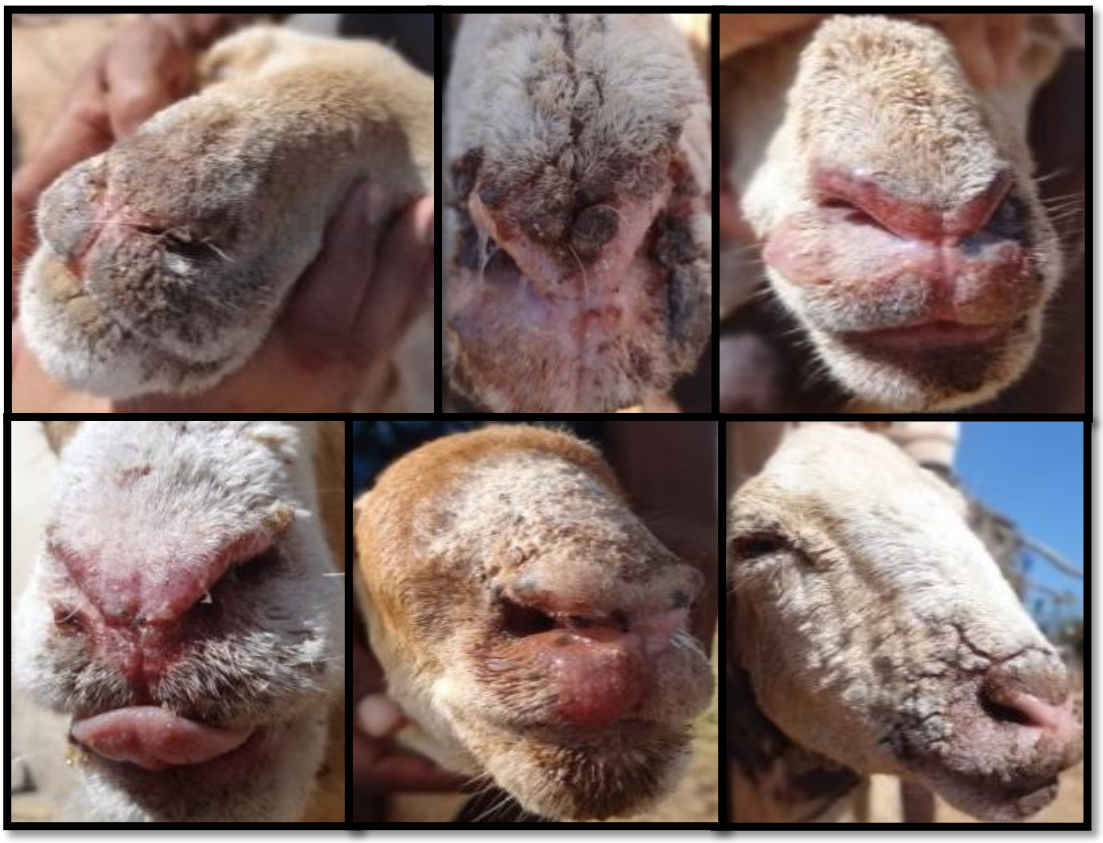


Figure 3.3 Erosion, crusting of muzzle and nasal discharge speckled with blood in BT suspected sheep, Karnataka 2015.

Figure 3.4 Vulvar lips haemorrhages & pyrexia in sheep suspected for bluetongue disease, Karnataka 2015.

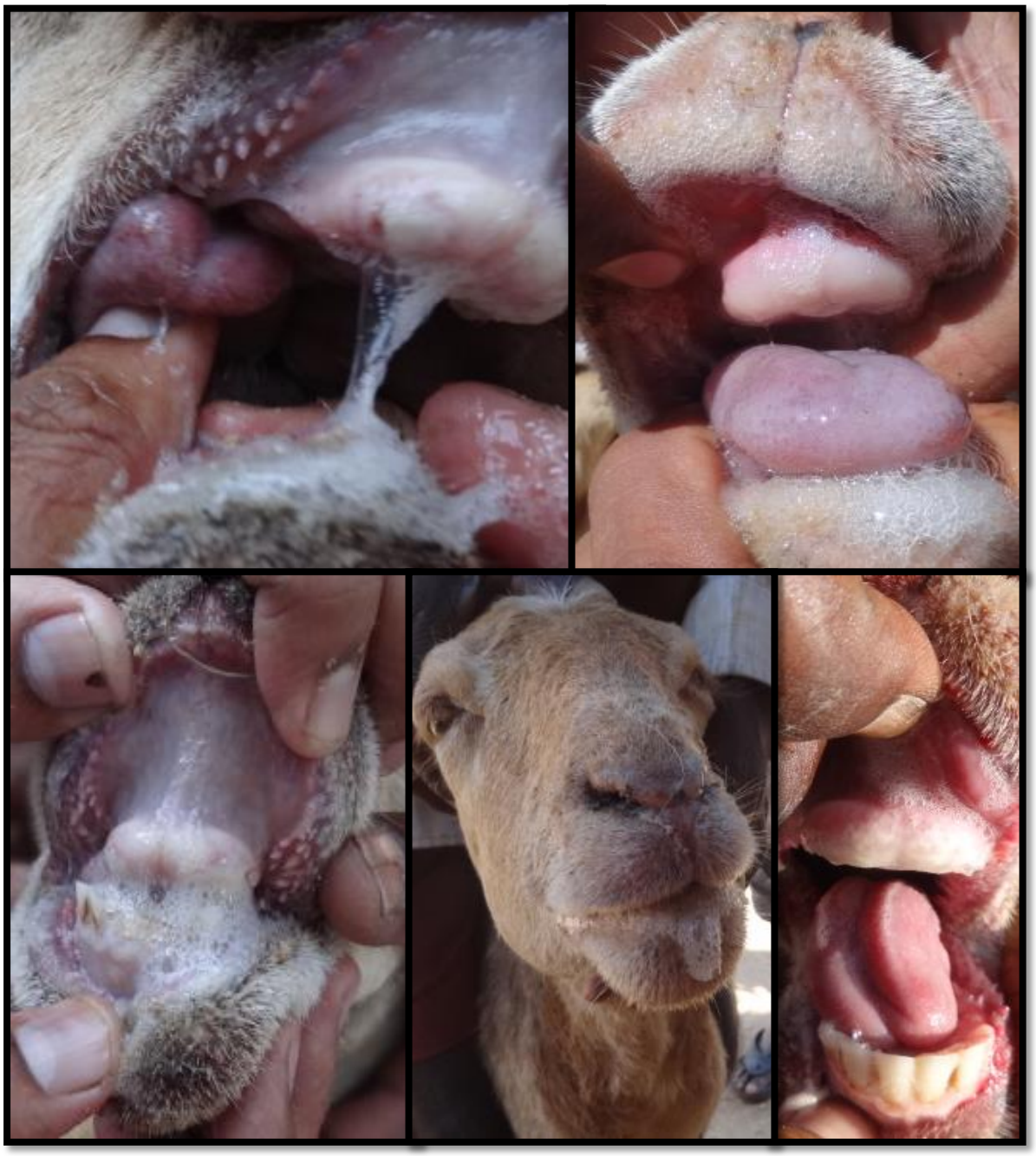


Figure 3.5 Excessive salivation, frothing at the mouth and swollen cyanotic tongue in BT suspected sheep, Karnataka 2015.

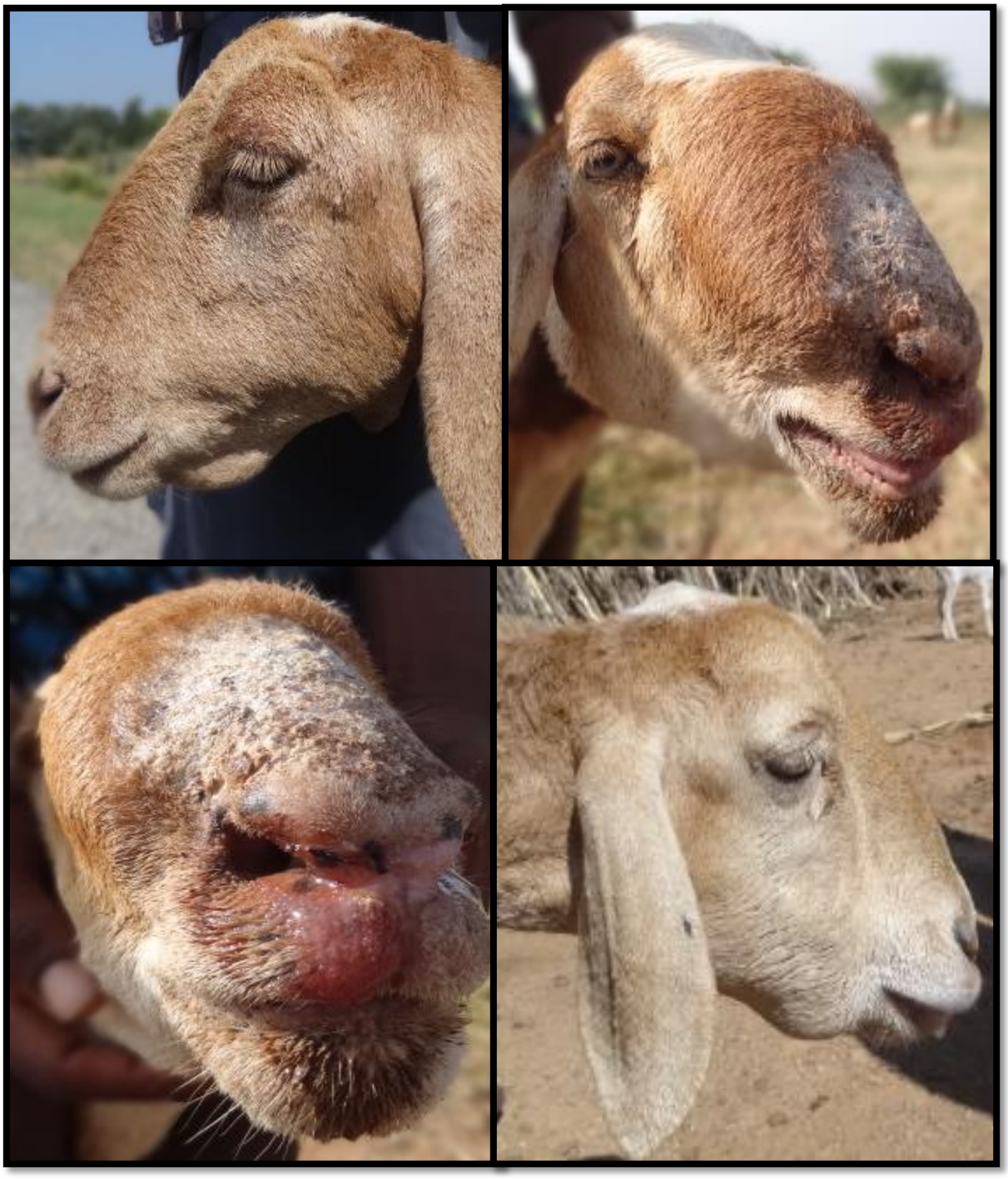


Figure 3.6 Submandibular and head oedema in BT suspected sheep, Andhra Pradesh 2015.

Figure 3.7 Coronitis resulting in lameness and break in wool in BT suspected sheep,

A: Sheep suspected for BT infection from Tamil Nadu state, 2015; B, C, D, E & F from Karnataka state, 2015

Figure 3.8 BT suspected cases from Tamil Nadu, 2015.

A, B, C, D: Erosion of muzzle, nasal discharge and oral erosions in BT suspected sheep, Tamil Nadu 2015; E: coronitis; F: mortality

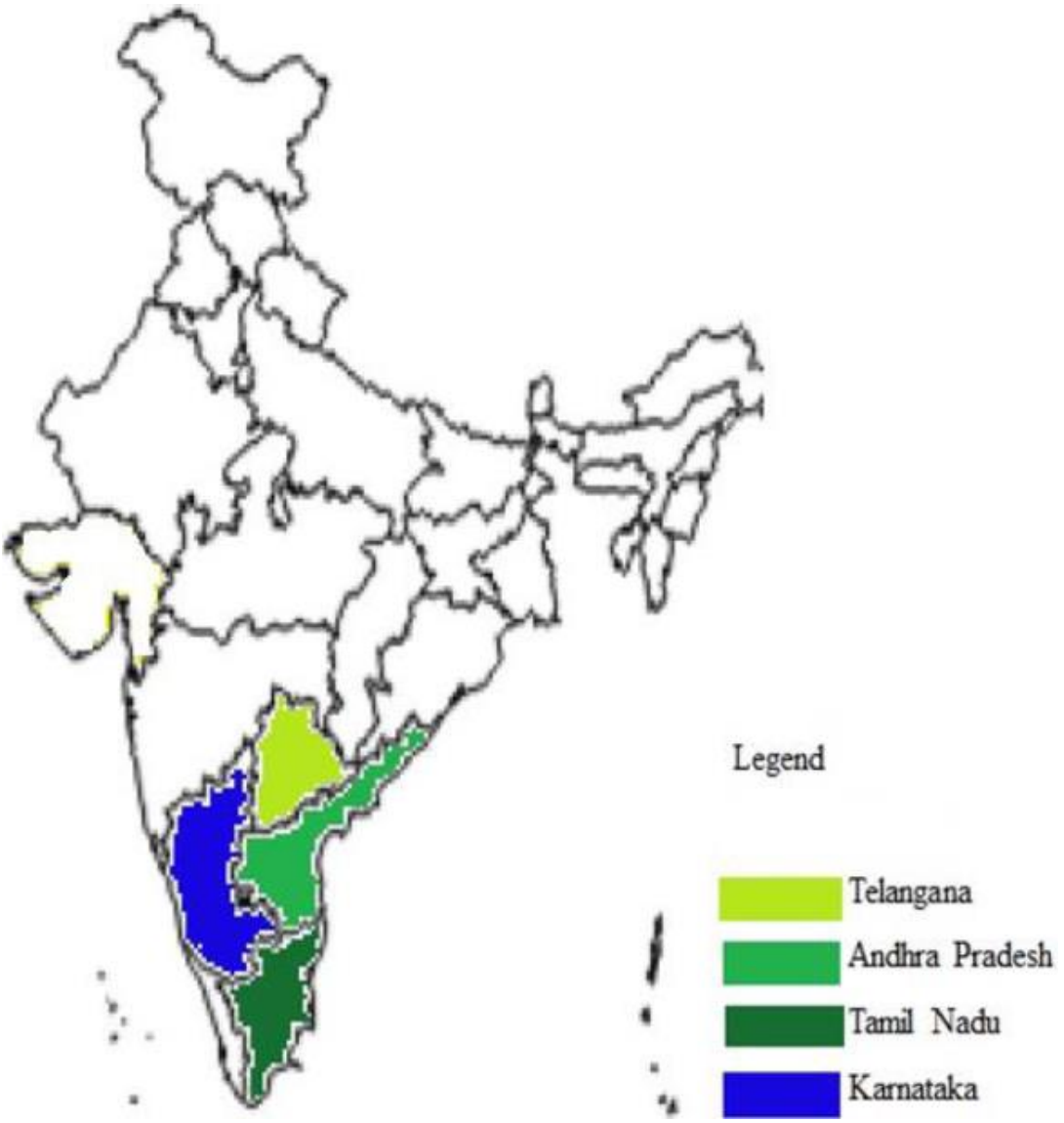


Figure 3.9 Map of India showing the geographical location of the study, 2014-15.

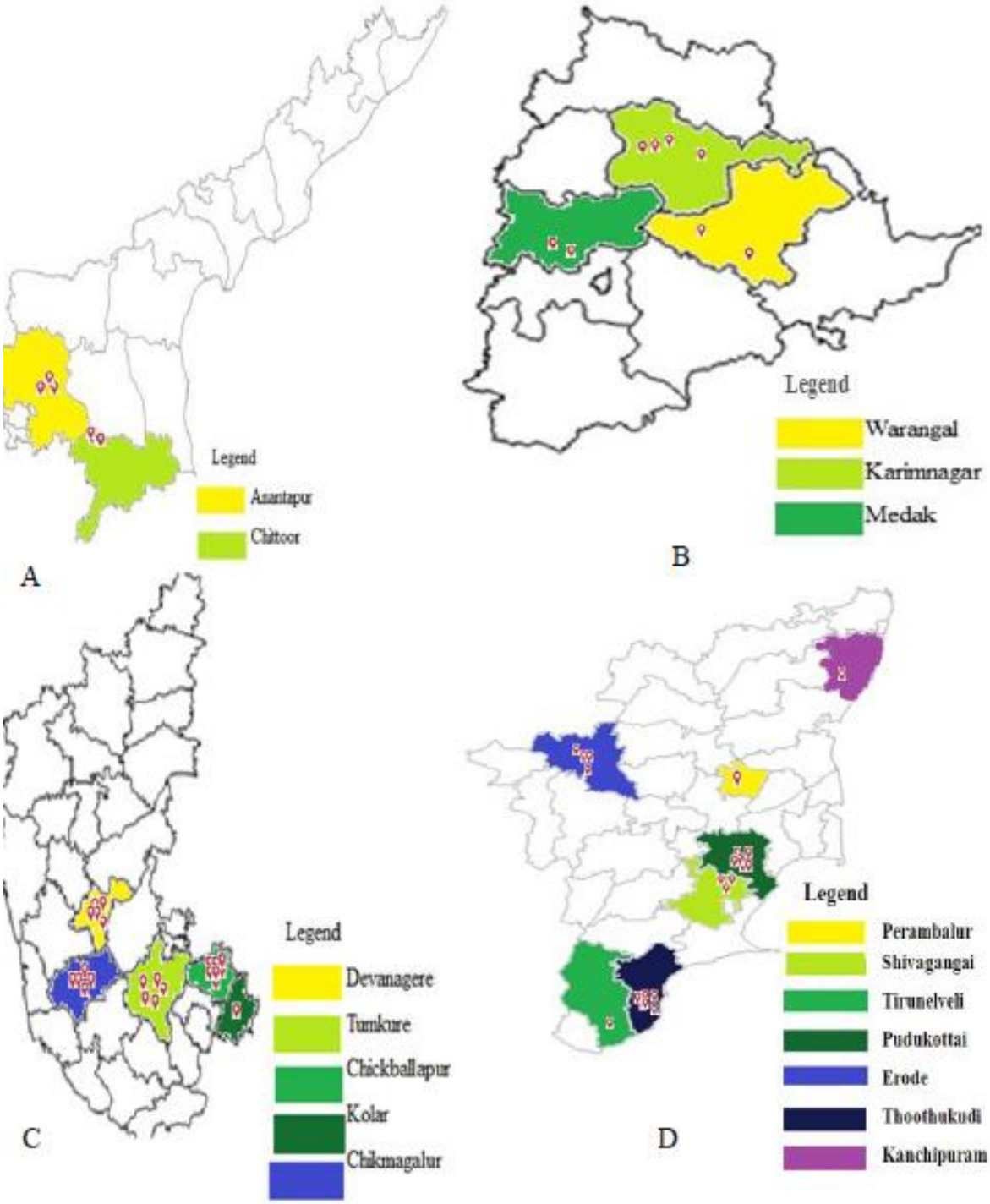


Figure 3.10 Locations of bluetongue suspected outbreaks in this study from Southern India, 2015

A) Andhra Pradesh; B) Telangana; C) Karnataka; D) Tamil Nadu. Locations of outbreaks are depicted in dots.

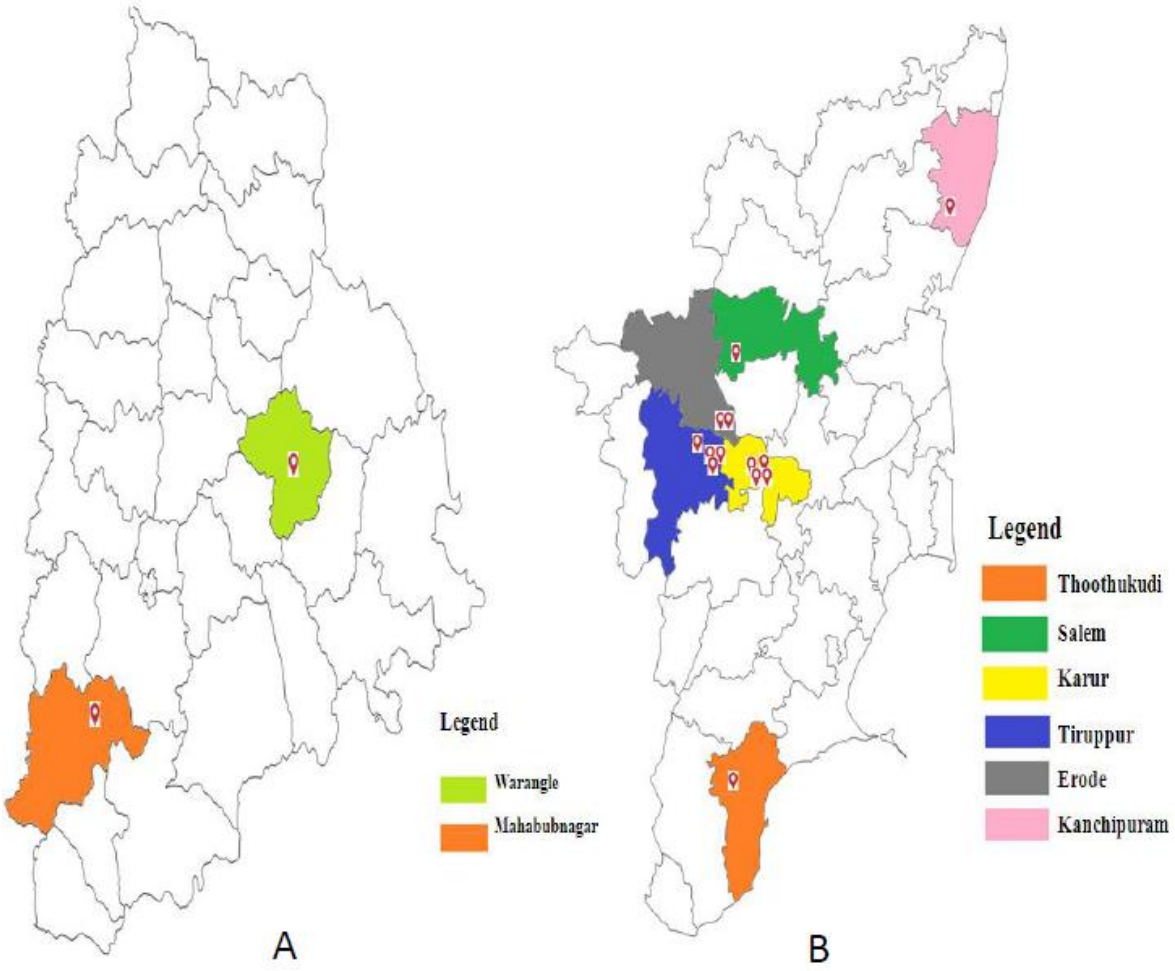


Figure 3.11 Locations of bluetongue suspected outbreaks in this study from Southern India, 2014

A) Telangana; B) Tamil Nadu. Locations of outbreaks are depicted in dots.

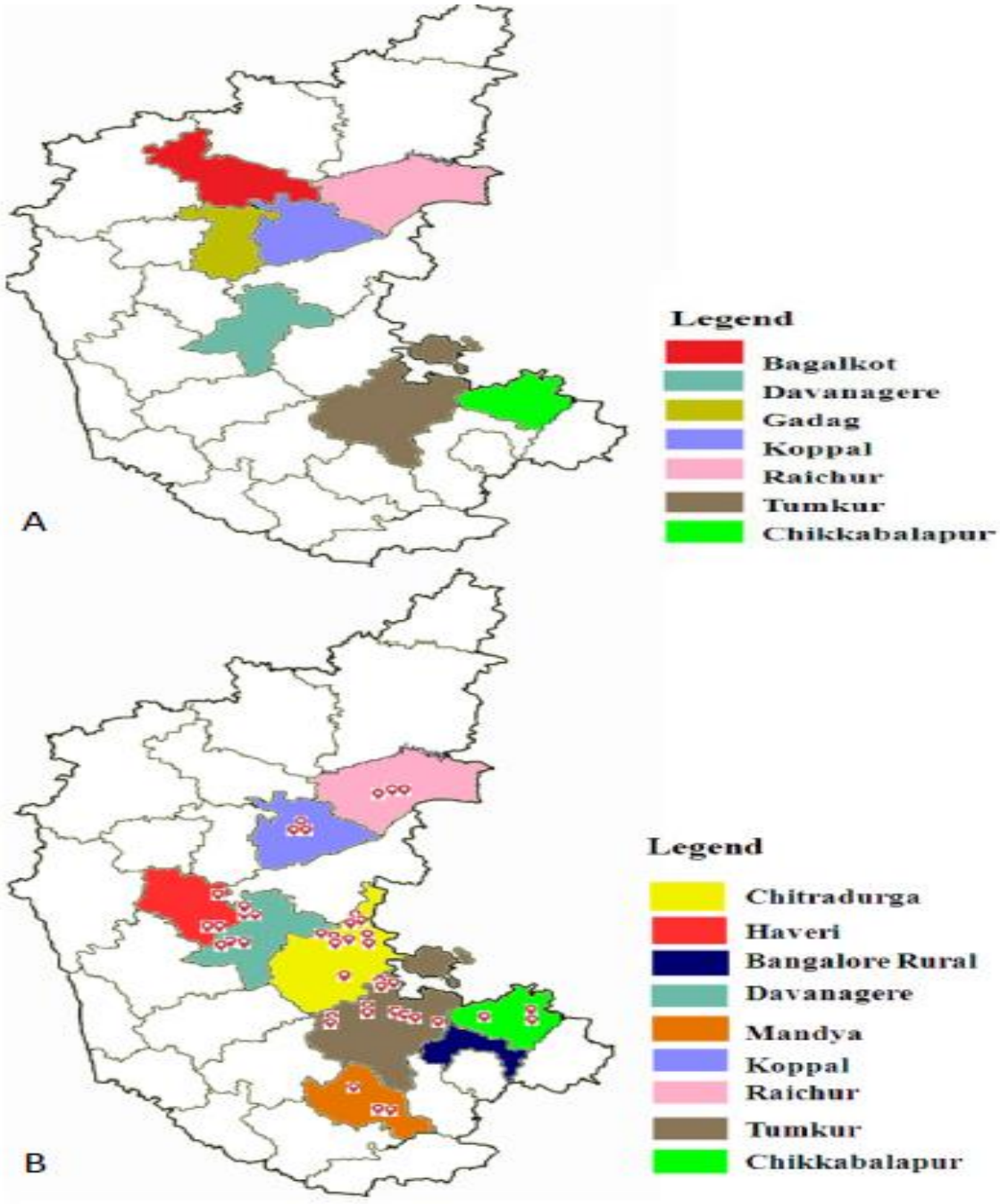


Figure 3.12 Locations of bluetongue suspected outbreaks in Karnataka recorded by ICAR-NIVEDI, 2014-15.

A) Karnataka, 2014; B) Karnataka, 2015BT outbreaks officially recorded by Indian Council of Agriculture Research-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI, 2015, ICAR-NIVEDI, 2016). Only the districts affected by BT outbreaks, 2014 were available in NIVEDI report 2014, not the exact location of outbreaks.

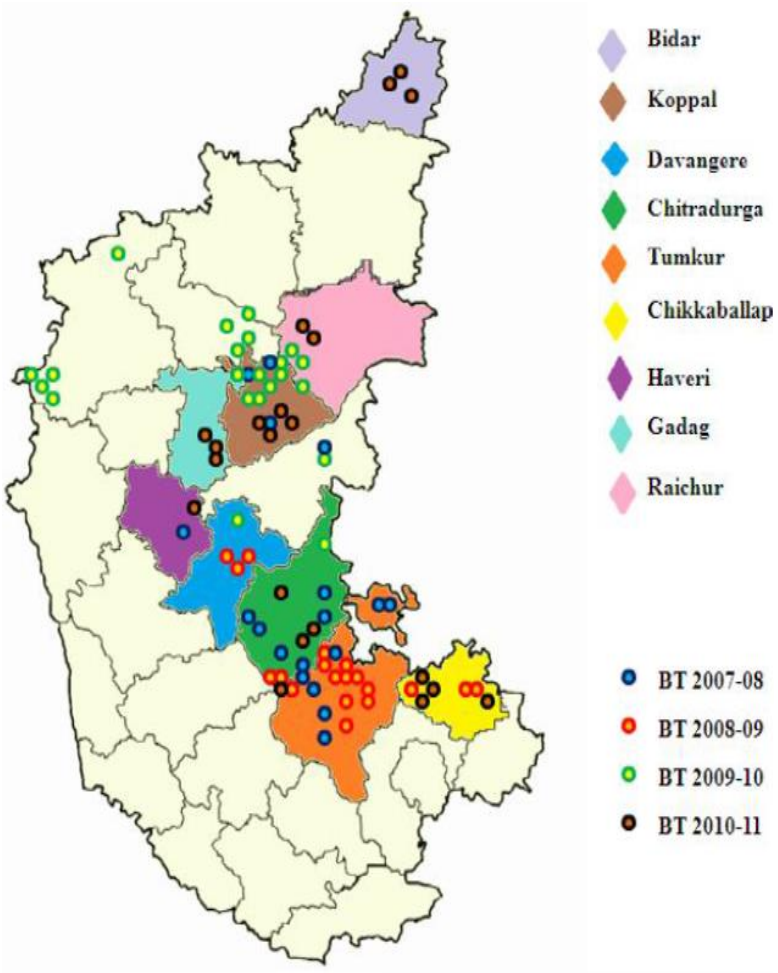


Figure 3.13 Locations of bluetongue suspected outbreaks in Karnataka recorded by ICAR-NIVEDI, 2007-11

BT outbreaks officially recorded by Indian Council of Agriculture Research-National Institute of Veterinary Epidemiology and Disease Informatics from 2007-2011 (ICAR-NIVEDI, 2013)

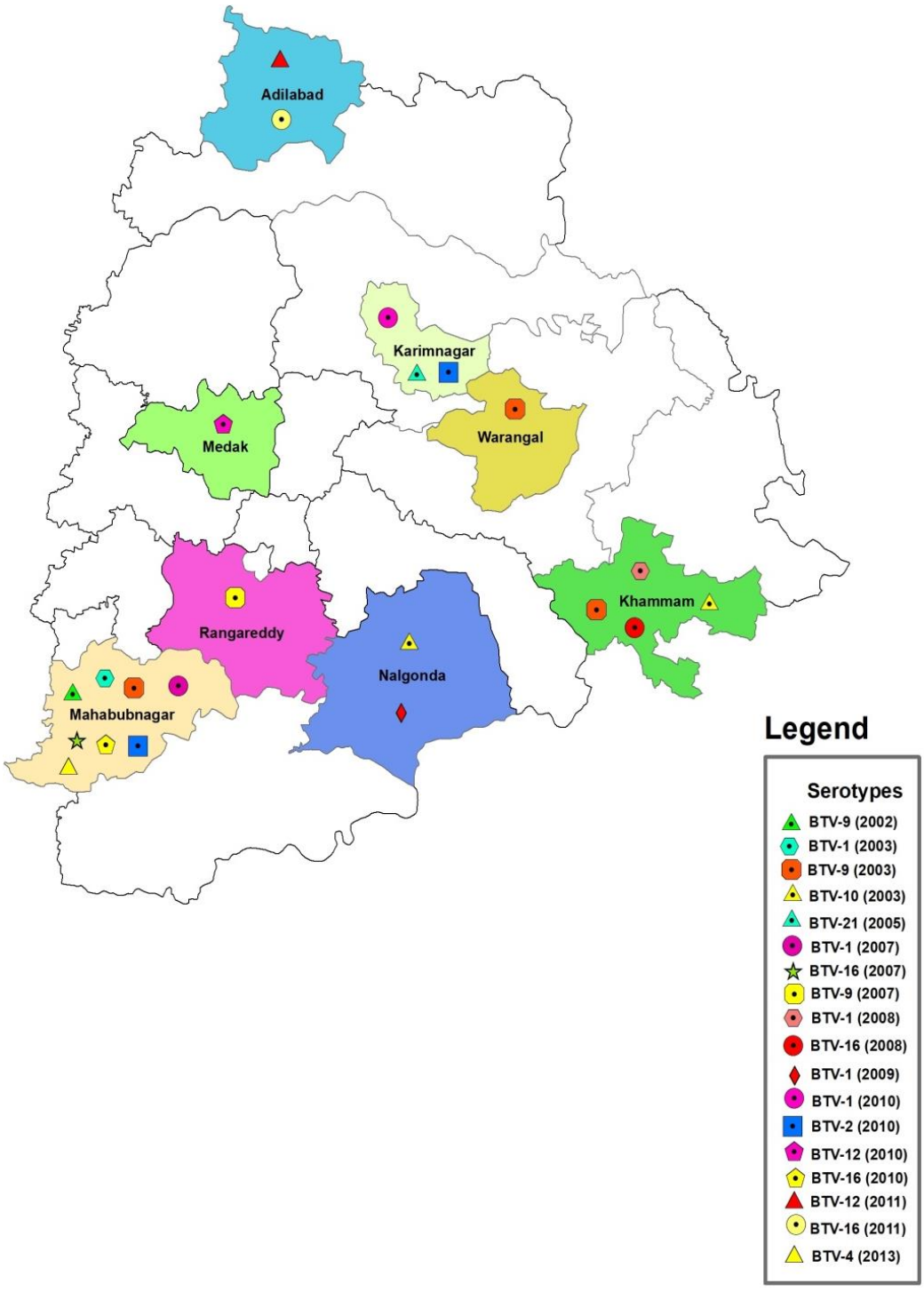


Figure 3.14 Different serotypes isolated from Telangana, 2002-2013.

The information was taken from (Reddy et al., 2016b). The serotypes BTV-1. BTV-2, BTV-4, BTV-9, BTV-10, BTV-12, BTV-16 and BTV-21 were isolated in different BT outbreaks from Telangana, 2002-2013. Recently, BTV-24 has been isolated from Medak, Telangana in 2010 (Krishnajyothi et al., 2016).

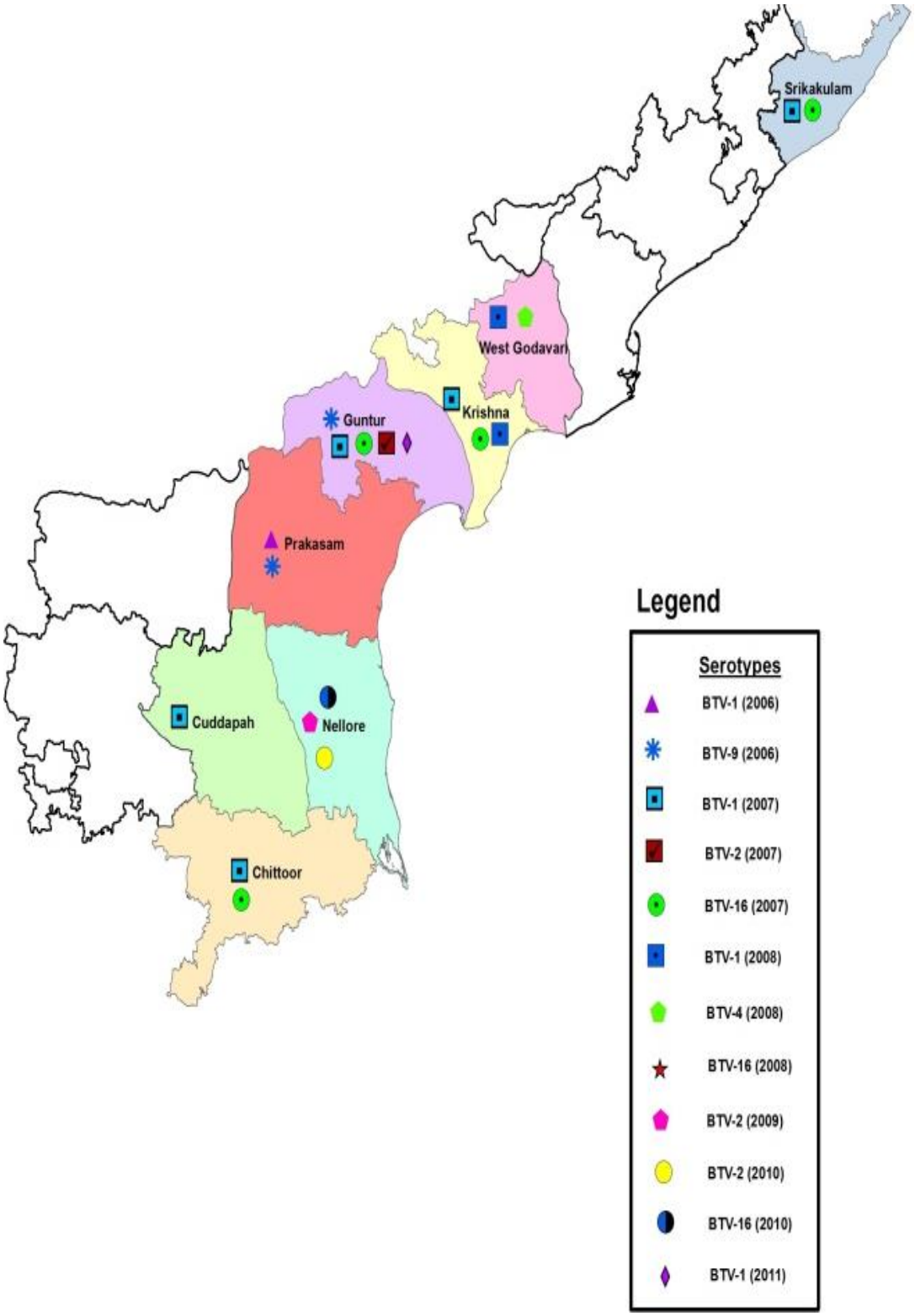


Figure 3.15 Different serotypes isolated from Andhra Pradesh, 2006-2011.

The information was taken from (Reddy et al., 2016b). The serotypes BTV-1. BTV-2, BTV-4, BTV-9, BTV-16 were isolated in different BT outbreaks from Andhra Pradesh, 2002-2013.

Table 3.8 Detail of clinical samples positive for BTV and used for virus isolations.

S.No.	original sample collection number	*Ct value	Vaccination status	Symptoms	Place of sampling	Collection date	Host spp	Sample
1.	[IND2015/01]2	24	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	18/12/2015	S	B
2.	[IND2015/04]8	19	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	18/12/2015	S	B
3.	[IND2015/07]12	27	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	18/12/2015	S	B
4.	[IND2015/10]14	19	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	18/12/2015	S	B
5.	[IND2015/13]22	24	V	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	18/12/2015	S	B
6.	[IND2015/16]40	26	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	G	B
7.	[IND2015/19]36	24	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
8.	[IND2015/22]34	28	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
9.	[IND2015/24]42	28	V	Lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
10.	[IND2015/27]52	27	V	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
11.	[IND2015/30]54	25	V	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
12.	[IND2015/34]56	20	V	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
13.	[IND2015/37]62	33	V	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
14.	[IND2015/41]68	25	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
15.	[IND2015/45]70	22	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
16.	[IND2015/48]72	26	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
17.	[IND2015/51]76	23	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
18.	[IND2015/54]80	29	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
19.	[IND2015/57]98	28	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	S	B
20.	[IND2015/60]100	21	UV	Pyrexia, oral lesions, lameness	Kayathar,Thoothukudi, TN	22/12/2015	G	B
21.	[IND2015/62]530	32	UV	Salivation, limping	Ottadanpathi, Thoothukudi, TN	25/12/2015	C	B
22.	[IND2015/65]531	33	UV	Oral lesions, limping	Ottadanpathi, Thoothukudi, TN	25/12/2015	C	B
23.	[IND2015/68]532	34	UV	Excess salivation	Ottadanpathi, Thoothukudi, TN	25/12/2015	C	B
24.	[IND2015/71]533	34	UV	Limping , salivation	Ottadanpathi, Thoothukudi, TN	25/12/2015	C	B
25.	[IND2015/74]534	34	UV	Limping, mouth lesions	Ottadanpathi, Thoothukudi, TN	25/12/2015	G	B
26.	[IND2015/77]535	33	UV	Limping, mouth lesions	Ottadanpathi, Thoothukudi, TN	25/12/2015	G	B
27.	[IND2015/80]536	24	UV	Limping, fever	Ottadanpathi, Thoothukudi, TN	25/12/2015	G	B
28.	[IND2015/84]541	26	UV	Aborted on 10/12/2015, limping	Ottadanpathi, Thoothukudi, TN	25/12/2015	G	B
29.	[IND2015/87]543	22	UV	Mouth lesions, blood speckled nasal discharge	Ottadanpathi, Thoothukudi, TN	25/12/2015	S	B

30.	[IND2015/90]545	27	UV	Mouth lesions,	Ottadanpathi, Thoothukudi, TN	25/12/2015	S	B
31.	[IND2015/93]548	27	UV	Mouth lesions, limping	Ottadanpathi, Thoothukudi, TN	25/12/2015	C	B
32.	[IND2015/96]549	27	UV	Mouth lesions, limping	Ottadanpathi, Thoothukudi, TN	25/12/2015	C	B
33.	[IND2015/99]550	27	UV	Excess salivation	Ottadanpathi, Thoothukudi, TN	25/12/2015	C	B
34.	[IND2015/102]551	28	UV	Excess salivation	Ottadanpathi, Thoothukudi, TN	25/12/2015	C	B
35.	[IND2015/105]552	33	UV	Limping	Ottadanpathi, Thoothukudi, TN	25/12/2015	G	B
36.	[IND2015/108]479	29	V	Pyrexia, limping	Tirunelveli, TN	22/12/2015	G	B
37.	[IND2015/111]482	33	V	Blood stained nasal discharge	Tirunelveli, TN	22/12/2015	S	B
38.	[IND2015/114]481	28	V	Oral lesions, limping	Tirunelveli, TN	22/12/2015	S	B
39.	[IND2015/116]V1	30	V	Erosion and crusting of muzzle, hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break	Vemgal,Sidlaghattataluk, Chikballapur,K	26/11/2015	S	B
40.	[IND2015/119]V2	21	V	Erosion and crusting of muzzle,hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break	Vemgal, Sidlaghattataluk, Chikballapur,K	26/11/2015	S	B
41.	[IND2015/122]V13	30	V	Erosion and crusting of muzzle,hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break	Vemgal, Sidlaghattataluk, Chikballapur,K	26/11/2015	S	B
42.	[IND2015/125]V43	19	V	Erosion and crusting of muzzle,hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break	Genjigunte Sidlaghattataluk, Chikballapur, K	26/11/2015	S	B
43.	[IND2015/128]V40	22	V	Erosion and crusting of muzzle,hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break	Genjigunte, Sidlaghattataluk, Chikballapur K	26/11/2015	S	B
44.	[IND2015/131]V	18	V	Erosion and crusting of muzzle,hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break	Lakkenahalli, Sidlaghattataluk,Chikballapur,K	26/11/2015	S	B
45.	[IND2015/134]V41	18	V	Erosion and crusting of muzzle,hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break	Genjigunte, Sidlaghattataluk, Chikballapur,K	26/11/2015	S	B
46.	[IND2015/137]V42	20	V	Erosion and crusting of muzzle,hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break	Genjigunte, Sidlaghattataluk, Chikballapur,K	26/11/2015	S	B

47.	[IND2015/140]V44	25	V	Erosion and crusting of muzzle,hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break	Lakkenahalli, Sidlaghattataluk, Chikballapur,K	26/11/2015	S	B
48.	[IND2015/143]V44	19	V	Erosion and crusting of muzzle,hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break	Lakkenahalli, Sidlaghattataluk, Chikballapur,K	26/11/2015	S	B
49.	[IND2015/146]491	34	UV	Rhinitis, lameness, footrot, high mortality group	Kanchipuram, TN	25/12/2015	S	B
50.	[IND2015/149]492	34	UV	Rhinitis, lameness, footrot, high mortality group	Kanchipuram, TN	25/12/2015	S	B
51.	[IND2015/152]493	32	UV	Rhinitis, lameness, footrot, high mortality group	Kanchipuram, TN	25/12/2015	S	B
52.	[IND2015/155]494	34	UV	Rhinitis, lameness, footrot, high mortality group	Kanchipuram, TN	25/12/2015	S	B
53.	[IND2015/158]CS4	25	V	Healthy BTV vaccinated sheep	Tirunehveli, TN	25/12/2015	S	B
54.	[IND2015/161]CS6	26	V	Healthy BTV vaccinated sheep	Tirunehveli, TN	25/12/2015	S	B
55.	[IND2015/164]CS9	32	V	Healthy BTV vaccinated sheep	Tirunehveli, TN	25/12/2015	S	B
56.	[IND2015/167]470	28	V	Slight oral lesions and limping	Tirunehveli, TN	25/12/2015	S	B
57.	[IND2015/170]471	25	UV	Slight oral lesions and limping	Tirunehveli, TN	25/12/2015	C	B
58.	[IND2015/173]472	28	UV	Slight oral lesions and limping	Tirunehveli, TN	25/12/2015	S	B
59.	[IND2015/176]473	29	V	Slight oral lesions and limping	Tirunehveli, TN	25/12/2015	S	B
60.	[IND2015/179]474	28	UV	Slight oral lesions and limping	Tirunehveli, TN	25/12/2015	C	B
61.	[IND2015/182]475	32	V	Slight oral lesions and limping	Tirunehveli, TN	25/12/2015	S	B
62.	[IND2015/185]476	28	V	Slight oral lesions, abortion and limping	Tirunehveli, TN	25/12/2015	S	B
63.	[IND2015/188]477	30	V	Slight oral lesions and limping	Tirunehveli, TN	25/12/2015	S	B
64.	[IND2015/191]478	29	V	Slight oral lesions and limping	Tirunehveli, TN	25/12/2015	G	B
65.	[IND2015/194]480	25	V	Oral lesions	Tirunehveli, TN	25/12/2015	S	B
66.	[IND2015/197]483	32	V	Blood speckled nasal discharge	Tirunehveli, TN	25/12/2015	S	B
67.	[IND2015/200]484	33	V	limping	Tirunehveli, TN	25/12/2015	G	B
68.	[IND2015/203]486	34	V	Healthy BTV vaccinated sheep	Tirunehveli, TN	25/12/2015	S	B
69.	[IND2015/206]404	30	V	Oral lesions were there few days back but now recovered.	Erode, TN	23/12/2015	S	B
70.	[IND2015/209]406	24	UV	Oral lesions were there few days back but now recovered.	Erode, TN	23/12/2015	S	B
71.	[IND2015/212]408	26	UV	Oral lesions , head oedema and limping	Erode, TN	23/12/2015	S	B
72.	[IND2015/2015]547	30	UV	Mortality	Thoothukudi, TN	25/12/2015	S	Sp
73.	[IND2015/218]228	32	UV	Mortality	Tirunelveli, TN	25/12/2015	S	Sp
74.	[IND2015/221]34	23	UV	Mortality	Thoothukudi,TN	25/12/2015	S	Sp
75.	[IND2015/224]PPR S	30	UV	Mortality	Medak, T	14/09/2015	S	Sp

76.	[IND2015/227]555	32	UV	Excessive salivation, blood speckled nasal discharge	Thoothukudi, TN	25/12/2015	C	Sa
77.	[IND2015/230]556	33	UV	Excessive salivation, blood specked nasal discharge	Thoothukudi, TN	25/12/2015	C	Sa
78.	[IND2015/233]BTV14	30	UV	Mortality	Thoothukudi, TN	26/11/2015	S	LN
79.	[IND2015/236]6PK	24	UV	Temperature 104 ^{0}F, nasal discharge, oral lesions	Jukal, Parkal, Warangal,T	07/09/2015	S	B
80.	[IND2015/239]K3	22	UV	Temperature 102.1 ^{0}F,oral lesions	Medaram, Dharmaram, Karimnagar,T	10/09/2015	S	B
81.	[IND2015/242]K4	27	UV	Temperature 102 ^{0}F,oral lesions	Mallesham, Khilavanaparthi , Karimnagar,T	10/09/2015	S	B
82.	[IND2015/245]K8	28	UV	Temperature 103.1 ^{0}F,oral lesions	Oongathurthi, Karimnagar,T	10/09/2015	S	B
83.	[IND2015/248]K9	21	UV	Temperature 101 ^{0}F	Oongathurthi, Karimnagar, T	10/09/2015	S	B
84.	[IND2015/251]K10	30	UV	Temperature 103^{0}F	Madhurampally,Pegadapally, Karimnagar, T	12/09/2015	S	B
85.	[IND2015/254]K14	20	UV	Temperature 105 ^{0}F,nasal discharge, oral lesions	Madhurampally,Pegadapally Karimnagar, T	12/09/2015	S	B
86.	[IND2015/257]K15	25	UV	Temperature 104 ^{0}F,nasal discharge, oral lesions	Madhurampally,Pegadapally, Karimnagar, T	12/09/2015	S	B
87.	[IND2015/260]PPR2	30	UV	Temperature 103 ^{0}F, nasal discharge	Nalthur , Medak, T	14/09/2015	G	B
88.	[IND2015/263]PPR1	30	UV	Temperature 103 ^{0}F,nasal discharge,ocular discharge	Rauiapalli, Medak,T	14/09/2015	G	B
89.	[IND2015/266] 6A	22	UV	Nasal discharge, oral lesions, temperature 102 ^{0}F, swollen head, salivation	Open air jail,Bukkarayasamundram,Anantpur, AP	08/11/2015	S	B
90.	[IND2015/269] 7A	18	UV	Temperature 103 ^{0}F,mucocutaneous lesions near lips	Open air jail, Bukkarayasamundram,Anantpur, AP	08/11/2015	S	B
91.	[IND2015/272] 9A	26	UV	Temperature 102 ^{0}F,nasal discharge	Ammavarapalli,Bukkarayasamundram,Anantpur,AP	08/11/2015	S	B
92.	[IND2015/275]11A	18	UV	Temperature 103.2 ^{0}F,nasal discharge	Ammavarapalli,Bukkarayasamundram,Anantpur,AP	08/11/2015	S	B
93.	[IND2015/278]12A	27	UV	Temperature 104.2 ^{0}F,oral lesions, blood tinged nasal discharge	Ammavarapalli,Bukkarayasamundram,Anantpur,AP	08/11/2015	S	B
94.	[IND2015/281]25A	21	UV	Temperature 103.2 ^{0}F	Ammavarapalli,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
95.	[IND2015/284]27A	20	UV	Temperature 102 ^{0}F nasal discharge, salivation	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
96.	[IND2015/287]28A	22	UV	Temperature 104 ^{0}F, nasal discharge	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
97.	[IND2015/290]29A	20	UV	Temperature 103 ^{0}F	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
98.	[IND2015/293]30A	21	UV	Temperature 104 ^{0}F, salivation	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
99.	IND2015/296 40A	31	UV	Temperature 103 ^{0}F	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
100.	[IND2015/299]12Karn	27	UV	Erosion and crusting of muzzle, hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break.	Gonimoradahalli,Sidlaghattataluk,Chikballapur,K	25/11/2015	S	B
101.	[IND2015/302]18Karn	19	UV	Erosion and crusting of muzzle, hard palate ulcers. The coronary bands of the feet exhibit hyperemia, painful, wool break.	G.Kuruboahalli,Sidlaghattataluk,Chikballapur,K	25/11/2015	S	B
102.	[IND2015/305]31Karn	25.8	UV	Erosion and crusting of muzzle, hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break.	Vemgal,Sidlaghattataluk,Chikballapur,K	26/11/2015	S	B

103.	[IND2015/308]35Karn	27.28	UV	Erosion and crusting of muzzle, hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break.	Genjigunte, Sidlaghattataluk, Chikballapur, K	26/11/2015	S	B
104.	[IND2015/311]40Karn	26	UV	Erosion and crusting of muzzle, hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break.	Lakkenahalli,Sidlaghattataluk, Chikballapur, K	26/11/2015	S	B
105.	[IND2015/314]K1-K	29	UV	Erosion and crusting of muzzle, hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break.	Vemgal,Sidlaghattataluk, Chikballapu, K	26/11/2015	S	B
106.	[IND2015/317]K2-K	26	UV	Erosion and crusting of muzzle, hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break.	Vemgal Sidlaghattataluk, Chikballapur, K	26/11/2015	S	B
107.	[IND2015/320]K3-K	26	UV	Erosion and crusting of muzzle, hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break.	Vemgal,Sidlaghattataluk, Chikballapur, K	26/11/2015	S	B
108.	[IND2015/323]K16-K	19	UV	Erosion and crusting of muzzle, hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break.	Burudugun, chintamani, Chikballapur, K	26/11/2015	S	B
109.	[IND2015/326]K20-K	26	UV	Erosion and crusting of muzzle, hard palate ulcers. The coronary bands of the feet exhibit hyperaemia (Coronitis) and painful, wool break.	Gangireddypalya , chintamani, Chikballapur, K	26/11/2015	S	B
110.	[IND2015/]A14	28	UV	Temperature 103.8^{0}F,blood tinged nasal discharge , eye discharge, excess salivation	Madhurampally,Pegadapally,Karimnagar,T	12/11/2015	S	B
111.	[IND2015/]26A	21	UV	Temperature 102 ^{0}F	Ammavarapalli,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
112.	IND2015/296]31A	26	UV	Temperature 102.3 ^{0}F, salivation	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
113.	[IND2015/]32A	24	UV	Temperature 103.2 ^{0}F	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
114.	[IND2015/]33A	19	UV	Temperature 104 ^{0}F	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
115.	[IND2015/]34A	19	UV	Temperature 103 ^{0}F	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
116.	[IND2015/]35A	28	UV	Temperature 102.5 ^{0}F	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
117.	[IND2015/]36A	21	UV	Temperature 103 ^{0}F,Frothy salivation	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
118.	[IND2015/]37A	21	UV	Temperature 102 ^{0}F	Bomnalatapaly,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
119.	[IND2015/]38A	30	UV	Temperature 103 ^{0}F	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B
120.	[IND2015/]39A	30	UV	Temperature 103 ^{0}F,Head swelling	Bomnalatapally,Bukkarayasamundram,Anantpur,AP	12/11/2015	S	B

121.	[IND2015/]07Karn	21	UV	Erosion and crusting of muzzle,hard palate ulcers.The coronary bands of the feet exhibit hyperaemia Coronitis) and painful, wool break.	Gowdanahalli, Chikballapur, K	25/11/2015	S	B
122.	[IND2015/]08Karn	20	UV	Hyperemic oral mucosa .swollen lips . BT disease course just started. Very high temp 105^0F	Gowdanahalli , Chikballapur, K	25/11/2015	S	B
123.	[IND2015]32Karn	24	UV	Erosion and crusting of muzzle,hard palate ulcers.The coronary bands of the feet exhibit hyperaemia Coronitis) and painful, wool break	Vemgal,Sidlaghattataluk,Chikballapur,K	26/11/2015	S	B
124.	[IND2015/]33Karn	25	UV	Erosion and crusting of muzzle,hard palate ulcers.The coronary bands of the feet exhibit hyperaemia Coronitis) and painful, wool break	Vemga,Sidlaghattataluk,Chikballapur,K	26/11/2015	S	B
125.	[IND2015/]K1	30	UV	Temperature 103.7 ^{0}FOral lesions	Medaram,Dharmaram,Karimnagar,T	10/09/2015	S	B
126.	[IND2015/]K11	30	UV	Temperature 103.2^{0}F	Madhurampally,Pegadapally,Karimnagar, T	12/09/2015	S	B
127.	[IND2015/]K12	30	UV	Temperature 104 ^{0}FNasal discharge, Oral lesions	Madhurampally,Pegadapally, Karimnagar, T	12/09/2015	S	B
128.	[IND2015/]K13	30	UV	Temperature 103.8 ^{0}FNasal discharge, Oral lesions	Madhurampally,Pegadapally, Karimnagar ,T	12/09/2015	S	B
129.	[IND2015/]PPR1	30	UV	Temperature 102 ^{0}F, Nasal discharge	Nalthur, Medak, T	14/09/2015	G	B
130.	[IND2015/] 4A	26	UV	Temperature 102.8^{0}FSwollen head, salivation, erosions on upper palate	Open air jail, Bukkarayasamundram, Anantpur, AP	08/11/2015	S	B
131.	[IND2015/]spleen K	30	UV	Mortality	Gowdanahalli, MandikalMandal, Chikballapur, K	26/11/2015	S	Sp
132.	[IND2015/]3 organ	30	UV	Mortality	Thoothukudi,TN	16/12/2015	S	Sp
133.	[IND2015/]19Karn	24	UV	Erosion and crusting of muzzle,hard palate ulcers.The coronary bands of the feet exhibit hyperaemia Coronitis) and painful, wool break.	G.Kuruboahalli,Sidlaghattataluk,Chikballapur,K	25/11/2015	S	B
134.	[IND2015/]20Karn	26	UV	Erosion and crusting of muzzle,hard palate ulcers.The coronary bands of the feet exhibit hyperaemia Coronitis) and painful, wool break.	Chokkanahalli,Sidlaghatta,Chikballapur,K	25/11/2015	S	B
135.	[IND2015/]21Karn	28	UV	Erosion and crusting of muzzle,hard palate ulcers.The coronary bands of the feet exhibit hyperaemia Coronitis) and painful, wool break.	Chokkanahalli,Sidlaghatta,Chikballapur,K	25/11/2015	S	B
136.	[IND2014/]CHE2	28	UV	Pyrexia, oral lesions	Erode,TN	22/11/2014	S	B
137.	[IND2014/]CHE5	23	UV	Pyrexia, oral lesion	Tirupur,TN	22/11/2014	S	B
138.	[IND2014/]CHE12	30	UV	Oral lesions, coronitis	Karur,TN	22/11/2014	S	B
139.	[IND2014/]CHE14	29	UV	Oral lesion, coronotis	Karur, TN	22/11/2014	S	B
140.	[IND2014/]CHE3	28	UV	Pyrexia, oral lesions	Karur, TN	22/11/2014	S	B
141.	[IND2014/]4WGL/14	28	UV	Nasal discharge	Warangal, TN	13/10/2014	S	B

*based on BTV specific qRT-PCR targeting BTV Seg- 10 (Hofmann et al; 2008) for 2015 outbreak and BTV specific qRT-PCR targeting BTV Seg-9 for 2014 outbreaks (Maan et al., 2015a)

Abbreviations: TN, Tamil Nadu; T, Telangana; K, Karnataka; AP, Andhra Pradesh; V, Vaccinated; UV, Unvaccinated; S, Sheep; G, Goat; C, Cattle; B, Blood; Sp, Spleen; LN, Lymph node; Sa, Saliva

3.3 Discussion

BTV is endemic in India, with regular hyper-endemic outbreaks occurring in the Southern states. To date it has been reported that bluetongue is positively associated with climatic factors in various parts of India, as well as the local size / density of the sheep population (Prasad et al., 2009, Rao et al., 2016b, Rao et al., 2016a, ICAR-NIVEDI, 2010). Rao and colleagues reported that in Southern India, there was a correlation between BT outbreaks and climatic conditions, with BT particularly following the pattern of monsoons(Rao et al., 2016a). Severe BT outbreaks were reported during 2005 in Southern India because of flooding and heavy south west monsoon as well as north east monsoon in India (Rao et al., 2016b, Reddy et al., 2016a, ICAR-NIVEDI, 2013). Johnson and colleagues noted more BT outbreaks when temperature, relative humidity, and wind speed were 26°C, 60-65% and 5 kmph, respectively (Johnson et al. 2006) because this climate is very congenial for *Culicoides* breeding and survival.

Climatic changes can affect the incidence, circulation and evolution of infectious diseases, especially arboviral diseases (Jimenez-Clavero, 2012). In Telangana, BT outbreaks occur mostly during the south west monsoons with highest occurrence during August and September. The south west monsoon as a whole was deficient in 2014 (Bhan et al., 2015) which led to few sporadic BT outbreaks in Telangana during 2014 (AICRP, 2014). It is considered likely that the deficit of monsoon conditions in Telangana generated an environment that was less-suitable for *Culicoides.*

In contrast, the north east monsoon is generally responsible for the rainfall in most of Tamil Nadu from October to December (Rao et al., 2016b) and hence, the normal north east monsoon contributed to BT outbreaks in Tamil Nadu during 2014. However, in 2015, a strong northeast monsoon, together with cyclonic storms, caused heavy rainfall in November and December and resulted floods in Tamil Nadu (Gaitonde and Gopichandran, 2016). This climatic and environmental change generated a climate that was suitable for the breeding of *Culicoides,* which also resulted in severe outbreaks of BT in Tamil Nadu compared to 2014.

Due to the heavy north east monsoon during 2015, there was also a higher incidence of BT outbreaks in the southern interior of Karnataka state. ICAR-National Institute of Veterinary

Epidemiology and Disease Informatics (NIVEDI) reported large number of BT outbreaks (n=56) in months of November and December, Karnataka, 2015(ICAR-NIVEDI, 2016, AICRP, 2016) as compared to 13 BT outbreaks reported in previous year (AICRP, 2014) (Figure 3.12, 3.13). Studies based on reliable and precise meteorological data will show a correlation between rainfall patterns and BT outbreaks, which will further help in the development of a predictive model for the disease, and in planning the strategies to forecast BT outbreaks well in advance.

A pentavalent inactivated BTV vaccine containing BTV-1, BTV-2, BTV-10, BTV-16 and BTV-23 is currently used in Southern India. Although BT is endemic in India, particularly in the Southern states, BT vaccination is not carried on a large scale. Furthermore, only sheep and goats are currently vaccinated while cattle are ignored in the vaccination campaigns. Cattle vaccination against BT should be given due importance, as cattle are usually considered to play a more important role in the transmission of BTV than sheep (Elbers et al., 2008a).

According to the 2012 Livestock Census, distribution of livestock between species in India was 37.28% cattle, 26.40% goats, 21.23% buffaloes, 12.71% sheep and 2.01% pigs and 0.37 % other species (camel, mithun ,yak, horse, ponies, mule and donkey). State-wise the percentage of the national cattle population was: 5.03% for Andhra Pradesh, 4.98% Karnataka and 4.62% Tamil Nadu. However, BT disease is frequently asymptomatic or inapparent in cattle and goats, with severe disease mostly restricted to sheep and some wildlife species (Verwoerd and Erasmus, 2004). Vaccination of cattle against BT is not therefore considered essential in these BT endemic areas. However, cattle may be preferentially bitten by adult *Culicoides* and can serve as a reservoir host for BTV (Brewer and MacLachlan, 1994). BT disease may be less severe in cattle but present as a prolonged subclinical infection and associated viraemia, with potential to infect *Culicoides*, causing transmission of the virus and disease (Alpar et al., 2009).

The clinical manifestation of BT in cattle includes a mild form of the disease showing viremia, anorexia, oral and nasal mucosae congestion, serous or mucopurulent nasal discharge, excessive frothy salivation, erosions, coronitis and decrease in milk production (Williamson et al., 2008). These clinical signs can be confused with FMD, which is also an endemic disease in India, highlighting importance of differential diagnosis of disease.

Thirteen suspected cases of BT were observed in cattle during the outbreaks in Tamil Nadu during 2015, showing symptoms of profuse salivation, lameness and nasal discharge. These cattle were being reared along with sheep and goat as a mixed population. This is the first time that samples have been taken simultaneously from suspected clinical cases of BT in cattle, sheep and goat in India. Mixed populations of these species in Thoothukudi and Tirunelveli districts, Tamil Nadu, were shown to be positive for BT infections by BTV serogroup-specific qRT-PCR targeting Seg-10, with Ct value ranging from 25 to 34. These samples were not tested for differential diagnosis of FMD or other multifactorial etiology because of the scarcity of the required logistics and resources for this project. Although, the sheep in these mixed populations showed severe clinical manifestations of BT disease, the only clinical signs in goats were lameness and pyrexia. Thirteen of the cattle in a mixed population that included sheep and goats in Tamil Nadu: ten cattle in Thoothukudi and three in Tirunelveli, Tamil Nadu, 2015 showed clinical signs of profuse salivation and lameness. Other cattle in these mixed populations showed no clinical signs of the disease. Generally, BT is asymptomatic in cattle, however clinical cases may be evident particularly when new serotypes affect naive population (Dedolli et al., 2017).

The detection of 12 BTV serotypes in Southern India since 2001 emphasizes the need for regular virological surveillance, to ensure a regular re-evaluation of the serotype and strain composition of the available vaccines. The well characterised and documented BTV strains may provide seed stock for relevant vaccine development. It is therefore important to collect samples from both unvaccinated and BT-vaccinated animals showing clinical signs of BT, so that the circulating field serotypes can be detected and identified. In this study, only three small flocks that had been vaccinated against BT in the Chikballapur district were available for observation and sampling during the 2015 outbreak in Karnataka state. One flock in village Vemgal, Sidlaghatta, Chikballapur district of Karnataka State had been vaccinated with a bivalent inactivated BT vaccine containing serotypes 1 and 23, while the other two flocks, one from village Lakkenahalli, Sidlaghatta and another from village Genjigunte, Sidlaghatta of Chikballapur district of Karnataka State, 2015 were vaccinated with the pentavalent inactivated BT vaccine, containing serotypes BTV-1, BTV-2, BTV-10, BTV-16 and BTV-23. These animals were vaccinated in the month of August with pentavalent BTV vaccine and booster dose of vaccine was given after 28 days of primary vaccination. The vaccinated and non-vaccinated sheep showed BT suspected

clinical signs of same severity and were diagnosed positive for BT by BTV serogroup-specific qRT-PCR targeting Seg-10. Similarly, six other flocks vaccinated with BT pentavalent inactivated vaccine were observed in Tirunelveli and one flock from Erode (Tamil Nadu) were observed, highlighting the fact that vaccination was not done on a massive scale.

As well as BT, India is also endemic for *Foot and Mouth Disease Virus* (FMDV), *Peste des Petits Ruminants Virus* (PPRV), *Sheeppox Virus* (SPPV), *Goatpox Virus* (GTPV) and *Orf Virus* (ORFV) with natural hosts that include small ruminants and cattle. The clinical signs of BT disease can be easily confused with these diseases and thus diagnosis requires differential diagnosis confirmation by laboratory testing. There are many reports of BT infection in small ruminants from India, but mixed infections or multifactorial aetiologies have not been frequently reported (Maan et al., 2017, Biswas et al., 2010) and few diagnostic facilities have been developed for differential diagnosis.

Historically, clinical diagnosis has been the standard method for the detection and monitoring of BT but this not only opens the possibility of false positives and false negatives, it also requires a well experienced veterinary service for the differential diagnoses of BT (Bexiga et al., 2007). Sub-clinical BTV infections, particularly in cattle and goats are likely to go unnoticed or unrecognized, particularly in situations where outbreaks are infrequent or previously unknown. Consequently, rapid, efficient and reliable laboratory diagnostic tests are important especially during an epidemic in order to confirm a clinical suspicion of BT and provide veterinary health authorities with adequate time to develop and deploy appropriate control measures.

The most frequent clinical signs observed in this study were oral lesions, congested mucosa, coronitis and pyrexia, followed by nasal discharge and salivation. The severity and range of clinical signs of BTV infection are influenced by the age, breed, species, immune status of the host, as well as environmental stress and the pathogenicity of the BTV strain involved (Mertens et al., 2009). It is therefore unsurprising that the clinical manifestation of the disease observed in this study varied from severe and acute to subclinical forms.

In the present study, the prevalence of BTV in one hundred seventy nine (n=179) field samples, tested by Seg-10 based qRT-PCR was 44.7% (80/179) from Karnataka BT outbreak, 2015 whereas it was 50% (75/150) in one hundred and fifty (n=150) samples tested from Tamil Nadu, 2015 BT outbreaks. The low prevalence of BTV in these samples may be due to involvement of multifactorial etiology and thus highlighting the importance of differential diagnosis of BT from other endemic diseases prevalent in the region. Moreover, some of the published BTV serogroup-specific, qRT-PCR diagnostic assays cannot detect all BTV strains belonging to different BTV serotypes or topotypes, with the exception of the Seg-10 based assay (Hofmann et al., 2008a) which appears to detect most BTV strains (Hoffmann et al., 2009). However as this assay is also RNA sequence dependent, it is still possible that some other variant strains may still go undetected.

In this study, the BTV RNA detection efficiencies and mean Ct values generated were not directly compared for Indian field samples using the Seg-9 based and Seg-10 based group-specific qRT-PCR assays. However in this study, the Seg-10 based assay was clearly more effective than the Seg-9 based assay, for the detection of BTV RNA.

It is possible that different BTV topotypes have sequence differences in the target footprints for either primers or probes in the Seg-9 assay, significantly decreasing its relative efficiency for detection of Indian field strains. Full length sequence data for the genome segments of the BTV isolates generated in this study, will be added to databases to support the development, evaluation of alternative primers and probes required for BTV serogroup specific and serotype specific RT-PCR assays to ensure their relevance for the typing and detection of Indian BTV field strains.

Poor infrastructure for veterinary clinics highlights a gap in diagnostic provision, which results in unreported and undiagnosed clinical cases in the field. The serotypes involved in BT outbreaks in India may change every year. A robust and timely virological surveillance is needed for effective control of BT, so that circulating serotypes can be identified and incorporated in vaccine formulations in a timely manner. An initial step in this process is to attend BT field outbreaks from Southern India and to collect well documented samples for virus isolations which will subsequently help us understand the epidemiology of the disease in the endemic regions and in the country as a whole.

This study has involved the collection of well documented diagnostic samples, with records of collection date, geographical location, coordinates, and vaccination status of host species. These well documented samples will be used to make virus isolation, for typing and subsequent sequence analysis.

The recent trends of BT outbreaks in India emphasize the importance of an organized mass vaccination campaign to break the epidemiological cycle of BT in the endemic areas. Veterinary hospitals, polyclinics and Veterinary dispensaries, mostly have an inadequate infrastructure, equipment, and technically qualified and/or experienced manpower to support important health issues for the world's largest national livestock population. There is therefore a requirement to extend veterinary hospital facilities at the field level for prompt diagnosis, surveillance, monitoring and treatment of animal diseases in India. There is also a need to improve and extend mobile veterinary services to provide 'doorstep' veterinary support.

3.4 Summary

This study shows that:

- There is a correlation between BT outbreaks and climatic conditions, particularly in Southern India with BT following the pattern of the monsoons, especially in Southern India.
- Although, BT is endemic particularly in Southern India, BT vaccination is not done on a large scale, with only sheep and goats currently being vaccinated, Cattle are ignored in vaccination campaign.
- Thirteen suspected cases of BT disease were observed in cattle showing symptoms of profuse salivation, lameness and nasal discharge during outbreaks in Tamil Nadu during 2015. These cattle which were positive for BT by BTV serogroup-specific qRT-PCR targeting Seg-10 with Ct value ranging from 25 to 34, were being reared along with sheep and goats as mixed populations.
- Vaccinated sheep were diagnosed positive for BT by BTV serogroup-specific qRT-PCR targeting Seg-10 and showed clinical signs of suspected BT with the same severity as unvaccinated sheep. These observations indicate that the

vaccination campaign did not prevent BTV infection or the disease in individual animals.

- The most frequent clinical signs in this study were oral lesions (61%), congested mucosa (52%), coronitis (46%) and pyrexia (41%) followed by nasal discharge (17%) and salivation (13%). The clinical manifestations of the disease observed during this study varied from acute to subclinical forms.
- Prevalence of BTV in four hundred and forty seven (447) samples collected from four Southern states during 2014-2015 outbreaks was 53% (447/236). The negative samples might be from the animals that may not actually had BTV infection, in spite of presenting with BT- like symptoms. This highlights the importance of differential diagnosis of BT from other endemic diseases prevalent in the region to address the involvement of multifactorial aetiology in field cases. Moreover, it is still possible that some of the variant BTV strains may go undetected by Seg-10 based assay (Hofmann et al., 2008a).
- Of the 447 field samples, 236 were positive for BTV RNA. Total 141 BTV virus isolations were made from positive samples and deposited with virus collection number in Veterinary Biotechnology lab, LUVAS, Haryana under the Indo-UK project titled “Development of diagnostic systems, reference collections and molecular epidemiology studies for important arboviral pathogens of livestock in India".
- Sixty three BTV isolates were serotyped by qRT-PCR assay based on Seg-2 (Maan et al., 2016) reported in later chapters of this thesis. Seg-2 sequencing of 46 BTV isolates by NGS was done at Centre for Virus Research (MRC-CVR), University of Glasgow reported in chapter 5 and the full genome sequencing and analyses is still ongoing in Glasgow.
- This current study was undertaken to collect the well documented field samples including sample collection date, geographical location of sample collection, geographical coordinates, host species affected, vaccination status against BT and symptoms of disease.
- All India Network Project on Bluetongue, supported by the Indian Council of Agricultural Research (ICAR-AINPB) at eleven centres in various states of India is

functioning on BT in India. This is first time that large number of samples (n=447) were collected simultaneously from all four hyperendemic Southern states for BT during 2014-15, 141 BTV isolations were attempted from these clinical samples and 63 BTV isolates were typed and finally 46 were sequenced. Till date, only 46 well documented whole genome sequences of Indian BTV isolates were available in Genbank.

Chapter 4: Isolation and Molecular typing of BTV from Southern India in 2014-2015.

4.1 Introduction

Prior to 2000, BTV isolation and serotyping in India was mainly carried out at the World Reference Laboratories for Bluetongue, in the Onderstepoort Veterinary Institute, South Africa and at the Pirbright Institute, UK .(Prasad et al., 2009). Since 2001, the ICAR-All India Network Project on BT (ICAR-AINPBT) and individual research groups have been working on the isolation and typing of BTV (AINPBT, 2013). Although BT is endemic and a notifiable disease in India, the majority of the state diagnostic laboratories are not well-equipped to diagnose and type BTV (Rao et al., 2016b).

Virus isolation represents a gold standard for the identification of BT. This can be acheived by yolk sac (Mason et al., 1940) or intravenous inoculation (Goldsmit and Barzilai, 1985) of clinical samples into embryonated chicken eggs, followed by subpassaging in mammalian cells, usually BHK-21 or Vero cells. BTV can also be isolated by directly inoculating clinical samples into an insect cell line, such as *Culicoides sonorensis* cells (KC cells) (Wechsler and McHolland, 1988) or Aedes albopictus cells (C6-36), followed by adaptation to BHK-21 or Vero cells. The clinical samples suitable for BTV isolation include EDTA treated blood, semen or tissue samples including liver, spleen, brain, lymph nodes or mucosal epithelium (Mertens et al., 2009).

BTV serotype has been used as an important identifier of BTV strains that has traditionally been used to follow BT outbreaks. It is also a major factor in the selection of an appropriate vaccine strains to combat the disease. BTV serotype is determined by outer capsid proteins VP2 and VP5, particularly VP2, which primarily controls the specificity of interactions between VP2 and neutralizing antibodies generated during infection of the host (Mertens et al., 1989a). Traditionally, the typing of BTV strains has involved serum neutralization tests (SNT), or virus isolation followed by virus neutralization tests (VNT). SNTs require reference strains of each of the BTV serotypes, to determine if they can be neutralised by the serum sample being tested (Parsonson and Snowdon, 1985). In contrast VNTs require the initial isolation of the outbreak virus, which is then assayed for its

susceptibility to neutralisation by reference antisera for each of the BTV serotypes (Afshar, 1994).

These serological typing methods are labour intensive, expensive, time consuming and unreliable, particularly if the test serum contains antibodies to more than one BTV serotype, or if the diagnostic tissue samples contain more than one BTV serotype. Consequently nucleic acid-based assays which do not require either reference virus strains or reference antisera are increasingly being used for the typing of BTV, although they do require a well-equipped laboratory.

As the least conserved of the BTV genome segments, sequence variations in Seg-2 (which encodes VP2), present a basis for the design of primers and probes for conventional and real time RT-PCR assays that can be used to identify different BTV serotypes and topotypes (Mertens et al., 2007a, Maan et al., 2016). However, the geographical separation of BTV strains over an extensive period of time has allowed BTV strains in different regions to evolve independently within their respective local ecosystems, resulting in the appearance of distinctive regional variants (namely eastern and western topotypes) for all of the BTV genome segments. The genetic diversity of Seg-2 between different BTV strains within individual BTV serotypes, presents a significant challenge for the development of molecular serotyping assays that will reliably detect and identify of all BTV strains within each serotype and may require the use of multiplex primers and probes.

The BTV serotype can also be determined directly by sequencing and phylogenetic analysis, of Seg-2 comparing it to the sequences of known reference strains (Maan et al., 2007a, Maan et al., 2004). These techniques will usually also identify the topotypes of Seg-2 and may indicate its geographic origin. Nucleic acid-based assays are increasingly being used for the characterisation and typing BTV but require a well-equipped laboratory. The RT-PCR assays using serotype-specific primers have the potential to reduce the need for BTV serotype-specific antisera for serological typing methods.

Since 2001, twelve BTV-serotypes (BTV-1, 2, 3, 4, 5, 9, 10, 12, 16, 21, 23 and 24) have been isolated in India. However, serological evidence suggests that a total of 21 different BTV serotypes exist in India (Maan et al., 2015c). A pentavalent inactivated BTV vaccine

containing serotypes 1, 2, 10, 16 and 23 is currently being used in Southern India since 2010, although not on a large scale. No live BTV vaccine has been used in India till date. Experimental monovalent, bivalent or trivalent inactivated BT vaccines were developed under the All India Network Program on BT which started in 2001. The experimental inactivated monovalent vaccine inactivated with binary ethylenimine and saponified: BTV-1 and BTV-2 have been reported from India. Experimental bivalent inactivated BT vaccines incorporating BTV-1 and BTV-23 have also been reported from Tamil Nadu Veterinary and Animal Science University (TANUVAS) and Institute of Animal Health and Veterinary Biologicals (IAHVB), Hebbal, Bangaluru (Bhanuprakash et al., 2009). None of these except pentavalent inactivated vaccine have been commercialized.

The design of an appropriate vaccine requires regular virological surveillance for accurate identification of the serotypes currently circulating in the region. By comparing BTV isolates from before, during and after implementation of the current pentavalent inactivated-vaccine campaign, it may be possible to determine if it has influenced the prevalence of different BTV serotypes.

The objectives for this chapter include:

- Isolation of BTV from suspected field BTV outbreaks in four Southern states of India during 2014-15.
- Identification of serotype in these Indian BTV field isolates by serotype-specific TaqMan based qRT-PCR assay targeting BTV Seg-2 (Maan et al., 2016) .
- Typing of BTV isolates obtained from various collaborators under the Indo-UK project by serotype-specific TaqMan based qRT-PCR assay targeting BTV Seg-2 (Maan et al., 2016).

4.2 Results

4.2.1 Isolation of BTV

Overall, one hundred and forty one BTV isolations were made from suspected BTV outbreaks in Southern India during 2014-2015 under the present study (Table 4.1, Appendix 2). One hundred and thirty five (135) of these were derived from EDTA blood (n=126), tissues [spleen (n=6), lymph node (n=1)] or saliva (n=2) during 2015 (Table 4.1,

Appendix 2). Six isolates were made from EDTA blood samples during 2014, one from Telangana and five from Tamil Nadu. Briefly, clinical blood sample was washed thrice with Dulbecco's phosphate buffered saline and washed blood cells were then lysed with sterile water. For tissue sample, the homogenization of tissue was done in 10 % PBS supplemented with 1000 units/mL penicillin and 1000 µg streptomycin/mL. The supernatant of processed samples were then passed through a 0.22 µm sterile syringe filter to infect KC cells (200 µL of filtered inoculum) in 25 cm^2 culture flask (Chapter 2, Section 2.12, 2.15). Primary isolation of virus from clinical samples (blood, tissue and saliva) was attempted by KC passage 1(KC1), followed by KC1 and BHK passage 3 (KC1-BHK3). Following successful isolation, the presumptive positive isolates in BHK-21 cell culture were confirmed as bluetongue virus (BTV) by group-specific real time RT-PCR targeting genome Seg-10, to detect BTV RNA (Hofmann et al., 2008a), or after dsRNA extraction from cell pellet by 1% agarose gel electrophoresis, to visualize the typical dsRNA migration pattern of BTV RNA. Virus isolations used 25 cm^2 culture flask containing KC cells (KC1), followed by three passages on BHK-21 cells.

Seg-9 based serogroup-specific real-time RT-PCR targeting genome segment 9 (Maan et al., 2015a) and Seg-10 based assay (Hofmann et al., 2008a) were used to detect BTV in clinical samples from 2014 and 2015 outbreaks respectively. One hundred and seventeen of the clinical samples from 2015, gave Ct value <30, with eighteen with Ct value > 30. The virus was successfully isolated from these (141) one hundred and forty one clinical samples (KC1-BHK3) (Table 4.1, Appendix 2). Characteristic cytopathic effects (CPE) indicated by cell rounding and cell detachment, were usually observed at 3-4 days post inoculation in BHK-21 cells (Figure 4.1). Each BTV isolate was tested for BTV RNA by group specific real-time RT-PCR targeting genome segment 10 (NS3) (Table 4.1). Details concerning BTV virus isolates from Southern India, 2014-15 are given in appendix 2.

4.2.2 Assigning unique collection number to BTV field strains

After testing positive for BTV RNA by group specific real-time RT-PCR targeting genome segment 10, the BTV field isolates (KC1 and BHK3) were given unique virus-collection numbers, consisting of country code, year / sample number (Appendix 2), prior to storage at -80^{0}C.

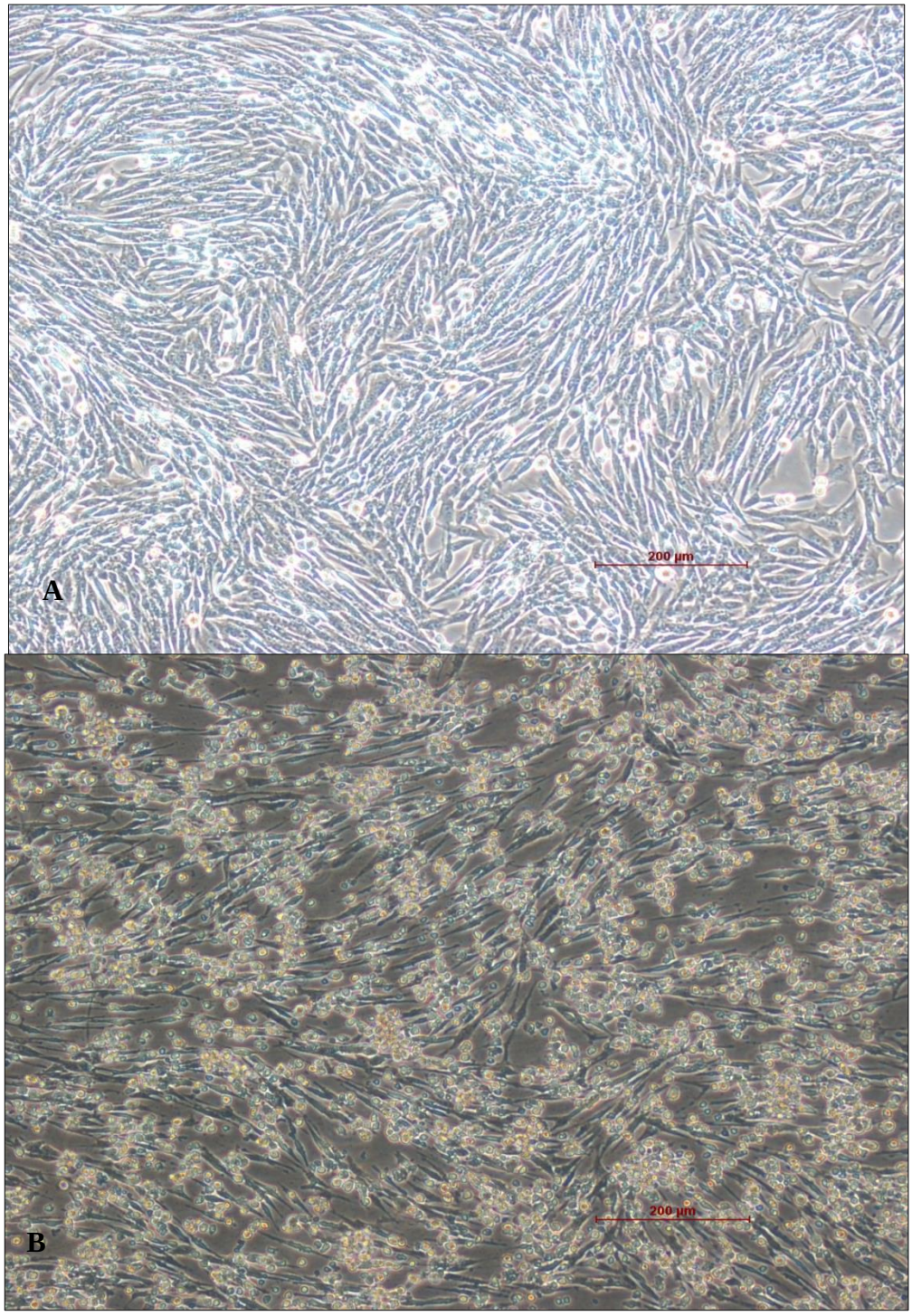


Figure 4.1 Cytopathic changes induced by the BTV isolate from sheep in BHK-21

A: healthy uninfected BHK-21 cells two days old monolayers; B: cytopathic effects induced in BHK-21 by BTV isolate [IND2015/169]470 from sheep on day 2 post infection.

Table 4.1 Isolation of BTV from qRT-PCR positive clinical samples (n=141)

Indian State	qRT-PCR positive clinical samples[$] (Ct<30)	qRT-PCR positive clinical samples[$] (Ct>30)	qRT-PCR positive in KC1-BHK3[$] (Ct<30)	qRT-PCR positive KC1-BHK3[$] (Ct>30)
Telangana,2014	1[*]	-	1	-
Tamil Nadu,2014	5[*]	-	5	-
Telangana, 2015	16[¥]	-	16	-
Andhra Pradesh, 2015	23	-	23	-
Karnataka, 2015	28[µ]	-	27	1
Tamil Nadu, 2015	50[£]	18	38	30
Overall	123	18	110	31

* Clinical samples collected from field outbreaks, 2014 were subjected to serogroup-specific real-time RT-PCR targeting genome segment 9 (Maan et al., 2015a).

[¥] including one necropsy spleen sample.

[µ] including one necropsy spleen sample.

[£] including four necropsy spleen and one lymph node samples

[$] All samples except 2014 outbreaks were subjected to BTV RNA detection using BTV serogroup-specific qRT-PCR targeting Seg-10 (Hofmann et al., 2008a).

4.2.3 Virus propagation for typing

Sixty three (n=63) of the cell culture adapted Southern Indian field strains of BTV from 2014-2015 (Table 4.2), were cultivated in BHK-21 cells using 175 cm^2 culture flasks, prior to extraction of viral dsRNA for further typing and sequencing work. The details of these cultivated field strains, along with their passage history are given in Table 4.2. Viral RNA from these BTV isolates was extracted from pelleted infected BHK-21 cells (175 cm^2 culture flask) using TRIzol (Invitrogen) (Chapter 2, Section 2.17), then tested using a BTV serogroup-specific qRT-PCR targeting Seg-10. These isolates gave Ct values ranging from 5.0 to 31.0 (Table 4.2). The dsRNA migration patterns of isolates with Ct < 20, were analysed by 1% agarose gel electrophoresis (AGE) generating a 3-3-3-1 migration pattern, typical of BTV (Figure 4.2). The RNAS of isolates with Ct>20 were not detectable by AGE.

Ten additional cell culture adapted BTV isolates (Table 4.3) were obtained under the Indo-UK project titled "Development of diagnostic systems, reference collections and molecular epidemiology studies for important arboviral pathogens of livestock in India" and were cultivated in BHK-21 cells using 175 cm^2 culture flasks. dsRNAs extracted from the infected BHK-21 cell pellets, were used to confirm that these isolates were positive for BTV, by BTV serogroup-specific qRT-PCR targeting Seg-10, generating cycle threshold (Ct) value ranging from 10 to 19 (Table 4.3).

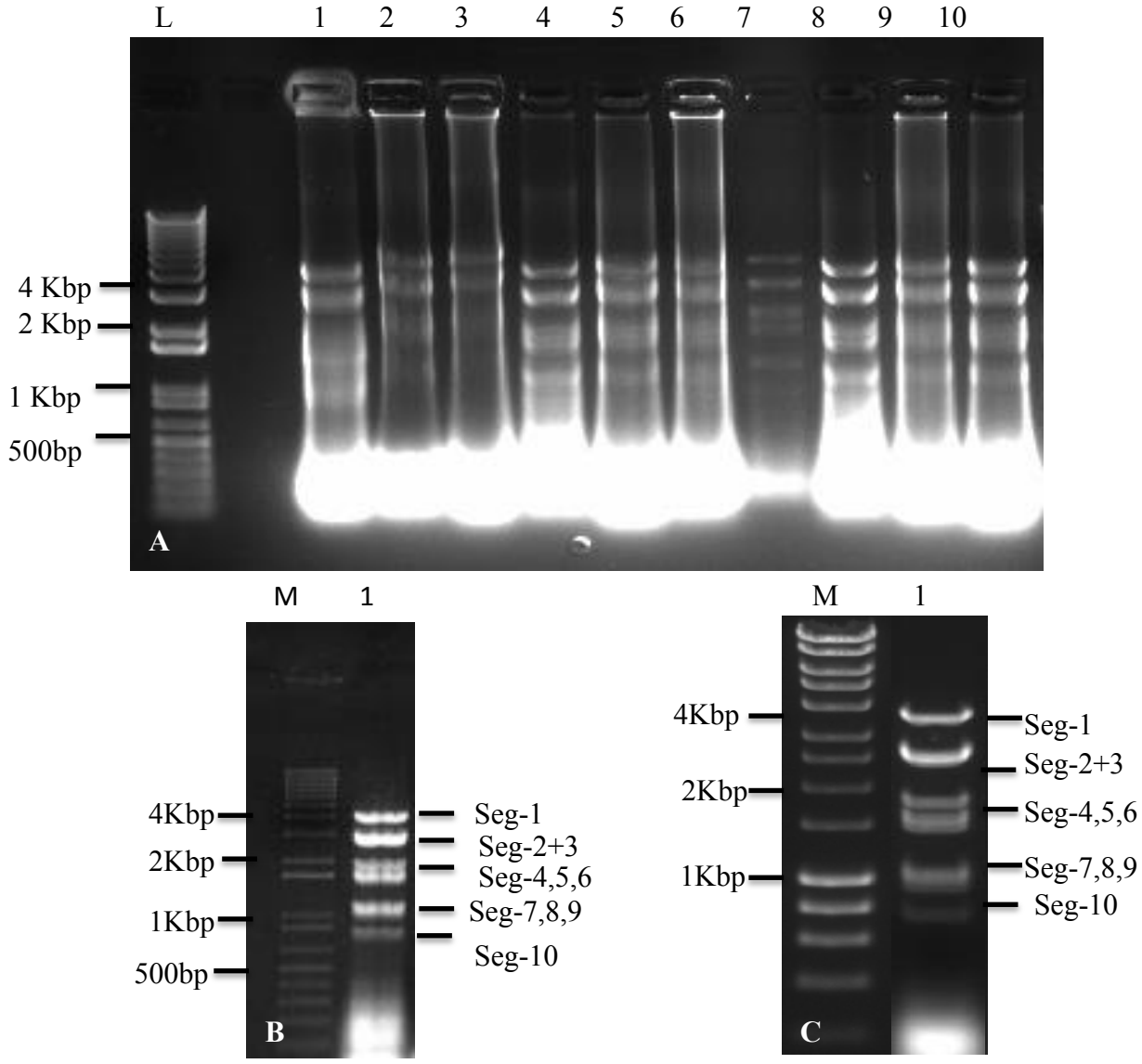


Figure 4.2 Electrophoretic analysis of BTV dsRNA in a 1 % agarose gel.

A) Lane M: 1Kb plus DNA Ladder (Invitrogen); Lanes 1-10 contained dsRNAs from the following viral isolates: [IND2015/15]22; [IND2014/34]BTV-1; [IND2014/35] BTV-2;

[IND2015/118]V1; [IND2015/121]V2; [IND2015/124]V13; [IND2015/238]6PK; [IND2015/184]475; [IND2015/187]476; [IND2015/190]477; B) Lane M: 1Kb plus DNA Ladder (Invitrogen); Lanes 1: dsRNAs of [IND2015/196]480; C) Lane M: 1Kb plus DNA Ladder (Invitrogen); Lanes 1: dsRNAs of [IND2015/199]483. The ssRNA was not precipitated efficiently during dsRNA extraction (Chapter 2, section 2.17) and is visible as smeared bands at the bottom of the gel with different intensity for each isolate

Table 4.2: Indian field strains of BTV (n=63) serotyped by Seg-2 based qRT-PCR.

Virus collection number#	Place of origin	Species	Original sample	Vaccine status	Passage history	qRT-PCR$^{\$}$(Ct)
[IND2015/60]100	Thoothukudi,TN	Goat	Blood	UV	KC1/BSR6	16
[IND2015/15]22	Thoothukudi,TN	Sheep	Blood	V	KC1/BSR6	16
[IND2015/18] 40	Thoothukudi,TN	Goat	Blood	UV	KC1/BHK4	15
[IND2015/148]491	Kanchipuram,TN	Sheep	Blood	UV	KC1/BHK5	7
[IND2015/151]492	Kanchipuram,TN	Sheep	Blood	UV	KC1/BHK6	18
[IND2015/154]493	Kanchipuram,TN	Sheep	Blood	UV	KC1/BSR5	5
[IND2015/157]494	Kanchipuram,TN	Sheep	Blood	UV	KC1/BHK6	6
[IND2015/208]404	Erode,TN	Sheep	Blood	UV	KC1/BHK4	9
[IND2015/211]406	Erode,TN	Sheep	Blood	UV	KC1/BHK5	6
[IND2015/214]408	Erode,TN	Sheep	Blood	UV	KC1/BHK4	18
[IND2015/169]470	Tirunehveli,TN	Sheep	Blood	V	KC1/BHK4	6
[IND2015/172]471	Tirunehveli,TN	Cow	Blood	UV	KC1/BHK5	8
[IND2015/175]472	Tirunehveli,TN	Cow	Blood	UV	KC1/BHK4	7
[IND2015/178]473	Tirunehveli,TN	Sheep	Blood	V	KC1/BHK4	7
[IND2015/181]474	Tirunehveli,TN	Cow	Blood	UV	KC1/BHK4	5
[IND2015/184]475	Tirunehveli,TN	Sheep	Blood	V	KC1/BHK4	6
[IND2015/187]476	Tirunehveli,TN	Sheep	Blood	V	KC1/BHK5	7
[IND2015/190]477	Tirunehveli,TN	Sheep	Blood	V	KC1/BHK5	8
[IND2015/193]478	Tirunehveli,TN	Goat	Blood	V	KC1/BHK4	7
[IND2015/196]480	Tirunehveli,TN	Sheep	Blood	V	KC1/BHK5	5
[IND2015/199]483	Tirunehveli,TN	Sheep	Blood	V	KC1/BHK4	7
[IND2015/202]484	Tirunehveli,TN	Goat	Blood	V	KC1/BHK4	20
[IND2015/205]486	Tirunehveli,TN	Sheep	Blood	V	KC1/BHK4	9
[IND2015/160]CS4	Tirunehveli,TN	Sheep	Blood	V	KC1/BHK4	6
[IND2015/163]CS6	Tirunehveli,TN	Sheep	Blood	V	KC1/BHK4	8
[IND2015/166]CS9	Tirunehveli,TN	Sheep	Blood	V	KC1/BHK5	17
[IND2015/76]534	Thoothukudi,TN	Goat	Blood	UV	KC1/BHK4	20
[IND2015/2017]547	Thoothukudi,TN	Sheep	spleen	UV	KC1/BHK4	10
[IND2015/220]228	Tirunehveli	Sheep	spleen	UV	KC1/BHK4	5
[IND2015/223]34	Thoothukudi,TN	Sheep	spleen	UV	KC1/BHK4	15
[IND2015/232]556	Thoothukudi,TN	Cattle	saliva	UV	KC1/BHK5	7
[IND2015/118]V1	Chikballapur,K	Sheep	Blood	V	KC1/BHK5	8
[IND2015/121]V2	Chikballapur,K	Sheep	Blood	V	KC1/BHK5	6
[IND2015/124]V13	Chikballapur,K	Sheep	Blood	V	KC1/BHK5	7
[IND2015/136]V41	Chikballapur,K	Sheep	Blood	V	KC1/BHK5	5
[IND2015/139]V42	Chikballapur,K	Sheep	Blood	V	KC1/BHK5	10

[IND2015/142]V44	Chikballapur,K	Sheep	Blood	V	KC1/BHK4	7
[IND2015/145]V44 L	Chikballapur,K	Sheep	Blood	V	KC1/BHK4	8
[IND2015/133]V S	Chikballapur,K	Sheep	Blood	V	KC1/BHK4	9
[IND2015/]07Karn	Chikballapur,K	Sheep	Blood	UV	KC1/BHK6	6
IND2015/241]K3	Karimnagar,T	Sheep	Blood	UV	KC1/BHK4	7
[IND2015/244]K4	Karimnagar,T	Sheep	Blood	UV	KC1/BHK4	5
[IND2015/250]K9	Karimnagar,T	Sheep	Blood	UV	KC1/BHK6	21
[IND2015/253](K10)	Karimnagar,T	Sheep	Blood	UV	KC1/BHK7	25
[IND2015/]K13	Karimnagar,T	Sheep	Blood	UV	KC1/BHK6	16
[IND2015/256]K14	Karimnagar,T	Sheep	Blood	UV	KC1/BHK4	7
[IND2015/238]6PK	Warangal,T	Sheep	Blood	UV	KC1/BHK4	7
[IND2015/271]7A	Anantpur,AP	Sheep	Blood	UV	KC1/BHK4	9
[IND2015/]14A	Anantpur,AP	Goat	Blood	UV	KC1/BHK4	24
[IND2014/34]BTV-1*	Thanjavur,TN	Sheep	Blood	UV	KC1/BHK4	8
[IND2014/35]BTV-2*	Thanjavur,TN	Sheep	Blood	UV	KC1/BHK4	9
[IND2014/36] 3NLG€	Nalgonda,T	Sheep	Blood	UV	KC1/BHK6	6
[IND2014/37] 10NLG€	Nalgonda,T	Sheep	Blood	UV	KC1/BHK4	7
[IND2015/289]28A	Anantpur,AP	Sheep	Blood	UV	KC1/BHK5	30
[IND2015/224]PPR spleen	Medak, Telangana	Sheep	Blood	UV	KC1/BHK5	29
[IND2015/335]33A	Anantpur,AP	Sheep	Blood	UV	KC1/BHK5	30
[IND2015/292]29A	Anantpur,AP	Sheep	Blood	UV	KC1/BHK5	29
[IND2015/70]532	Thoothukudi,TN	Cow	Blood	UV	KC1/BHK5	31
CHE 14¥	Karur,TN	Sheep	Blood	UV	KC1/BHK4	7
CHE 2¥	Erode,TN	Sheep	Blood	UV	KC1/BHK4	8
CHE 12¥	Karur,TN	Sheep	Blood	UV	KC1/BHK4	25
CHE5¥	Tirupur,TN	Sheep	Blood	UV	KC1/BHK4	25
WGL4¥	Warangal,T	Sheep	Blood	UV	KC1/BHK4	28

Virus collection number: [Country code, year / sample number]

$All samples except 2014 outbreaks were subjected to BTV RNA detection using BTV serogroup-specific qRT-PCR targeting Seg-10 (Hofmann et al., 2008a).

* These BTV field isolates from BTV suspected outbreaks in Tamil Nadu, 2014 were provided by Dr. K.M.N Reddy.

€ These BTV field isolates from BTV suspected outbreaks in Telangana, 2014 were provided by Dr. Y.V. Reddy.

¥ These BTV field isolates were from samples collected during BTV suspected outbreaks in Tamil Nadu, 2014.

Abbreviations: TN, Tamil Nadu; T, Telangana; K, Karnataka; AP, Andhra Pradesh; V, vaccinated and NV, nonvaccinated.

Table 4.3 Details of Indian field strains of BTV (n=10) obtained from collaborators for typing by Seg-2 based qRT-PCR

BTV isolate	Geographic origin	Serogroup specific qRT-PCR[$] (Ct value)
BTV2DH	Karnataka	14
BTV1 IVRI	Uttar Pradesh	15
BTV2 IVRI	Uttar Pradesh	14
BTV10 IVRI	Uttar Pradesh	19
BTV 23 IVRI	Uttar Pradesh	17
BTV MP	Telangana	14
BTV WG103	Telangana	19
BTV IPGR/10	Telangana	13
BTV VJW 764	Telangana	15
BTV KMN 07/05	Telangana	16

[$] BTV RNA detection using BTV serogroup-specific qRT-PCR targeting Seg-10 (Hofmann et al., 2008a).

4.2.4 Typing of BTV isolates

Typing assays for BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 were performed on RNA samples from Southern Indian BTV field isolates 2014-15 (n=63) using serotype-specific TaqMan based qRT-PCR assays (Maan et al., 2016). For BTV-1, -2, -4, -9 and -16, two sets of qRT-PCR primers and probes were used in a duplex format in order to detect both eastern and western topotypes/strains of each serotype.

Multiple co-circulating BTV serotypes, including BTV-1, BTV-2, BTV-4, BTV-5, BTV-9, and BTV-10 & BTV-12 were identified circulating during BTV outbreaks in four states of Southern India, Telangana, Andhra Pradesh, Karnataka and Tamil Nadu during 2014 and 2015 (Table 4.4).

Infection involving only one serotype was found in thirty six of the BTV isolates (Table 4.6 and 4.7) with BTV-12 in fourteen isolates [IND2015/169]470; [IND2015/175]472; [IND2015/178]473; [IND2015/199]483; [IND2015/202]484; [IND2015/160]CS4; [IND2015/163]CS6; [IND2015/136]V41; CHE5; [IND2015/232]556 saliva; [IND2015/223]34 tissue; [IND2015/211]406; [IND2015/145]V44lamb and [IND2015/133]V severe, from Tamil Nadu and Karnataka, 2014-15. BTV-5 was detected

in nine of the single type BTV isolates, [IND2015/172]471; [IND2015/184]475; [IND2015/193]478; [IND2015/166] CS9; [IND2015/205]486; [IND2015/142] V44; [IND2015/76]534; [IND2015/208]404; [IND2015/214]408 from Tamil Nadu and Karnataka, 2015. Serotype BTV-2 was identified in five BTV isolates, [IND2015/241] K3; [IND2015/250] K9; [IND2015/253] K10; [IND2015/] K13; [IND2015/256] K14 from Telangana, 2015, one each [IND2015/] 14A from Andhra Pradesh, 2015 and [IND2015/18] 40 from Tamil Nadu, 2015 (Table 4.6 and 4.7). BTV-1 was detected in three BTV isolates from Andhra Pradesh, 2015: [IND2015/289]28A ; [IND2015/292]29A; [IND2015/335]33A and BTV-10 in one BTV isolate [IND2015/2017]547 tissue from Tamil Nadu, 2015. Dual and triple BTV co-infections were also detected in twenty two and four BTV isolates respectively, of sixty three total BTV isolates typed. (Table 4.8 and 4.9).

One of the field BTV isolate, [IND2015/] 07Karn from unvaccinated sheep in Karnataka state, 2015 BT outbreak could not be typed using qRT-PCR assay against BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 serotypes (Maan et al., 2016) and may therefore contains another serotype.

4.2.4.1 BTV isolates from vaccinated flock of Tamil Nadu state, 2015

Fourteen BTV isolations (Table 4.2, 4.4) were made from animals vaccinated with pentavalent inactivated BTV vaccine (BTV-1, BTV-2, BTV-10, BTV-16 and BTV-23) in outbreaks in Tamil Nadu during 2015 BT. These viruses were typed as BTV-4, BTV-5, BTV-9 and BTV-12, with Ct values ranging from 7 to 27 from extracted RNA of cell culture adapted BTV isolates. The animals were vaccinated during August 2015, with pentavalent BTV vaccine and booster dose of vaccine was given after 28 days of primary vaccination. Clinical samples were taken in December, 2015 during BT outbreak and after four months of primary vaccination. Six BTV isolates [IND2015/169]470; [IND2015/178]473; [IND2015/199]483; [IND2015/202]484; [IND2015/160] CS4 and [IND2015/163] CS6 typed as BTV-12, with Ct value ranging from 7 to 23. Four isolates typed as BTV-5: [IND2015/184]475; [IND2015/193]478; [IND2015/205]486 and [IND2015/166] CS9, with Ct value ranging from 15 to 23. Interestingly, mixed infections were identified in four BTV isolates: [IND2015/15]22 (BTV-12, Ct 10 and BTV-4, Ct20); [IND2015/187]476 (BTV-5, Ct15 and BTV-12, Ct32); [IND2015/190]477 (BTV-4, Ct 30 and BTV-9, Ct25) and [IND2015/196]480 (BTV-4, Ct23 and BTV-12, Ct9) (Table 4.8).

4.2.4.2 BTV isolates from vaccinated flock of Karnataka state, 2015

Five BTV isolates from animals vaccinated with the pentavalent inactivated BTV vaccine (BTV-1, BTV-2, BTV-10, BTV-16 and BTV-23) and three from animals vaccinated with the bivalent inactivated BTV vaccine (BTV-1 and BTV-23) during BT outbreaks in Karnataka state during 2015, were typed as BTV-4, BTV-5 and BTV-12 with Ct value ranging from 8 to 25 (Table 4.2, 4.4). These animals were vaccinated with the pentavalent BTV vaccine during August 2015 with booster doses of the vaccine given after 28 days. Clinical samples were taken in late November, 2015 during BT outbreak and nearly after three months of primary vaccination. One BTV isolate [IND2015/142] V44 from a vaccinated animal was typed as BTV-5 with a Ct of 14. Three BTV isolates were typed as BTV-12: [IND2015/136] V41 with Ct 9-16. Eight BTV isolations were made from a vaccinated flock during BT outbreaks in Karnataka state during 2015, four of which typed as mixed infections: [IND2015/118] V1 (BTV-5, Ct15 and BTV-12, Ct25); [IND2015/121] V2 (BTV-5, Ct14 and BTV-12, Ct22); [IND2015/124] V13 (BTV-5, Ct19 and BTV-12, Ct21) and [IND2015/139] V42 (BTV-4, Ct21 and BTV-12, Ct8) (Table 4.8).

4.2.4.3 BTV isolates from unvaccinated flock of Tamil Nadu state, 2014

Six BTV isolates from an unvaccinated flock during BT outbreaks in Tamil Nadu in 2014 were typed as BTV-2, BTV-10, and BTV-12, with Ct value ranging from 10 to 34 (Table 4.2, 4.4). Of these, one isolate CHE5 was typed as BTV-2 with a Ct value of 27. Mixed infections were detected in five BTV isolates [IND2014/34] BTV-1 (BTV-10, Ct 19 and BTV-12, Ct28); [IND2014/35] BTV-2 (BTV-2, Ct26, BTV-10, Ct15, and BTV-12, Ct34); CHE 2 (BTV-2, Ct11 and BTV-12, Ct30); CHE 12 (BTV-2, Ct30 and BTV-12, Ct25) and CHE 14 (BTV-2, Ct32 and BTV-12, Ct10) (Table 4.8). One BTV isolate (IND2014/01) collected from the Karur district, Tamil Nadu, 2014, was typed by Dr. Lalit Kumar, College of Veterinary Sciences, LLR University of Veterinary and Animal Sciences, Haryana, India as BTV-16 (Kumar et al., 2016).

4.2.4.4 BTV isolates from unvaccinated flock of Tamil Nadu state, 2015

Eighteen BTV isolates taken from unvaccinated flocks during BT outbreaks in Tamil Nadu 2015, were typed as BTV-2, BTV-5, BTV-9, BTV-10, and BTV-12, with Ct values ranging from 6 to 33 (Table 4.2, 4.4). Of these, one isolate [IND2015/18] 40 from sheep

was typed as BTV-2 with a Ct value 30. One isolate from sheep [IND2015/2017]547 tissue was typed as BTV-10 with a Ct value 25. Four isolates, [IND2015/172]471 from cattle [IND2015/76]534, [IND2015/208]404 and [IND2015/214]408 from a mixed flock were typed as BTV-5 with Ct value 18-27. Four isolates [IND2015/232]556 saliva from cattle) and [IND2015/223]34 tissue, [IND2015/211]406 & [IND2015/175]472 from mixed flock were typed as BTV-12 with Ct values of 8 to 20. Mixed infections were found in eight isolates [IND2015/60]100 (BTV-10, Ct 23 and BTV-12, Ct14); [IND2015/70] 532 (BTV-2, Ct19 and BTV-12, Ct16); [IND2015/148] 491 (BTV-2, Ct25, BTV-5, Ct10 and BTV-12, Ct23); [IND2015/151]492 (BTV-5, Ct13 and BTV-12, Ct24); [IND2015/154] 493 (BTV-2, Ct35 and BTV-12, Ct 25); [IND2015/157] 494 (BTV-2, Ct30 and BTV-12, Ct 6); [IND2015/181]474 (BTV-12, Ct19 and BTV-10, Ct 33) and [IND2015/220]228 tissue (BTV-5, Ct20 and BTV-9, Ct22) (Table 4.8). One isolate (IND2014/01) collected from the Karur district of Tamil Nadu, in 2014 was typed by Dr. Lalit Kumar, College of Veterinary Sciences, LLR University of Veterinary and Animal Sciences, Haryana, India as BTV-16.

4.2.4.5 BTV isolates from unvaccinated flock of Telangana state, 2014

Three BTV isolates derived from unvaccinated flocks during BT outbreaks in Telangana, 2014, were typed as BTV-2, BTV-5, BTV-9 and BTV-12, with Ct value ranging from 16 to 32 (Table 4.2, 4.4). All were mixed infections: BTV isolate WGL4 (BTV-2, Ct32 and BTV-12, Ct 30); [IND2014/36] 3NLG (BTV-2, Ct26; BTV-5, Ct30 and BTV-9, Ct 27) and [IND2014/37] 10NLG (BTV-2, Ct30; BTV-5, Ct16 and BTV-12, Ct 22) from mixed flock (Table 4.8)

4.2.4.6 BTV isolates from unvaccinated flock of Telangana state, 2015

Eight BTV isolates derived from unvaccinated flocks during BT outbreaks in Telangana, 2015, were typed as BTV-2, BTV-4 and BTV-12 with Ct value ranging from 10 to 35 (Table 4.2, 4.4). Of these, five isolates IND2015/241] K3, [IND2015/250] K9, [IND2015/253] K10, [IND2015/] K13 and [IND2015/256] K14 were typed as BTV-2 with Ct values of 10 to 35. Two isolates [IND2015/244] K4 and [IND2015/224] PPR spleen typed as BTV-4, with Ct values of 10 to 16. Mixed infection were found in one isolate [IND2015/238]6PK containing BTV-4, Ct13 and BTV-12, Ct 23 (Table 4.8).

4.2.4.7 BTV isolates from unvaccinated flock of Andhra Pradesh state, 2015

Five BTV isolates derived from unvaccinated flocks of sheep during BT outbreaks in Andhra Pradesh, 2015, were typed as BTV-1, BTV-2, BTV-4 and BTV-12, with Ct values ranging from 10 to 35 (Table 4.2, 4.4). Of these, three isolates [IND2015/289]28A, [IND2015/292]29A and [IND2015/335]33A were typed as BTV-1 with Ct values of 35 to 36. One isolate [IND2015/] 14A was typed as BTV-2 with a Ct of 8. A mixed infection was found in one BTV isolate [IND2015/271]7A containing BTV-4, Ct20 and BTV-12, Ct 23 (Table 4.8).

4.2.4.8 Mixed infections

Of sixty three BTV isolates, dual and triple BTV co-infections were detected in twenty two and four isolates respectively (Table 4.8 and 4.9). The dominant BTV serotype in these dual and triple mixed infection BTV isolates based on a low Ct value were: BTV-12, with Ct of 6 to 30 in eleven BTV isolates; BTV-5, with Ct of 10 to 20 in eight BTV isolates; BTV-2, with Ct of 11 to 26 in three isolates; BTV-9, with a of Ct 25 in one isolate; BTV-10, with Ct values of 15 and 19 in two isolates and BTV-4, with a Ct value of 13 in one isolate (Table 4.9). The second BTV serotype in these mixed infection BTV isolates, based on Ct values were: BTV-12, with Ct values of 21 to 32 in eleven isolates, BTV-2, with Ct values of 22 to 27 in seven isolates; BTV-9, with Ct values of 22 to 27 in two isolates; BTV-10, with Ct values of 32 to 33 in two isolates and BTV-4, with Ct values of 20 to 33 in four mixed isolates (Table 4.9).

Table 4.4 ®Serotyping of Indian field isolates of BTV (n=63) from 2014-15 BT outbreaks in Southern India.

#Virus collection number	Place of sampling	Passage history	π qRT-PCR	BTV-1	BTV-2	BTV-4	BTV-5	BTV-9	BTV-10	BTV-12
				Ct values$						
[IND2015/15]22£	Thoothukudi ,TN	KC1/BSR6	16	-	-	20	-	-	-	10
[IND2015/18] 40*	Thoothukudi ,TN	KC1/BHK4	24	-	30	-	-	-	-	-
[IND2015/60]100*	Thoothukudi ,TN	KC1/BSR6	16	-	-	-	-	-	23	14
[IND2015/76]534*	Thoothukudi,TN	KC1/BHK4	17	-	-	-	18	-	-	-
[IND2015/70]532∞	Thoothukudi,TN	KC1/BHK5	31	-	19	-	-	-	-	16

[IND2015/2017]547 tissue[&]	Thoothukudi,TN	KC1/BHK4	20	-	-	-	-	-	25	-
[IND2015/223]34 tissue[&]	Thoothukudi,TN	KC1/BHK4	5	-	-	-	-	-	-	8
[IND2015/232]556 saliva[∞ ©]	Thoothukudi,TN	KC1/BHK5	15	-		-	-	-	-	20
[IND2015/148]491	Kanchipuram,TN	KC1/BHK5	5	-	25	-	10	-	-	23
[IND2015/151]492	Kanchipuram,TN	KC1/BHK6	7	-		-	13	-	-	24
[IND2015/154]493	Kanchipuram,TN	KC1/BSR5	18	-	35	-	-	-	-	25
[IND2015/157]494	Kanchipuram,TN	KC1/BHK6	5	-	30	-	-	-	-	6
[IND2015/208]404	Erode,TN	KC1/BHK4	6	-	-	-	24	-	-	-
[IND2015/211]406	Erode,TN	KC1/BHK5	9	-	-	-	-	-	-	19
[IND2015/214]408	Erode,TN	KC1/BHK4	6	-	-	-	19	-	-	
[IND2015/169]470[£]	Tirunehveli,TN	KC1/BHK4	18	-	-	-	-	-	-	21
[IND2015/172]471[∞]	Tirunehveli,TN	KC1/BHK5	6	-	-	-	27	-	-	
[IND2015/175]472[∞]	Tirunehveli,TN	KC1/BHK4	8	-	-	-	-	-	-	16
[IND2015/178]473[£]	Tirunehveli,TN	KC1/BHK4	7	-	-	-	-	-	-	12
[IND2015/181]474[∞]	Tirunehveli,TN	KC1/BHK4	7	-	-	-	-	-	33	19
[IND2015/184]475[£]	Tirunehveli,TN	KC1/BHK4	5	-	-	-	21	-	-	-
[IND2015/187]476[£]	Tirunehveli,TN	KC1/BHK5	6	-	-	-	15	-	-	32
[IND2015/190]477[£]	Tirunehveli,TN	KC1/BHK5	7	-	-	30		25	-	-
[IND2015/193]478*[£]	Tirunehveli,TN	KC1/BHK4	8	-	-	-	18	-	-	-
[IND2015/196]480[£]	Tirunehveli,TN	KC1/BHK5	7	-	-	23	-	-	-	9
[IND2015/199]483[£]	Tirunehveli,TN	KC1/BHK4	5	-	-	-	-	-	-	11
[IND2015/202]484*[£]	Tirunehveli,TN	KC1/BHK4	7	-	-	-	-	-	-	7
[IND2015/205]486[£]	Tirunehveli,TN	KC1/BHK4	20	-	-	-	23	-	-	-
[IND2015/160]CS4[£]	Tirunehveli,TN	KC1/BHK4	9	-	-	-	-	-	-	19
[IND2015/163]CS6[£]	Tirunehveli,TN	KC1/BHK4	6	-	-	-	-	-	-	23
[IND2015/166]CS9[£]	Tirunehveli,TN	KC1/BHK5	8	-	-	-	18	-	-	-
[IND2015/220]228 tissue[&]	Tirunehveli,TN	KC1/BHK4	10	-	-	-	20	22	-	-
[IND2014/34]BTV-1	Thanjavur,TN	KC1/BHK4	7	-	-	-	-	-	19	28
[IND2014/35]BTV-2	Thanjavur,TN	KC1/BHK4	8	-	26	-	-	-	15	34
CHE 2[¥]	Erode,TN	KC1/BHK4	8	-	11	-	-	-	-	30
CHE5[¥]	Tirupur,TN	KC1/BHK4	25	-	27	-	-	-	-	-
CHE 12[¥]	Karur,TN	KC1/BHK4	25	-	30	-	-	-	-	25
CHE 14[¥]	Karur,TN	KC1/BHK4	7	-	32	-	-	-	-	10
[IND2015/118]V1[€]	Chikballapur,K	KC1/BHK5	9	-	-	-	15	-	-	25
[IND2015/121]V2[€]	Chikballapur,K	KC1/BHK5	8	-	-	-	14	-	-	22
[IND2015/124]V13[€]	Chikballapur,K	KC1/BHK5	6	-	-	-	18	-	-	21
[IND2015/136]V41[£]	Chikballapur,K	KC1/BHK5	7	-	-	-	-	-	-	11
[IND2015/139]V42[£]	Chikballapur,K	KC1/BHK5	5	-	-	21	-	-	-	8

[IND2015/142]V44[£]	Chikballapur,K	KC1/BHK4	0	-	-	-	14	-	-	-
[IND2015/145]V44[£] lamb	Chikballapur,K	KC1/BHK4	7	-	-	-	-	-	-	9
[IND2015/133]V[£] Severe	Chikballapur,K	KC1/BHK4	8	-	-	-	-	-	-	16
[IND2015/]07Karn	Chikballapur,K	KC1/BHK6	9	-	-	-	-	-	-	-
WGL4[β]	Warangal,T	KC1/BHK4	28	-	32	-	-	-	-	30
IND2015/241]K3	Karimnagar,T	KC1/BHK4	6	-	10	-	-	-	-	-
[IND2015/244]K4	Karimnagar,T	KC1/BHK4	7	-		10	-	-	-	-
[IND2015/250]K9	Karimnagar,T	KC1/BHK6	5	-	10	-	-	-	-	-
[IND2015/253]K10	Karimnagar,T	KC1/BHK6	21	-	30	-	-	-	-	-
[IND2015/]K13	Karimnagar,T	KC1/BHK6	25	-	35	-	-	-	-	-
[IND2015/256]K14	Karimnagar,T	KC1/BHK4	16	-	23	-	-	-	-	-
[IND2015/238]6PK	Warangal,T	KC1/BHK4	7	-	-	13	-	-	-	23
[IND2015/224]PPR spleen[&]	Medak, T	KC1/BHK5	29	-	-	16	-	-	-	-
[IND2014/36] 3NLG	Nalgonda,T	KC1/BHK6	7	-	26	-	30	-	27	-
[IND2014/37] 10NLG	Nalgonda,T	KC1/BHK4	6	-	30	-	16	-	-	22
[IND2015/271]7A	Anantpur,AP	KC1/BHK4	7	-	-	20	-	-	-	23
[IND2015/]14A*	Anantpur,AP	KC1/BHK4	9	-	8	-	-	-	-	-
[IND2015/289]28A	Anantpur,AP	KC1/BHK5	30	35	-	-	-	-	-	-
[IND2015/335]33A	Anantpur,AP	KC1/BHK5	30	36	-	-	-	-	-	-
[IND2015/292]29A	Anantpur,AP	KC1/BHK5	29	36	-	-	-	-	-	-

[#] Virus collection number: [Country code, year / sample number]

[$] Ct values were depicted below corresponding serotype detected by Seg-2 based qRT-PCR (Maan et al., 2016).

[*] Host species for these samples were goat.

[∞] Host species for these samples were Cattle.

[&] These were necropsy spleen samples.

[©] This was saliva sample from cattle.

[π] All samples except 2014 outbreaks were subjected to BTV RNA detection using BTV serogroup-specific qRT-PCR targeting Seg-10 (Hofmann et al., 2008a).

[¥] These BTV field isolates from samples collected during BTV suspected outbreaks in Tamil Nadu, 2014.

[β] These BTV field isolates from BTV suspected outbreaks in Telangana, 2014 were provided by Dr. Y.V. Reddy.

[€] Bivalent inactivated BTV vaccine (BTV-1 and 23) was used to vaccinate animals.

[£] Pentavalent inactivated BTV vaccine (BTV-1, BTV-2, BTV -10, BTV -16 and BTV -23)

[®] Typing assays for BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 were performed on RNA samples from Southern Indian BTV field isolates 2014-15 (n=63) using serotype-specific TaqMan based qRT-PCR assays (Maan et al., 2016).

Abbreviations: TN, Tamil Nadu; T, Telangana; K, Karnataka; AP, Andhra Pradesh

Table 4.5: Serotyping of BTV isolates provided by various collaborators.

BTV isolates	Place of sampling	qRT-PCR*	**BTV-1**	**BTV-2**	**BTV-5**	**BTV-9**	**BTV-10**	**BTV-16**	**BTV-23**
			Ct value$						
BTV2DH	K	7	-	14	24	24	-	-	-
BTV1IVRI	U.P	10	15	-	-	-	32	25	-
BTV2IVRI	U.P	8	-	14	-	-	-	-	-
BTV10IVRI	U.P	12	-	-	-	-	19	-	-
BTV23IVRI	U.P	10	-	-	-	-	-	-	17
BTVMP	T	8	-	-	-	-	14	-	-
BTVWG	T	12	-	-	-	-	-	19	-
BTVIPGR	T	12	-	-	-	13	-	-	-
BTVVJW	T	10	-	-	-	-	-	15	-
BTVKMN	T	10	-	-	-	16	-	38	-

[*] BTV RNA detection was done using BTV serogroup-specific qRT-PCR targeting Seg-10 (Hofmann et al., 2008a).

[$] Ct values were depicted below corresponding serotype detected by Seg-2 based qRT-PCR (Maan et al., 2016).

Abbreviations: T, Telangana; K, Karnataka; UP, Uttar Pradesh

Table 4.6 BTV isolates (n=36*) with single serotype infections during BT outbreaks in Southern India, 2014-15.

Virus collection number	BTV-1	BTV-2	BTV-4	BTV-5	BTV-9	BTV-10	BTV-12
	Ct value$						
[IND2015/18] 40	-	30	-	-	-	-	-
[IND2015/76]534	-	-	-	18	-	-	-
[IND2015/2017]547 tissue	-	-	-	-	-	25	-
[IND2015/223]34 tissue	-	-	-	-	-	-	8
[IND2015/232]556 saliva	-	-	-	-	-	-	20
[IND2015/208]404	-	-	-	24	-	-	-
[IND2015/211]406	-	-	-	-	-	-	19
[IND2015/214]408	-	-	-	19	-	-	-
[IND2015/169]470	-	-	-	-	-	-	21
[IND2015/172]471	-	-	-	27	-	-	-
[IND2015/175]472	-	-	-	-	-	-	16
[IND2015/178]473	-	-	-	-	-	-	12
[IND2015/184]475	-	-	-	21	-	-	-
[IND2015/193]478	-	-	-	18	-	-	-
[IND2015/199]483	-	-	-	-	-	-	11
[IND2015/202]484	-	-	-	-	-	-	7
[IND2015/205]486	-	-	-	23	-	-	-
[IND2015/160]CS4	-	-	-	-	-	-	19
[IND2015/163]CS6	-	-	-	-	-	-	23
[IND2015/166]CS9	-	-	-	18	-	-	-
CHE5		27	-	-	-	-	-
[IND2015/136]V41	-	-	-	-	-	-	11
[IND2015/142]V44	-	-	-	14	-	-	-
[IND2015/145]V44lamb	-	-	-	-	-	-	9
[IND2015/133]V Severe	-	-	-	-	-	-	16
[IND2015/]07Karn*	-	-	-	-	-	-	-
IND2015/241]K3	-	10	-	-	-	-	-
[IND2015/244]K4	-	-	10	-	-	-	-
[IND2015/250]K9	-	10	-	-	-	-	-
[IND2015/253]K10	-	30	-	-	-	-	-
[IND2015/]K13	-	35	-	-	-	-	-
[IND2015/256]K14	-	23	-	-	-	-	-
[IND2015/224]PPR spleen	-	-	16	-	-	-	-
[IND2015/]14A	-	8	-	-	-	-	-
[IND2015/289]28A	35	-	-	-	-	-	-
[IND2015/335]33A	36	-	-	-	-	-	-
[IND2015/292]29A	36	-	-	-	-	-	-

$ Ct values were depicted below corresponding serotype detected by Seg-2 based qRT-PCR (Maan et al., 2016)

* [IND2015/] 07Karn from unvaccinated sheep in Karnataka state, 2015 BT outbreak could not be typed using serotype-specific TaqMan based qRT-PCR assay against BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 serotypes (Maan et al., 2016).

Table 4.7 Serotype detected in BTV isolates (n=36) involving single serotype during BT outbreaks in Southern India, 2014-15.

Serotype	Number of BTV isolates (n=36)	Ct$ value
BTV-1	3	35-36
BTV-2	7	8-35
BTV-4	2	10-16
BTV-5	9	14-27
BTV-10	1	25
BTV-12	14	7-23

Table 4.8 BTV isolates with mixed infections(n=26) during BT outbreaks in Southern India, 2014-15.

Virus collection number	**BTV-1**	**BTV-2**	**BTV-4**	**BTV-5**	**BTV-9**	**BTV-10**	**BTV-12**
	Ct value$						
[IND2015/15]22	-	-	20	-	-	-	10
[IND2015/60]100	-	-	-	-	-	23	14
[IND2015/70]532	-	19	-	-	-	-	16
[IND2015/148]491	-	25	-	10	-	-	23
[IND2015/151]492	-	-	-	13	-	-	24
[IND2015/154]493	-	35	-	-	-	-	25
[IND2015/157]494	-	30	-	-	-	-	6
[IND2015/181]474	-	-	-	-	-	33	19
[IND2015/187]476	-	-	-	15	-	-	32
[IND2015/190]477	-	-	30	-	25	-	-
[IND2015/196]480	-	-	23	-	-	-	9
[IND2015/220]228 tissue	-	-	-	20	22	-	-
[IND2014/34]BTV-1	-	-	-	-	-	19	28
[IND2014/35]BTV-2	-	26	-	-	-	15	34
CHE 2	-	11	-	-	-	-	30
CHE 12	-	30	-	-	-	-	25
CHE 14	-	32	-	-	-	-	10
[IND2015/118]V1	-	-	-	15	-	-	25
[IND2015/121]V2	-	-	-	14	-	-	22
[IND2015/124]V13	-	-	-	18	-	-	21
[IND2015/139]V42	-	-	21	-	-	-	8
WGL4		32	-	-	-	-	30
[IND2015/238]6PK	-	-	13	-	-	-	23
[IND2014/36] 3NLG	-	26	-	30	-	27	-

[IND2014/37] 10NLG	-	30	-	16	-	-	22
[IND2015/271]7A	-	-	20	-	-	-	23

$ Ct values were depicted below corresponding serotype detected by Seg-2 based qRT-PCR (Maan et al., 2016)

Table 4.9 Dominant serotype detected in mixed co-infection BTV isolates (n=26).

	First dominant serotype		Second dominant serotype	
Serotype	Number of BTV isolates (n=26)	Ct$ value	Number of BTV isolates(n=26)	Ct value$
BTV-1	-	-	-	-
BTV-2	3	11-26	7	19-35
BTV-4	1	13	4	20-33
BTV-5	8	10-20	-	-
BTV-9	1	25	2	22-27
BTV-10	2	15-19	2	32-33
BTV-12	11	6-30	11	21-32

$ Ct values were depicted below corresponding serotype detected by Seg-2 based qRT-PCR (Maan et al., 2016).

Table 4.10 Distribution of BTVserotypes in BTV isolates (n=62) during 2014-15 BT outbreaks in Southern India.

	Single serotype infection	Mixed serotype infection		
Serotype	Number of BTV isolates (n=36)	Number of BTV isolates(n=26)	Total BTV isolates* (n=62)	Percentage (%)
BTV-1	3	0	3	4.8
BTV-2	7	12	19	30.6
BTV-4	2	5	7	11.3
BTV-5	9	9	18	29.0
BTV-9	-	3	3	4.8
BTV-10	1	4	5	8.1
BTV-12	14	23	37	59.7

* [IND2015/] 07Karn from unvaccinated sheep in Karnataka state, 2015 BT outbreak could not be typed using serotype-specific TaqMan based qRT-PCR assay against BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 serotypes (Maan et al., 2016).

4.2.4.9 BTV serotype distribution

Of sixty two BTV isolates, BTV-1 was detected in three (4.8%), BTV-2 in nineteen (30.6%), BTV-4 in seven (11.3%), BTV-5 in eighteen (29%), BTV-9 in three (4.8%), BTV-10 in five (8.1%) and BTV-12 in thirty seven BTV isolates (59.7%) (Table 4.10). Thus, BTV-12 was found to be predominant serotype (59.7%) and a substantial proportion of 62 samples were infected with serotype BTV-2 (30.6%) and BTV-5 (29%) as well. BTV isolate [IND2015/] 07Karn, derived from an unvaccinated sheep during a BT outbreak in Karnataka state, 2015, could not be typed using serotype-specific TaqMan based qRT-PCR assay against BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 serotypes (Maan et al., 2016), suggesting that it belongs to a different serotype, or contains point mutations in the primer or probe footprints.

4.3 Discussion

BTV is endemic in India and serotypes BTV-1, BTV-2, BTV-4, BTV-9, BTV-10, BTV-12, BTV-16 and BTV-23 have been prevalent across the four states of Telangana, Andhra Pradesh, Karnataka and Tamil Nadu since 2007 (Reddy et al., 2016a). To date, 15 BTV-serotypes i.e. BTV-1, 2, 3, 4, 5, 6, 9, 10, 12, 16, 17, 18, 21, 23 and 24 have been isolated by the framework project of the ‘’All India Network Program on Bluetongue’’ (AINPBT, 2010, AINPBT, 2013) and by individual research laboratories (Krishnajyothi et al., 2016, Hemadri et al., 2016). During the last decade, 12 serotypes have been isolated namely BTV-1, BTV-2, BTV-3, BTV-4, BTV-5, BTV-9, BTV-10, BTV-12, BTV-16, BTV-21, BTV-23 and BTV-24, mainly from peninsular India (Maan et al., 2015c). Recently, Maan and colleagues have reported isolation of BTV-12 from Haryana State of Northern India (Maan et al., 2017). BTV-1 was also isolated from goats in Uttar Pradesh State, during 2008 (Biswas et al., 2010, Mondal et al., 2009), and from the Pithoragarh hills of Uttarakhand, 2009 (Mondal et al., 2013) and has previously been reported from the spleen of an aborted goat foetus in Gujarat, Western India, 2007 (Chauhan et al., 2014).

In the present study, seven different serotypes of BTV (BTV-1, BTV- 2, BTV- 4, BTV-5, BTV-9, BTV-10 and 12) were identified using serotype-specific real time RT-PCR targeting Seg-2 of BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 (Maan et al., 2016), in sixty two Indian isolates (n=62) from BTV outbreaks from Southern India during 2014-

2015. This study confirms the circulation of seven BTV serotypes, including BTV-1, BTV-2, BTV-4, BTV-5, BTV-9, BTV-10 & BTV-12, with predominance of BTV-12 (59.7%) followed by BTV-2 (30.6%) and BTV-5 (29%) in 62 BTV isolates, in four southern states of India during 2014 and 2015 BTV outbreaks.

In Tamil Nadu state, serotypes BTV-2, BTV-5, BTV-9, BTV-10, BTV-12 and BTV-16 (Kumar et al., 2016) were identified in unvaccinated animals (n=24) during 2014-15 BT outbreaks, whereas serotypes BTV-4, BTV-5 and BTV-12 were identified in flocks (n=14) vaccinated with the pentavalent inactivated vaccine. Overall, multiple co-circulating BTV serotypes BTV-2, BTV-4, BTV-5, BTV-9, BTV-10, BTV-12 and BTV-16 were identified during the 2014-15 BT outbreaks in Tamil Nadu. Reddy and colleagues have reported the isolation of serotypes BTV-1, BTV-2, BTV-16 and BTV-23 from Tamil Nadu between 2003-2011 (Reddy et al., 2016a).

The pentavalent inactivated BTV vaccine, currently being used in the Southern India, contains serotypes 1, 2, 10, 16 and 23. Interestingly, none of these serotypes were found in the vaccinated animals, including serotypes BTV-2, BTV-10, and BTV-16 that were detected in unvaccinated animals, suggesting protection against these serotypes by vaccination. For the first time, BTV-4, BTV-5 and BTV-9 serotype were identified during BT outbreaks in Tamil Nadu during 2014-15.

In Telangana state, serotypes BTV-2, BTV-4, BTV-5, BTV-9 and BTV-12 were identified in unvaccinated animals (n=11) during the 2014-15 BT outbreaks. Between 2002 - 2013, serotypes BTV-1, BTV-2, BTV-4, BTV-9, BTV-10, BTV-12, BTV-16, BTV-21 and BTV-24 had been isolated from Andhra Pradesh and Telangana States (Gollapalli et al., 2012, Krishnajyothi et al., 2016, Reddy et al., 2016b, Susmitha et al., 2012, Reddy et al., 2018).

In Karnataka state, serotypes BTV-4, BTV-5 and BTV-12 were identified in vaccinated animals (n=8) during the 2015 BT outbreaks. BTV-4 was also identified for the first time, in Karnataka state during the 2015 BT outbreaks. Recently BTV-2 and BTV-5 have also been isolated as a dual infection in sheep from Karnataka state, 2010 (Hemadri et al., 2016).

In Andhra Pradesh state, serotypes BTV-1, BTV-2, BTV-4 and BTV-12 were identified in unvaccinated animals (n=5) during the 2015 BT outbreaks. During 2002-2013, serotypes BTV-1, BTV-2, BTV-4, BTV-9, BTV-10, BTV-12, BTV-16, BTV-21 and BTV-24 had been isolated from Andhra Pradesh (Rao et al., 2016b).

The clinical presentation and disease severity during the 2014-15 BT outbreaks sampled in this study, varied from less to more severe, although the severity of disease did not differ substantially in vaccinated and non-vaccinated field cases.

One of the BTV field isolates, [IND2015/] 07Karn, from unvaccinated sheep during the 2015 BT outbreak in Karnataka state, could not be typed using the serotype-specific TaqMan based qRT-PCR assay against BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 serotypes (Maan et al., 2016). The failure of these assays to identify the virus type may be due to variations in the sequence of the VP2 gene of the strains currently circulating in I dia, leading to a mismatch in the footprints of the either the primers or probes used in the typing assays, compared to the reference strains of the relevant serotype. It is also possible, although less likely that this virus represents an entirely novel BTV serotype, or a perhaps novel topotypes of one of the known serotypes. However, it is likely that the typing of this isolate can be achieved by next generation sequencing of Seg-2.

Significant sequence variations occurs in Seg-2 of BTV strains from different geographic origins but of the same serotype (topotypes) (Maan et al., 2009). Therefore, close monitoring of circulating BTV strains and BTV typing by serotype-specific TaqMan based qRT-PCR assay is important to ensure that the assays used in regular virological surveillance and to assess the molecular remain epidemiology of BT in India, remain relevant to currently circulating strains in India.

BTV-5, which is a rare serotype in India, was recently reported by Hemadri and colleagues during the 2010-11 BT outbreaks in Karnataka state (Hemadri et al., 2016). A significant observation from the present study is the identification of BTV-5 in a substantial proportion of samples (29%) analysed as shown in Table 4.10.

In the present study, more than one BTV serotype, including BTV-1, BTV-2, BTV-4, BTV-5, BTV-9, BTV-10 & BTV-12 was detected in several Indian field isolates (n=63) from the four states in southern India during the 2014 and 2015 outbreaks (Table 4.4).

From a total of sixty two BTV isolates, BTV-12 was found to be the predominant serotype (59.7%) along with a substantial proportion of samples infected with BTV-2 (30.6%) and BTV-5 (29%) (Table 4.10). The BTV isolate [IND2015/]07Karn, derived from unvaccinated sheep in Karnataka state, during the BT outbreak in 2015, could not be typed using the serotype-specific TaqMan based qRT-PCR assays against BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 serotypes.

BTV-12 predominated (with 14 isolates) among the BTV isolates involving only one serotype (n=36), including, [IND2015/169]470; [IND2015/175]472; [IND2015/178]473; [IND2015/199]483; [IND2015/202]484; [IND2015/160]CS4; [IND2015/163]CS6; [IND2015/136]V41; CHE5; [IND2015/232]556 saliva; [IND2015/223]34 tissue; [IND2015/211]406; [IND2015/145]V44lamb and [IND2015/133]V severe from Tamil Nadu and Karnataka, 2014-15; followed by serotype BTV-5 (9 BTV isolates), [IND2015/172]471; [IND2015/184]475; [IND2015/193]478; [IND2015/166]CS9; [IND2015/205]486; [IND2015/142]V44; [IND2015/76]534; [IND2015/208]404; [IND2015/214]408 from Tamil Nadu and Karnataka,2014-15.

A significant finding of this study is the co-circulation of multiple BTV serotypes within endemic areas, which provides an opportunity for the occurrence of bluetongue outbreaks involving multiple serotypes (Brenner et al., 2010, Reddy et al., 2010, Reddy et al., 2016b). Several investigators have previously described BT outbreaks involving multiple co-circulating BTV serotypes. Brenner and colleagues reported the co-circulation of BTV-4, BTV-8, BTV-16 and BTV-24 in Israel, 2008 (Brenner et al., 2010). Reddy and colleagues reported the occurrence of BTV-9 and BTV-10 from Andhra Pradesh, in 2010 (Reddy et al., 2010). The involvement of multiple circulating serotypes in the same outbreak may be a common phenomenon in subtropical regions, where a congenial climate exists for the multiplication of *Culicoides* throughout the year (Rao et al., 2016a). Detection of multiple co-circulating BTV serotypes in India emphasizes the need for regular virological surveillance, particularly to ensure the relevance of the ongoing pentavalent inactivated vaccination programme.

A second finding of this study is the occurrence of mixed serotype infections within the same animal. Guimaraes and colleagues reported co-circulation of multiple serotypes involving BTV-1, BTV-4 and BTV-17 in an outbreak in Brazil, 2014, as well as triple or

dual co-infection with these serotypes within the same animal (Guimaraes et al., 2017). Recently dual infection of BTV-2 and BTV-5 has been reported in a sheep from Karnataka, in 2010 (Hemadri et al., 2016). Of the 62 BTV isolates typed here from the 2014-2015 BT outbreaks in Southern India, dual and triple BTV co-infections were detected in twenty two and four of the BTV isolates respectively. Dual infection was detected in four of the BTV isolates from fourteen vaccinated flocks in Tamil Nadu, during 2015. Dual infections by BTV-12 and BTV-4 were found in two BTV isolates: [IND2015/15]22 and [IND2015/196]480. Dual infections with serotype BTV-5 and BTV-12 were detected in BTV isolate [IND2015/187]476, while BTV-4 and BTV-9 were detected in [IND2015/190]477 (Table 4.4).

Dual and triple co-infections were detected in eleven and two respectively of the BTV isolates derived from twenty-four unvaccinated flocks in Tamil Nadu, during 2014-15. Dual infection with BTV-2 and BTV-12 was detected in six BTV isolates: CHE 2; CHE 12; CHE 14; [IND2015/70] 532; [IND2015/154] 493; [IND2015/157] 494; while BTV-12 and BTV-10 were detected in three isolates: [IND2014/34] BTV-1; [IND2015/60]100; [IND2015/181]474. BTV-5 and BTV-12 were detected in [IND2015/151]492, while BTV-5 and BTV-9 were detected in [IND2015/220]228.

Two BTV isolates have triple co-infections: [IND2014/35]: BTV-2, BTV-10, BTV-12 and [IND2015/148] 491; BTV-2, BTV-5 and BTV-12. Mixed infections involving serotypes BTV-2 and BTV-10 (serotypes which are present in the BTV pentavalent inactivated vaccine) were not found in any of the vaccinated animals from Tami Nadu, although dual or triple co-infections involving serotype BTV-2, BTV-10 were detected in samples from the unvaccinated animals analysed in the present study.

Out of a total of eight BTV isolates from vaccinated flocks in Karnataka, during 2015, three isolates showed dual co-infections with BTV-5 and BTV-12: [IND2015/118] V1; [IND2015/121] V2; [IND2015/124] V13 and a dual infection involving BTV-4 and BTV-12 was detected in isolate [IND2015/139] V42 (Table 4.8, 4.9).

Out of eleven BTV isolates derived from unvaccinated mixed flocks in Telangana during the 2014 BT outbreaks, two BTV isolates were identified as dual mixed infections: WGL4 (BTV-2, BTV-12); [IND2015/238]6PK (BTV-4, BTV-12) and three isolates as triple

mixed infections: [IND2014/36]3NLG (BTV-2,BTV-5,BTV-9) and [IND2014/37]10NLG (BTV-2, BTV-5, BTV-12). Similarly, one BTV isolate [IND2015/271]7A from Andhra Pradesh during 2015 was detected as a dual mixed infection with serotype BTV-4 and BTV-12.

Five BTV isolates from cattle were typed as: mono serotype infection with BTV-12 in [IND2015/232]556 saliva and dual infection with BTV-2, BTV-12 in [IND2015/70]532 from Thoothukudi, Tamil Nadu, 2015; mon infection BTV-5 in [IND2015/172]471; BTV-12 in [IND2015/175]472 and dual inection with BTV-12, BTV-10 in [IND2015/181]474 from Tirunehveli, Tamil Nadu, 2015.

The clinical presentation and disease severity during the BT outbreaks of 2014-15 examined in this study varied from acute to less severe or even asymptomatic. So far, no investigations have been carried out to study the role of mixed BTV infections in clinical presentation and severity of BT in India. Further extensive studies involving large numbers of clinical cases that are suspected BT, are needed to understand the severity and clinical presentation of dual or multiple mixed BT infections. Two different BTV strains could potentially cause dual infections of an individual *Culicoides* vector by consecutive bites on two different infected hosts, or by a single bite of a co-infected host.

Moreover, a mixed BTV infection may occur in the mammalian host due to infection via a bite from a *Culicoides* that has a dual or triple co-infection with different BTV types. Alternatively bites from different *Culicoides*, each infected with a different BTV serotype could also cause dual/multiple co-infections. The chance of mixed infections with two or more BTV serotypes is more likely in endemic areas where multiple serotypes co-circulate. These mixed infections may affect the evolution of BTV in terms of infectivity and pathogenicity of BTV. The co-circulation of multiple BTV serotypes and strains in BTV endemic areas and their concurrent infection in the same host and cells also provide multiple opportunities for reassortment and suggest circulation of a complex quasi-species (McColl and Gould, 1994). Co-infection with multiple BTV serotypes pose the risk of emergence of novel reassortant BTV strains that could have distinct properties in terms of infectivity and pathogenicity (Maan et al., 2012d, Maan et al., 2015b, Shafiq et al., 2013). Rao and colleagues have reported that a few BTV serotypes dominate outbreaks in India for 4-5 years, followed by the domination by other types (Rao et al., 2016b, Rao et al.,

2016a). Therefore, continuous surveillance for identification of serotypes and genotypes circulating in the endemic region is required for proper identification and characterization of BT infections. This would further help with the design and implementation of control measures for BT outbreaks and towards development of relevant and effective vaccines.

A pentavalent inactivated BTV vaccine containing serotypes 1, 2, 10, 16 and 23, has been developed depending on local BTV epidemiology and is currently being used in southern part of India. In the present study, BTV serotypes, including BTV-1, BTV-2, BTV-4, BTV-5, BTV-9, BTV-10 & BTV-12 were detected in field isolates (n=62) from four southern states of India during 2014 and 2015 outbreaks. The pentavalent vaccination was commercialized in 2015 but is not used on a large scale. In this study, nineteen BTV isolates were derived from vaccinated flocks and were typed to detect serotype. Serotypes BTV-4, BTV-5 and BTV-12 were identified in vaccinated flocks from Tamil Nadu (n=14) and Karnataka state, (n=8) during the 2015 BT outbreaks. A future study performed on a larger scale would be helpful to further strengthen these findings to show the efficacy of vaccination against different BTV serotypes. The currently circulating serotypes BTV-4, BTV-5, BTV-9 and BTV-12 are not present in the pentavalent inactivated BT vaccine. However, no final conclusions can be made because of small sample size from vaccinated animals in the present study.

Immunization with one serotype does not elicit strong cross-protection against other serotypes, making the design of effective vaccination strategies more difficult, particularly in endemic areas where multiple BTV serotypes circulate (Eschbaumer et al., 2009). In India, the control of BT is therefore difficult to achieve where multiple serotypes are circulating. However, vaccination programmes using inactivated vaccines containing currently circulating serotypes, or subunit vaccines targeting current serotypes, may help to reduce the clinical signs caused by the disease in India and form a component of future control strategies. Studies to characterise circulating strains and virological surveillance in India would improve our understanding of the serotypes / strains causing disease and help to support the design of appropriate control strategies / vaccination programmes.

Since 2010, BTV12 has been shown to circulate in Telangana and Andhra Pradesh (Rao et al., 2016a). However, four to five years were dominated by a single serotype: BTV-1 and BTV-10 in 2003, BTV-1 again from 2006-11, BTV-2 in 2007 -10, BTV-4 from 2007-13,

BTV-9 from 2002-07, BTV-16 from 2007-11 (Rao et al., 2016a). The results presented here confirm the persistant circulation of BTV serotypes, BTV-1, BTV-2, BTV-4, BTV-5, BTV-9, BTV-10 and BTV-12 in Southern India during the 2014-15 BT outbreaks. Further monitoring will be essential to detect and identify the serotypes that continue to circulate in India, especially, as the introduction of the pentavalent vaccine in endemic areas of Southern India may affect / alter the identity of the BTV strains that are dominant in the region.

4.4 Summary

- One hundred and thirty five (135) BTV isolations were made from clinical samples, including EDTA blood, tissues (spleen, lymph node) and saliva from suspected BTV outbreaks in Southern India during 2015. BTV isolations were also made from one / five EDTA blood samples from suspected outbreaks in Telangana / Tamil Nadu respectively during 2014.
- Sixty three (n=63) cell culture adapted Indian field strains of BTV from 2014-2015 outbreaks in Southern India were used for further typing.
- The results presented here confirm the current circulation of seven BTV serotypes, including BTV-1, BTV-2, BTV-4, BTV-5, BTV-9, BTV-10 & BTV-12, with predominance of BTV-12 followed by BTV-2 and BTV-5 in 62 samples typed, from four southern states of India during the 2014 and 2015 BTV outbreaks.
- BTV infection involving only one serotype was found in 36 out of 62 BTV isolates from Tamil Nadu and Karnataka, 2014-15. BTV-12 predominated (14 isolates), followed by BTV-5 (9 isolates).
- Dual and triple BTV co-infections were detected in twenty two and four BTV isolates, respectively. The dominant BTV serotype in the dual and triple mixed isolates based on lower Ct values was: BTV-12, Ct values of 6 to 30 in eleven BTV isolates.
- Out of sixty two BTV isolates, BTV-12 was found to be predominant serotype (59.7%) with a substantial proportion of samples infected with BTV-2 (30.6%) and BTV-5 (29%).
- BTV isolate [IND2015/] 07Karn, from unvaccinated sheep in Karnataka state, 2015 BT outbreak could not be typed using serotype-specific TaqMan based qRT-PCR

assay against BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 serotypes (Maan et al., 2016).

- Serotypes BTV-2, BTV-5, BTV-9, BTV-10, BTV-12 and BTV-16 (Kumar et al., 2016) were identified in unvaccinated animals (n=24) during the 2014-15 BT outbreaks in Tamil Nadu state. Serotypes BTV-4, BTV-5 and BTV-12 were identified in flocks (n=14) vaccinated with pentavalent inactivated vaccine.
- In Karnataka state, serotypes BTV-4, BTV-5 and BTV-12 were identified in vaccinated animals (n=8) during 2015 BT outbreaks, BTV-4 was identified for the first time in Karnataka state during the 2014-15 BT outbreaks.
- None of the serotypes present in the pentavalent vaccine were identified in vaccinated animals, suggesting that the vaccine was indeed effective against the homologous serotype field strains.
- A significant observation of the present study is the identification of BTV-5, a previously rare serotype in India, in a substantial proportion of samples (29%).

Chapter 5: Phylogenetic analysis of genome segment 2 from bluetongue virus strains isolated in India during 2014-2015

5.1 Introduction

Twenty four distinct serotypes of BTV have been recognised for several decades. In addition Toggenburg virus which was detected during routine surveillance of goats in Switzerland during 2008 has been identified as BTV-25 (Hofmann et al., 2008b, Chaignat et al., 2009). BTV-26 was detected in clinical samples from sheep in Kuwait (Maan et al., 2011b) and BTV-27 was detected in asymptomatic goats on the island of Corsica, France (Zientara et al., 2014, Schulz et al., 2016, Savini et al., 2017a, Jenckel et al., 2015). Two further putative types have also been detected: BTV-28 in a Sheep pox vaccine preparation from the Middle East (Bumbarov et al., 2016), and BTV-29 in an Alpaca from South Africa (Wright, 2014, Posada, 2008). Recent studies of BTV strains circulating in Mongolia suggest the existence of several more novel types namely BTV-30, 31 and 32 (EPIZONE, 2017).

Serological evidence suggests that 22 BTV serotypes exist in India and at least 15 BTV serotypes (BTV-1, 2, 3, 4, 5, 8, 9, 10, 12, 16, 17, 18, 21, 23 and 24) have been isolated over the last five decades including BTV-5 & BTV-24 which were recently added to this list in 2016 (Maan et al., 2015c). Since 2001, 12 serotypes have been isolated, namely BTV-1, BTV-2, BTV-3, BTV-4, BTV-5, BTV-9, BTV-10, BTV-12, BTV-16, BTV-21, BTV-23 and BTV-24, mainly from Southern India(Bommineni et al., 2008, Chand et al., 2016, Desai et al., 2009, Hemadri et al., 2016, Krishnajyothi et al., 2016, Kumar et al., 2016, Maan et al., 2012a, Maan et al., 2012b, Maan et al., 2012c, Maan et al., 2012d, Maan et al., 2012e, Maan et al., 2015b, Maan et al., 2015c, Maan et al., 2017, Minakshi et al., 2012, Mondal et al., 2013, Prasad et al., 1992, Prasad et al., 1994, Pudupakam et al., 2017, Rao et al., 2012b, Rao et al., 2012c, Rao et al., 2015, Rao et al., 2016a, Rao et al., 2016b, Reddy et al., 2016a, Reddy et al., 2016b, Sreenivasulu et al., 2004, Susmitha et al., 2012)

BTV has seven structural proteins (VP1 to VP7) and at least 5 non-structural proteins (NS1 to NS5) (Chapter 1, Section 1.5). Genome segment 2 has the highest level of sequence variation, showing 29% to 59% nucleotide variation between different BTV 24 serotypes (Maan et al., 2004, Maan et al., 2007a, Batten et al., 2008). Genome segment 2 encodes outer-capsid protein VP2, the most variable of the BTV proteins, which determines BTV serotype based on the specificity of its interactions with the neutralizing antibodies generated during infection of the mammalian host (Mertens et al., 1989b, Inumaru and Roy, 1987, Cowley and Gorman, 1989, Gould and Eaton, 1990, Huismans and Van Dijk, 1990). The second most variable BTV protein, VP5 (encoded by genome segment 6) can also make a contribution to determination of the virus serotype, although it is less significant in this role than VP2 (Maan et al., 2008, Singh et al., 2004, Cowley and Gorman, 1989).

Phylogenetic analyses of Seg-2 from multiple BTV isolates have shown that Seg-2 divides into 27 distinct groups, precisely representing the different virus serotypes, with <33% variation in nucleotide sequence within each serotype and 29–59% nucleotide sequence variation between types(Maan et al., 2007a, Maan et al., 2010, Mertens et al., 2007a, Mertens et al., 2007b, Schulz et al., 2016). Sequencing studies also indicate that BTV strains in different geographic regions have evolved independently over an extensive period of time. This geographical separation has resulted in the accumulation of unique point mutations, some of which may enhance the transmission and survival of the viruses in their respective local ecosystems and vector populations (Maan et al., 2009). Genetic heterogeneity of BTV strains as a result of genetic drift, genetic shift and selection have gradually led to the appearance of distinctive regional variants, which can be divided into eastern and western topotypes for all segments of BTV(Maan et al., 2009).

The eastern BTV group generally constitutes viruses isolated from countries in Southern and South-Eastern Asia, India, China and Australia, while viruses that are mostly isolated from countries in Africa, America, Europe and Middle East, are included in the western BTV group(Maan et al., 2007a, Maan et al., 2008, Maan et al., 2009, Mertens et al., 2007a).As the least conserved of the BTV genome segments, the analysis of sequence variations in Seg-2 provide a basis for molecular evolutionary and phylogenetic studies, to identify the BTV serotype and the geographical origins of the Seg-2 lineage, and for the

design of RT-PCR assays to identify both virus-serotype and topotype (Mertens et al., 2007a).

The objectives for this chapter are:

- To generate full-length Seg-2 sequence data for BTV strains isolated during BT suspected outbreaks in Southern India during 2014-2015.
- To confirm the serotype of Indian field isolates, as previously identified by serotype-specific real-time RT-PCR assays (Maan et al., 2016) as described in the chapter 4, by comparing Seg-2 sequences with those of reference strains of the 27 BTV serotypes.
- Phylogenetic analyses of Seg-2 from different Indian BTV strains, to determine their geographic origins, movements of different virus lineages and evolutionary relationships with other isolates from different parts of the India and world.

5.2 Results

5.2.1 Phylogenetic characteristics of Seg-2/VP2

Genome segment 2 was sequenced from forty six (n=46) Indian BTV strains, isolated from BT outbreaks in Southern India during 2014-2015 (Table 5.1). Serotype-specific TaqMan based qRT-PCR assay on these Indian field isolates of BTV against BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 serotypes (Maan et al., 2016) identified BTV serotypes, including BTV-2, BTV-4, BTV-5, BTV-9, BTV-10 & BTV-12 (Chapter 4, Section 4.2.4 and Table 5.1). Seg-2 sequences from these forty six Indian BTV strains were initially compared with Seg-2 sequences of reference strains of BTV serotypes BTV-1 to BTV-27 available from GenBank (Figure 5.1).

Data for reference strains retrieved from Genbank are abbreviated with a serotype followed by a three-letter international country code (https://countrycode.org/) depicting geographical origin of each isolate, year of sample collection where accessible and Genbank accession number at the end of each sequence (serotype/three-letter country code/year of sample collection_Genbank accession number) e.g. 1/AUS/1979_JN881986 stands for serotype BTV-1 from Australia collected in 1979. Similarly, RSArrrr stands for a reference strain (rrrr) supplied to the Orbivirus Reference Collection by Onderstepoort Laboratory in the Republic of South Africa. The Indian strains sequenced in this study

were indicated as country code (IND) followed by year of sample collection, field sample ID number and date of sampling e.g. IND2015/40_2015-12-22 stands for an Indian isolate, with original sample ID number 40, dated 22 December 2015.

.

Table 5.1 Details of the BTV field strains sequenced in this study

Virus collection number*	Sequence ID	Collection date (day/month/year)	Place of origin (District)	State	Latitude	Longitude	Host species	Source	Vaccination status of the animal sampled	Passage history ($KC^{£}$/$BHK^{\$}$ or $BSR^{\&}$)	Serotype€
[IND2015/15]22	IND2015/22_2015-12-18	18/12/2015	Thoothukudi	TN	8°56'48.63"N	77°46'25.61"E	Sheep	Blood	V	KC1/BSR6	BTV-12
[IND2015/18] 40	IND2015/40_2015-12-22	22/12/2015	Tuticorin	TN	8°56'48.63"N	77°46'25.61"E	Goat	Blood	UV	KC1/BHK4	BTV-1
[IND2015/148]491	IND2015/491_2015-12-25	25/12/2015	Kanchipuram	TN	12°50'3.02"N	79°42'13.10"E	Sheep	Blood	UV	KC1/BHK5	BTV-5
[IND2015/151]492	IND2015/492_2015-12-25	25/12/2015	Kanchipuram	TN	12°50'3.02"N	79°42'13.10"E	Sheep	Blood	UV	KC1/BHK6	BTV-5
[IND2015/157]494	IND2015/494_2015-12-25	25/12/2015	Kanchipuram	TN	12°50'3.02"N	79°42'13.10"E	Sheep	Blood	UV	KC1/BHK6	BTV-12
[IND2015/208]404	IND2015/404_2015-12-25	25/12/2015	Erode	TN	11°20'27.73"N	77°43'1.79"E	Sheep	Blood	V	KC1/BHK4	BTV-5
[IND2015/211]406	IND2015/406_2015-12-25	25/12/2015	Erode	TN	11°20'27.73"N	77°43'1.79"E	Sheep	Blood	UV	KC1/BHK5	BTV-12
[IND2015/214]408	IND2015/408_2015-12-25	25/12/2015	Erode	TN	11°20'27.73"N	77°43'1.79"E	Sheep	Blood	UV	KC1/BHK4	BTV-5
[IND2015/169]470	IND2015/470_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Sheep	Blood	V	KC1/BHK4	BTV-12
[IND2015/172]471	IND2015/471_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Cow	Blood	UV	KC1/BHK5	BTV-5
[IND2015/175]472	IND2015/472_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Cow	Blood	UV	KC1/BHK4	BTV-12
[IND2015/178]473	IND2015/473_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Sheep	Blood	V	KC1/BHK4	BTV-12

[IND2015/181]474	IND2015/474_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Cow	Blood	UV	KC1/BHK4	BTV-12
[IND2015/184]475	IND2015/475_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Sheep	Blood	V	KC1/BHK4	BTV-5
[IND2015/187]476	IND2015/476_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Sheep	Blood	V	KC1/BHK5	BTV-5
[IND2015/190]477	IND2015/477_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Sheep	Blood	V	KC1/BHK5	BTV-9
[IND2015/193]478	IND2015/478_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Goat	Blood	V	KC1/BHK4	BTV-5
[IND2015/196]480	IND2015/480_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Sheep	Blood	V	KC1/BHK5	BTV-12
[IND2015/199]483	IND2015/483_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Sheep	Blood	V	KC1/BHK4	BTV-12
[IND2015/202]484	IND2015/484_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Goat	Blood	V	KC1/BHK4	BTV-12
[IND2015/205]486	IND2015/486_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Sheep	Blood	V	KC1/BHK4	BTV-5
[IND2015/160]CS4	IND2015/CS4_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Sheep	Blood	V	KC1/BHK4	BTV-12
[IND2015/163]CS6	IND2015/CS6_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Sheep	Blood	V	KC1/BHK4	BTV-12
[IND2015/166]CS9	IND2015/CS9_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Sheep	Blood	V	KC1/BHK5	BTV-5
[IND2015/76]534	IND2015/534_2015-12-25	25/12/2015	Thoothukudi	TN	8°45'51.00"N	78° 8'5.41"E	Goat	Blood	UV	KC1/BHK4	BTV-5
[IND2015/2017]547 tissue	IND2015/547_2015-12-25	25/12/2015	Thoothukudi	TN	8°45'51.00"N	78° 8'5.41"E	Sheep	Spleen	UV	KC1/BHK4	BTV-5
[IND2015/220]228 tissue	IND2015/228_2015-12-25	25/12/2015	Tirunehveli	TN	8°42'33.27"N	77°44'21.85"E	Sheep	Spleen	UV	KC1/BHK4	BTV-5
[IND2015/223]34 tissue	IND2015/34_tissue_2015-12-25	25/12/2015	Thoothukudi	TN	8°45'51.00"N	78° 8'5.41"E	Sheep	Spleen	UV	KC1/BHK4	BTV-12
[IND2015/232]556 saliva	IND2015/556_saliva_2015-12-25	25/12/2015	Thoothukudi	TN	8°45'51.00"N	78° 8'5.41"E	Cattle	Saliva	UV	KC1/BHK5	BTV-12

[IND2014/34]BTV-1	IND2014/BTV1_2014-12-06	06/12/2014	Thanjavur	TN	10°47'13.20"N	79° 8'16.18"E	Sheep	Blood	UV	KC1/BHK4	BTV-10
[IND2014/35]BTV-2	IND2014/BTV2_2014-12-06	06/12/2014	Thanjavur	TN	10°47'13.20"N	79° 8'16.18"E	Sheep	Blood	UV	KC1/BHK4	BTV-10
[IND2015/118]V1	IND2015/V1_2015-11-26	26/11/2015	Chikballapur	K	13°11'35.03"N	78° 1'14.86"E	Sheep	Blood	V	KC1/BHK5	BTV-5
[IND2015/121]V2	IND2015/V2_2015-11-26	26/11/2015	Chikballapur	K	13°11'35.03"N	78° 1'14.86"E	Sheep	Blood	V	KC1/BHK5	BTV-5
[IND2015/136]V41	IND2015/V41_2015-11-26	26/11/2015	Chikballapur	K	13°29'43.12"N	77°58'4.05"E	Sheep	Blood	V	KC1/BHK5	BTV-12
[IND2015/142]V44	IND2015/V44_2015-11-26	26/11/2015	Chikballapur	K	13°29'43.12"N	77°58'4.05"E	Sheep	Blood	V	KC1/BHK4	BTV-5
[IND2015/145]V44 lamb	IND2015/V44_lamb_2015-11-26	26/11/2015	Chikballapur	K	13°29'43.12"N	77°58'4.05"E	Sheep	Blood	V	KC1/BHK4	BTV-12
[IND2015/133]V Severe	IND2015/V44Severe_2015-11-26	26/11/2015	Chikballapur	K	13° 2'21.52"N	77°23'33.83"E	Sheep	Blood	V	KC1/BHK4	BTV-12
IND2015/241]K3	IND2015/K3_2015-09-10	10/09/2015	Karimnagar	T	18°19'20.63"N	80°14'29.70"E	Sheep	Blood	UV	KC1/BHK4	BTV-2
[IND2015/244]K4	IND2015/K4_2015-09-10	10/09/2015	Karimnagar	T	18°26'22.78"N	79° 7'44.26"E	Sheep	Blood	UV	KC1/BHK4	BTV-4
[IND2015/250]K9	IND2015/K9_2015-09-10	10/09/2015	Karimnagar	T	18°32'54.90"N	78°17'29.98"E	Sheep	Blood	UV	KC1/BHK6	BTV-2
[IND2015/]K13	IND2015/K13_2015-09-12	12/09/2015	Karimnagar	T	18°41'50.52"N	79° 4'26.61"E	Sheep	Blood	UV	KC1/BHK6	BTV-1
[IND2015/256]K14	IND2015/K14_2015-09-12	12/09/2015	Karimnagar	T	18°41'50.52"N	79° 4'26.61"E	Sheep	Blood	UV	KC1/BHK4	BTV-2
[IND2015/238]6PK	IND2015/6PK_2015-09-07	07/09/2015	Warangal	T	18°11'51.79"N	79°42'9.80"E	Sheep	Blood	UV	KC1/BHK4	BTV-4
[IND2014/37] 10NLG	IND2014/10NLG_2014-10-03	03/10/2014	Nalgonda	T	16°56'34.67"N	79°12'55.83"E	Sheep	Blood	UV	KC1/BHK4	BTV-5
[IND2015/271]7A	IND2015/7A_2015-11-08	08/11/2015	Anantpur	AP	14°41'37.97"N	77°38'3.41"E	Sheep	Blood	UV	KC1/BHK4	BTV-1
[IND2015/]14A	IND2015/14A_2015-11-08	08/11/2015	Anantpur	AP	14°41'37.97"N	77°38'3.41"E	Goat	Blood	UV	KC1/BHK4	BTV-2

[*] Virus collection number: [Country code, year / sample number]

[£] Number of passages in KC cell line

[$] Number of passages in BHK-21 cell line.

[&] Number of passages in BSR cell line.

[€] Based on Seg-2 phylogenetic analysis.

Abbreviations: TN, Tamil Nadu; K, Karnataka; T, Telangana; AP, Andhra Pradesh; V, Vaccinated; UV, Unvaccinated.

5.2.1.1 Seg-2 based phylogenetic analysis for confirmation of serotypes detected by real-time RT-PCR.

Phylogenetic analysis of the full open reading frame (ORF) of Seg-2 showed that the forty six Indian isolates were grouped into five Seg-2 nucleotypes A, E, G, H and I (Maan et al., 2007a) along with their corresponding reference strains confirming the serotypes (Figure 5.1). The majority of isolates were confirmed as BTV-5 and BTV-12; seventeen isolates each, while the remaining (twelve isolates) were identified as BTV-1 (three isolates), BTV-2 (four isolates), BTV-4 (two isolates), BTV-9 (one isolate) and BTV-10 (two isolates). However for isolates [IND2015/151]492, [IND2015/2017]547 tissue, [IND2015/] K13, [IND2015/271]7A and [IND2015/18] 40, the serotype detected by qPCR was different of that detected by NGS.

In addition, the full-length sequence of the ORF of Seg-2 from Indian BTV isolates (n=46), generated in this study were compared with genome Seg-2 sequences of Indian BTV field strains of eleven serotypes namely BTV-1, 2, 3, 4, 5, 9, 10, 12, 16, 21 and 23 available from GenBank (n=46) (accessed on 01.12.2017).This analyses showed that Indian BTV are grouped into eight “Seg-2 nucleotypes” A, B, C, D, E, G, H and I (Maan et al., 2007a) (Figure 5.2).

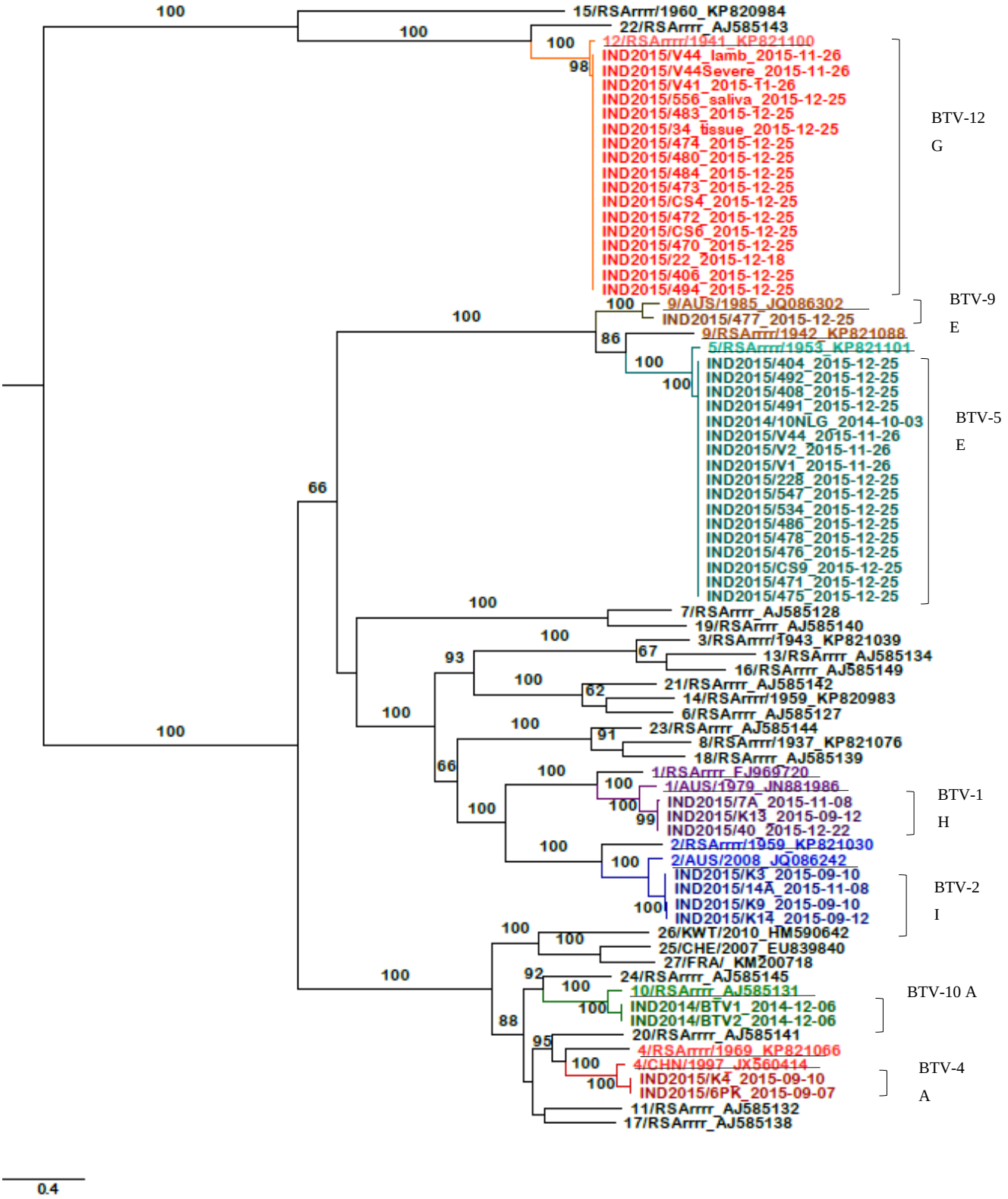


Figure 5.1 Unrooted Maximum Likelihood (ML) phylogenetic tree of the nucleotide sequences of Seg-2 coding region of Indian BTV isolates (n=46) and BTV reference strains of 27 serotypes isolated worldwide.

The Tree was constructed as described in Chapter 2, Section 2.23. Indian strains are indicated in different colours for each serotype in dark shade, whereas the corresponding reference serotypes are shown light coloured and underlined. Scale bar, 0.4 nucleotide substitutions per site.

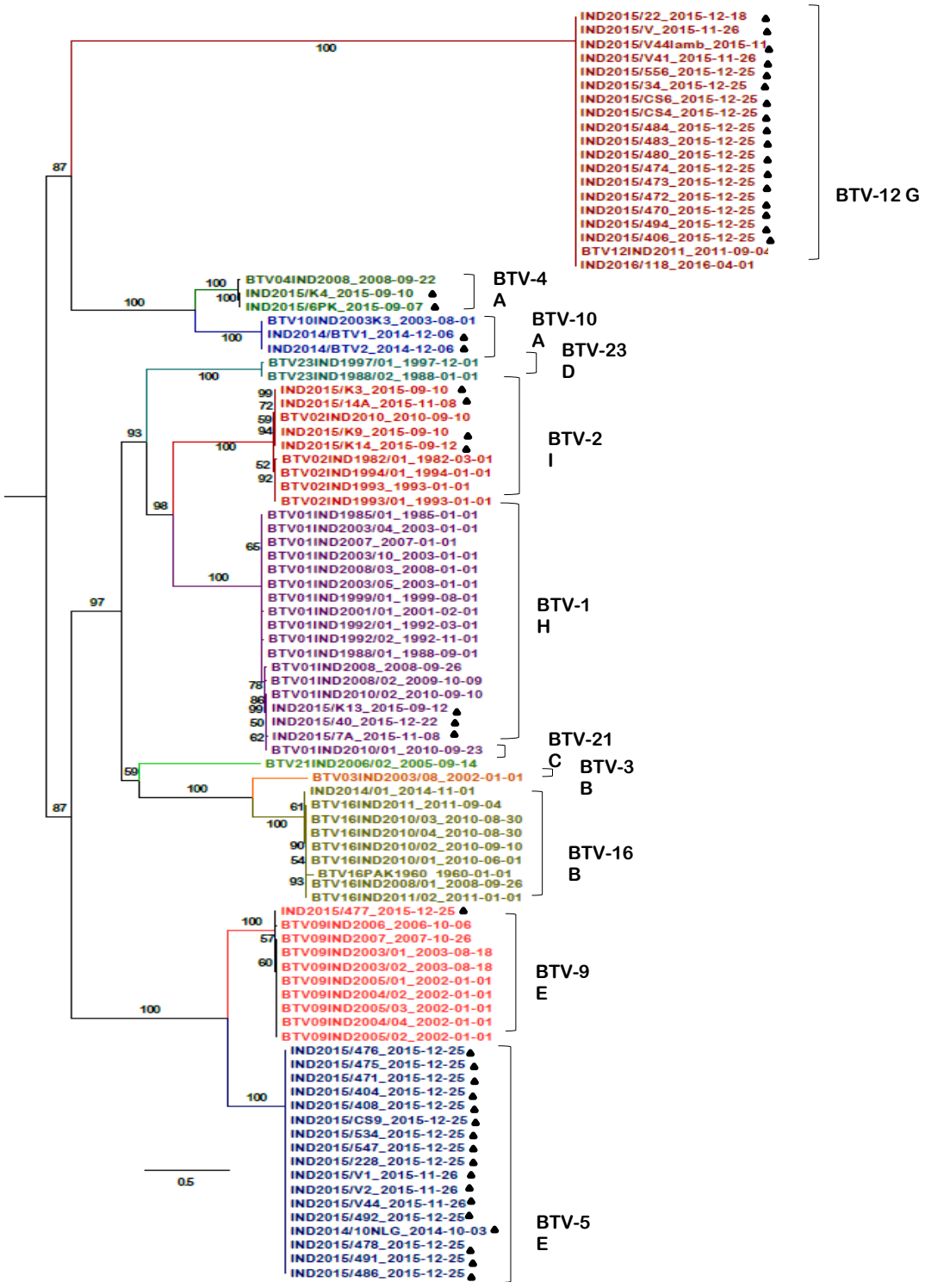


Figure 5.2 ML unrooted phylogenetic tree of Seg-2 coding sequences of Indian BTV isolates (n=46) during 2014-2015 (Table 5.1) and other Indian BTV field strains (n=46) available in GenBank (n=46).

The identified serotypes of Indian strains sequenced and analysed in this study were marked with bold triangle. The eight Seg-2 nucleotypes (A-E,G,H & I) were indicated (Maan et al., 2007a) with identified eleven serotypes (BTV-1, 2, 3, 4, 5, 9, 10, 12, 16, 21 and 23). Scale bar, 0.4 nucleotide substitutions per site

5.2.2 Phylogenetic analysis of genome segment 2 of BTV-1 within the Indian context

BTV-1: Three Indian isolates of BTV-1 (IND2015/K13_2015-09-12, IND2015/40_2015-12-22 and IND2015/7A_2015-11-08), were collected from Telangana, Tamil Nadu and Andhra Pradesh States respectively as part of this study during the 2015 BT outbreak. The length of Seg-2 obtained by NGS, ORF and size of VP2 protein were given in Table 5.2.

The phylogenetic analysis of the Seg-2 of these isolates showed that, they grouped with the BTV-1 reference strains (1/RSArrrr_FJ969720, 1/AUS/1979_JN881986) within the 'Seg-2-based nucleotype H' confirming their serotype (Figure 5.1). These three Indian isolates showed average nucleotide and amino acids (nt/aa) similarity of 74.69/83.71% with 1/RSArrrr_FJ969720, confirming as serotype BTV-1 (Appendix 3). These isolates also clustered more closely with 1/AUS/1979_JN881986 showing average nt/aa similarities of 87.25/94.24%, thus belonging to eastern topotype (Figure 5.1).

Comparison of the ORF nucleotide-sequences of Seg-2 from BTV-1 isolates from Telangana (IND2015/K13_2015-09-12) and Tamil Nadu (IND2015/40_2015-12-22) showed that they are identical, with 99.17/ 99.58 % nt/aa similarity to the BTV-1 isolate from Andhra Pradesh (IND2015/7A_2015-11-08). These high levels of sequence identity indicate that all three isolates represent extensions of a single outbreak. Comparisons of Seg-2/VP2 of these three BTV-1 isolates, with earlier BTV-1 isolates from Southern India during 2007-2008 (1/IND/2007_KF563933, 1/IND/2008_JX399149, 1/IND/2008_KF664124) showed nt and aa similarity of 98.72%-99.34% and 98.86%-99.9% respectively, while nt/aa similarities of 99.31%-99.72 % / 99.58-100 % were observed with BTV-1 strains from Southern India in 2010 (1/IND/2010_KP339145, 1/IND/2010_KP339135), indicating a recent common ancestral relationship and continuing circulation of the same BTV-1 lineage over time.

5.2.3 Phylogenetic analysis of BTV-1 Seg-2 in a global context

Phylogenetic analysis of the full coding region of Seg-2, from global BTV-1 isolates, clearly segregated them into eastern and western topotypes that reflect the geographical origin of the isolate. Furthermore, two major subclades (supported by high bootstrap value 96% and 71%) were clearly evident within the western topotype of BTV-1 (Figure 5.3). One subclade or lineage comprised strains from South Africa, Sudan, Italy, Spain, Nigeria, Cameroon, Libya, Morocco, Tunisia, Algeria, Gibraltar, Portugal, France-Corsica, (71 % bootstrap support) showing nt / aa identity of 74.53% to 75.36 % and 80.96% to 83.25 % with the Indian strains sequenced in this study.

The BTV-1 isolate from the United States (1/USA/2010_KX164020), along with isolates from French-Guyana (1/GUF/2011_JQ436736, 1/GUF/2011_KY049844 and 1/GUF/2015_KY049854) form a distinct lineage (96 % bootstrap support) that shares overall nt and aa identity of 73.56% to 73.80 % and 80.855% to 81.27 % respectively with the Indian isolates sequenced in this study (IND2015/K13_2015-09-12, IND2015/40_2015-12-22 and IND2015/7A_2015-11-08).

Four subgroups of Seg-2 were evident within the eastern topotype of BTV-1 (Figure 5.3). One subgroup comprised strains from Australia (supported by 91 % bootstrap value), sharing overall nt and aa identity of 86.66% to 87.35 % and 93.13% to 94.07 % respectively with the Indian BTV-1 strains sequenced in this study (IND2015/K13_2015-09-12, IND2015/40_2015-12-22 and IND2015/7A_2015-11-08). The Chinese BTV-1 isolate (1/CHN/1997_JN848760) along with the BTV-1 isolates from Malaysia and South Korea (1/MYS/1987_AJ585116, 1/KOR/2010_KC153300) formed another subgroup (97 % bootstrap support) that shared overall nt and aa identity of 90.515 to 91.09 % and 95.94% to 97.29 % respectively, with the Indian BTV-1 isolates (IND2015/K13_2015-09-12, IND2015/40_2015-12-22 and IND2015/7A_2015-11-08).

The three Indian BTV-1 isolates from the 2015 outbreak (IND2015/K13_2015-09-12, IND2015/40_2015-12-22 and IND2015/7A_2015-11-08) grouped with other Indian BTV-1 strains (supported by bootstrap values of 99%), sharing nt and aa identities of 96.29% to 99.72% and 96.98% to 100 % respectively, collectively forming an eastern sublineage of Indian BTV-1 strains.

A fourth subgroup was identified within the eastern BTV-1 Seg-2 topotype, comprising isolates from the Mediterranean region, Greece (1/GRC/2001_AJ585121, 1/GRC/2001_KP821005, 1/GRC/2001_KP821006, 1/GRC/2001_KP821007 and 1/GRC/2001_JN635334). The Indian BTV-1 isolates grouped closely with these Greek BTV-1 strains, showing overall nt and aa identity levels of 94.32% to 94.70 % and 96.25% to 97.4 %.

The average nt /aa identity levels were 94.75% / 96.27 % within the eastern group and 95.91% / 97.83 % within the western group.

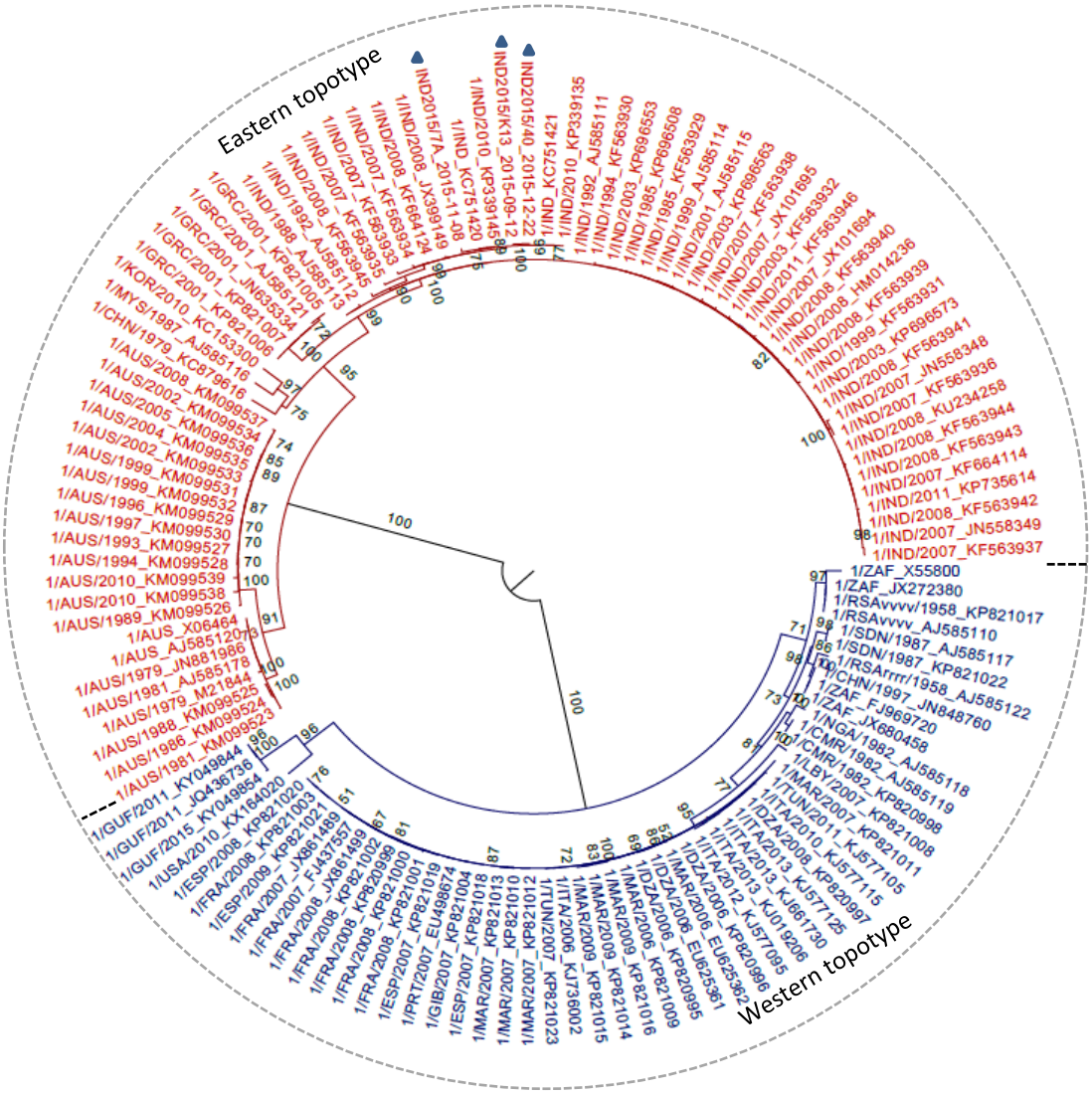


Figure 5.3 ML unrooted phylogenetic tree of coding region Seg-2/VP2 gene of Indian BTV-1 isolates (n=3) during 2015 (Table 5.1) with reference and field strains of BTV-1 from around the world.

The trees were represented as circular (top) and rectangular (bottom) tree. The Indian BTV-1 strains analysed in this study are shown coloured maroon, and marked by a bold triangle. Scale bar, 0.2 nucleotide substitutions per site.

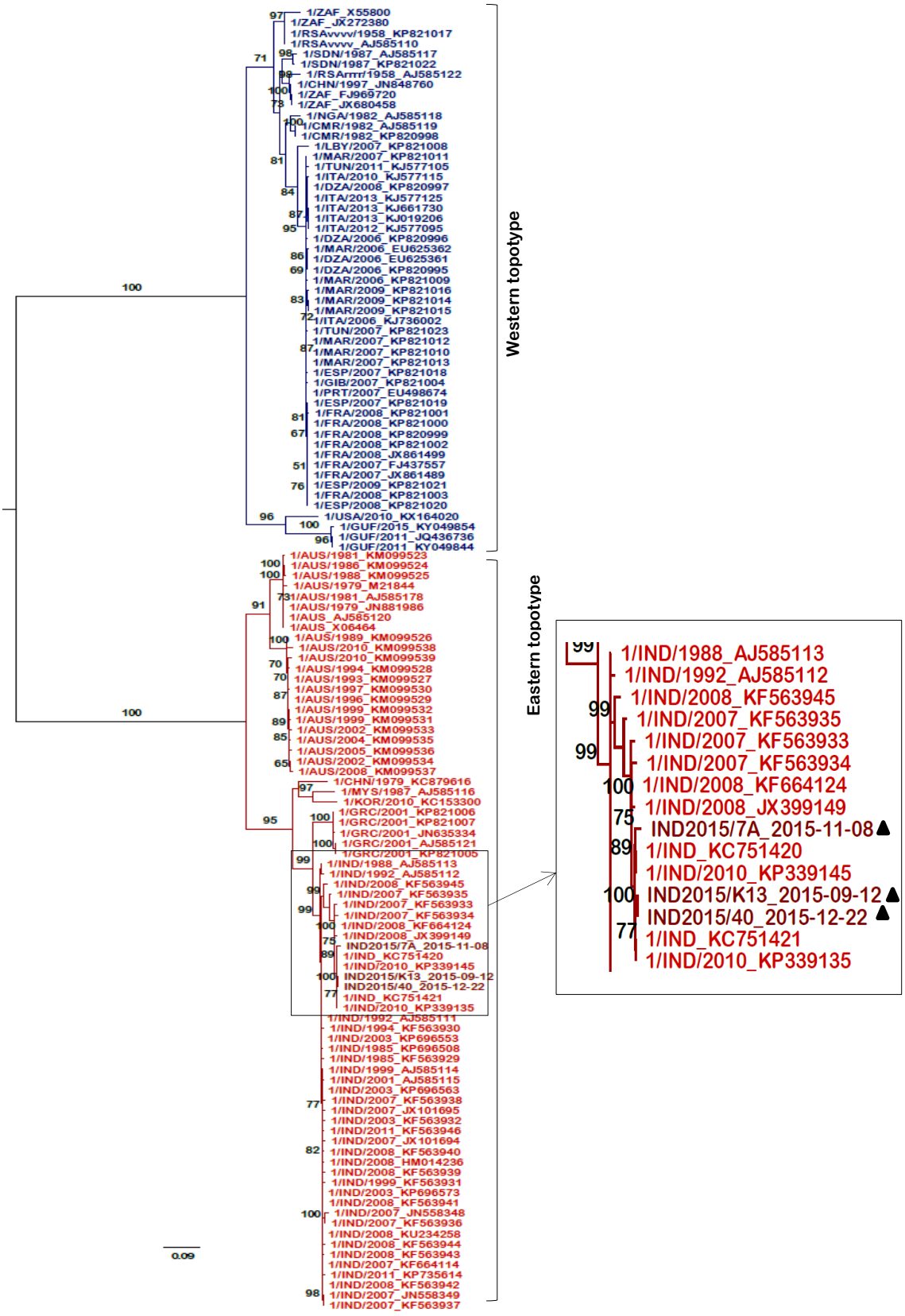


Table 5.2 Genetic characterization of Seg-2 from Indian BTV isolates sequenced.

Virus collection number	Coding region length (bp)	*Total length of Seg-2 (bp)	Size of VP2 (Da)	5'terminal sequences of the positive strand	3' terminal sequences of the positive strand
BTV- 1					
[IND2015/271]7A	2887	2934	961	ATAGTGTCGCGATG	TGACCGCGGGTCCGCGCGCTATTCCACTTAC
[IND2015/]K13	2886	2934	961	ATAGTGTCGCGATG	TGACCGCGGGTCCGCGCGCTATTCCACTTAC
[IND2015/18] 40	2886	2936	961	AAATAGTGTCGCGATG	TGACCGCGGGTCCGCGCGCTATTCCACTTAC
BTV- 2					
[IND2015/250]K9	2898	2938	962	AACATGCTCGCGATG	TGACTGCAGGCCCGCGATCTGTTACACTTAC
[IND2015/256]K14	2898	2939	962	AAACATGCTCGCGATG	TGACTGCAGGCCCGCGATCTGTTACACTTAC
IND2015/241]K3	2889	2938	961	AACAGTCTCGCGATG	TGACTGCAGGCCCGCGATCTGTTACACTTAC
[IND2015/]14A	2898	2937	962	ACATGCTCGCGATG	TGACTGCAGGCCCGCGATCTGTTACACTTAC
BTV- 4					
[IND2015/244]K4	2871	2926	955	GTTAAAAGAGTGTTCCATCATG	TGACACGGGTCGGTGGCCTC-TTACACTTAC
[IND2015/238] 6PK	2871	2926	956	GTTAAAAGAGTGTTCCATCATG	TGACACGGGTCGGTGGCCTC-TTACACTTAC
BTV- 5					
[IND2015/142]V44	2869	2908	955	GCTTCTCAGGATG	TGACATGAGACTCCTGGAGC-TTAC
[IND2015/121]V2	2868	2914	955	AGCTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTACACTTA
[IND2015/118]V1	2869	2910	954	AGCTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTACA
[IND2015/220]228	2868	2915	954	AAGCTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTACACTTA
[IND2015/2017]547	2868	2915	955	AAGCTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTACACTTA
[IND2015/76]534	2868	2911	954	AAAGCTTCTCAGGATG	TGACATGAGACTCCTGGAGCTAC
[IND2015/205]486	2868	2910	955	AGCTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTAC
[IND2015/193]478	2868	2910	955	AAAGCTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTA
[IND2015/187]476	2868	2912	955	CTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTACACTTA
[IND2015/172]471	2868	2910	955	CTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTACACT
[IND2015/214]408	2868	2910	955	AAAGCTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTA
[IND2015/151]492	2868	2914	955	AAAGCTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTACACT
[IND2015/148]491	2868	2913	955	CTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTACACTTAC
[IND2015/166]CS9	2868	2914	955	AAAGCTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTACACT

(IND2014/37) 10NLG	2868	2914	955	GTTAAAAGCTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTA
[IND2015/184]475	2868	2916	955	AAAGCTTCTCAGGATG	TGACATGAGACTCCTGGAGCTTACACTTA
[IND2015/208]404	2868	2916	955	GTTAAAAGCTTCTCAGGATG	TGACATGAGACTCCTGGAGC-TTACA
BTV- 9					
[IND2015/190]477	2868	2911	955	AAAGTTATCTAGGATG	TGACATGGGACTCCTAGAGCTTAC
BTV- 10					
[IND2014/34]BTV1	2871	2917	955	GTGTTCTACCATG	TGACATGGACCGGTAGCCTCTTACACTTAC
[IND2014/35]BTV2	2871	2917	955	GTGTTCTACCATG	TGACATGGACCGGTAGCCTCTTACACTTAC
BTV- 12					
[IND2015/133]VSevere	2853	2900	949	AAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/145]V44L	2853	2901	949	AAAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/136]V41	2853	2900	950	AAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/232]556	2853	2904	949	GTTAAAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/223]34	2853	2904	949	GTTAAAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/163]CS6	2853	2900	950	AAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/160]CS4	2853	2901	950	AAAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/202]484	2853	2901	950	AAAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/199]483	2853	2897	950	AAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACT
[IND2015/196]480	2853	2899	950	AAAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCAGCTTCAACTT
[IND2015/181]474	2853	2901	950	AAAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/178]473	2853	2899	950	AAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/175]472	2853	2901	950	AAAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/169]470	2853	2898	950	AGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/211]406	2853	2903	950	TTAAAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/15]22	2853	2901	950	AAAAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC
[IND2015/157]494	2853	2898	950	AAGTTGCGAGGATG	TGACAAGGCTCCCTCGCGGCTTCAACTTAC

* Total length of Seg-2 obtained by NGS.

5.2.4 Phylogenetic analysis of Seg-2 from Indian BTV-2 isolates

Phylogenetic analysis of Seg-2 revealed that the Indian BTV-2 strains isolated during the 2015 BT outbreaks from Telangana [IND2015/K3_2015-09-10, IND2015/K9_2015-09-10, and IND2015/K14_2015-09-12] and Andhra Pradesh [IND2015/14A_2015-11-08] grouped with the reference strains of BTV-2 (2/RSArrrr/1959_KP821030 and 2/AUS/2008_JQ086242) within nucleotype I (Figure 5.1). The length of Seg-2 obtained by NGS, ORF and size of VP2 protein were given in Table 5.2. These four Indian isolates showed average nt and aa identity of 71.56% to 80.47% with 2/RSArrrr_KP821030, confirming the results of serotype-specific real time RT-PCR targeting Seg-2, which identified these strains as BTV-2. They clustered more closely with 2/AUS/2008_JQ086242 showing average nucleotide and amino acid (nt/aa) identities of 88.85/95.01 %, thus belonging to eastern topotype of BTV-2 (Figure 5.1). They also show nt/aa identities of 99.07% to 100% and 99.48% to 100% to each other, reflecting a recent common ancestry, and suggesting that they represent extensions of a single outbreak (Appendix 3).

Seg-2 of the four Indian BTV-2 isolates sequenced as part of this study, is most closely related to those of BTV-2 strains from Southern India isolated during 1982 to 2010 (2/IND/2010_KP339165, 2/IND/1993_KP696583, 2/IND/1982_AJ585152, 2/IND/1994_KP268775, 2/IND/1993_KP339155 and 2/IND_KC751422) sharing 98.29% to 98.38 % and 99.08% to 99.20% nt and aa identities, respectively (Figure 5.4).The four Indian BTV-2 isolates were more tightly clustered with recent isolates of BTV-2 strains circulating in southern India during 2010 (2/IND/2010_KP339165), showing 99.45% to 99.48% and 99.58% to 99.69 % nt and aa identity. This supports the conclusion that these Indian BTV-2 strains share a common ancestry reflecting the circulation and persistence of same BTV-2 Seg-2 lineage over time.

5.2.5 Phylogenetic analysis of Seg-2 / VP2 of Indian BTV-2 isolates within a global context

Phylogenetic analysis of the nucleotide sequences of the Seg-2 ORF from global BTV-2 strains clearly identified two major topotypes that correspond to eastern and western subgroups (Figure 5.4). The overall percentage nucleotide identity was >81.17% within the

western group. However, two major subclades (supported by high bootstrap value 96% and 78%) were clearly evident within the western BTV-2 Seg-2 topotype (Figure 5.4). One subclade or lineage (bootstrap value 96%) comprised of strains from France-Martinique Island, Panama, United States of America, France-Guyane, which showed nt and aa identity of only 71.1-72.17 % and 79.31-80.25 % with the recent Indian strains sequenced in this study (IND2015/K3_2015-09-10, IND2015/K14_2015-09-12, IND2015/K9_2015-09-10 and IND2015/14A_2015-11-08), indicating that they belong to the eastern topotypes (Figure 5.4). The other western sub-group / lineage (78% bootstrap support) consisted of four earlier Indian BTV-2 isolates (2/IND/2008_JQ904072, 2/IND/2003_KP696593, 2/IND/2003_JQ681258 and 2/IND/2003_KP696603), along with isolates from France-La Reunion Island, Canada, Nigeria, Sudan, South Africa, United States, Italy, Spain, Israel, Portugal, France-Corsica, Tunisia which shared overall nucleotide and amino acid identity of only 71.3-72.86 % and 79.42-80.46% respectively with the recent Indian isolates sequenced in this study (IND2015/K3_2015-09-10, IND2015/K14_2015-09-12, IND2015/K9_2015-09-10 and IND2015/14A_2015-11-08).

The four Indian BTV-2 isolates from the 2015 outbreak analysed here (IND2015/K3_2015-09-10, IND2015/K14_2015-09-12, IND2015/K9_2015-09-10 and IND2015/14A_2015-11-08) showed only 71.44% to 71.79 % nt and 79.73% to 80.25 % aa identity respectively to the previous BTV-2 strains from India (2/IND/2003_KP696603, 2/IND/2003_KP696593, 2/IND/2003_JQ681258 and 2/IND/2008_JQ904072, which had shown very high nt and aa identities (>99%) with the South African BTV-2 vaccine strain (2/RSAvvvv_KP821031) .This indicated grouping of the earlier Indian BTV-2 strains within the western topotype of BTV-2 and reflects the introduction and spread of BTV-2 Seg-2 from the western vaccine strain in India, as previously reported by (Maan et al., 2015c)

Previous Indian BTV-2 isolates from 2003 to 2008 (2/IND/2003_KP696603, 2/IND/2003_KP696593, 2/IND/2003_JQ681258 and 2/IND/2008_JQ904072) collectively represent an Indian cluster, sharing overall nt and aa identities of 88.54% to 90.03% and 93.35% to 96.15% in Seg-2 / VP2 respectively, with eastern BTV-2 strains from Australia, Taiwan and Japan (2/AUS/2008_JQ086242, 2/AUS/2010_JQ240322, 2/JPN/2007_AB686224 and 2/TWN/2003_AY493687), but branched distinctly from these

strains (Figure 5.4). The overall percentage nucleotide identity was >88.7% within the eastern group.

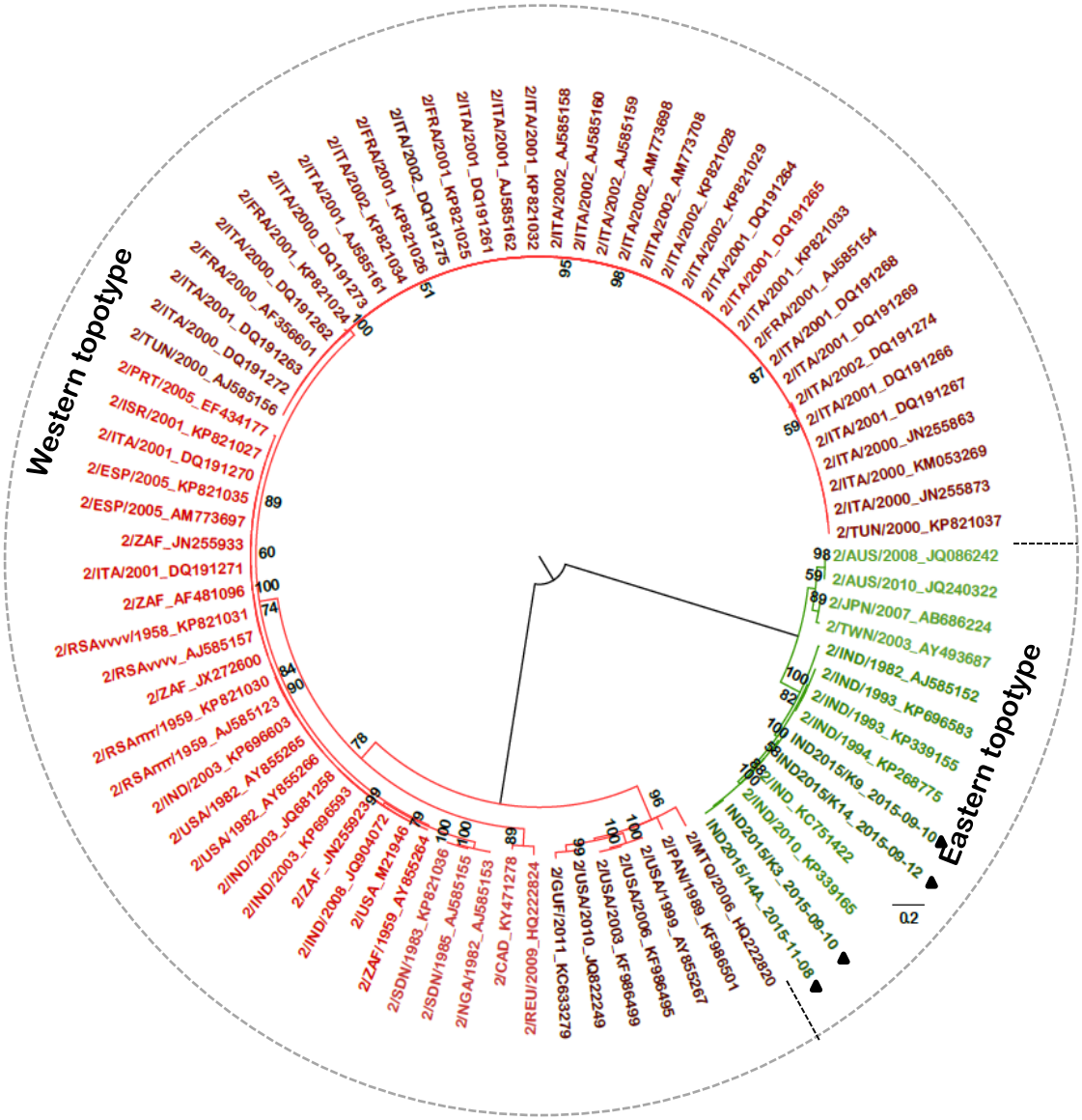
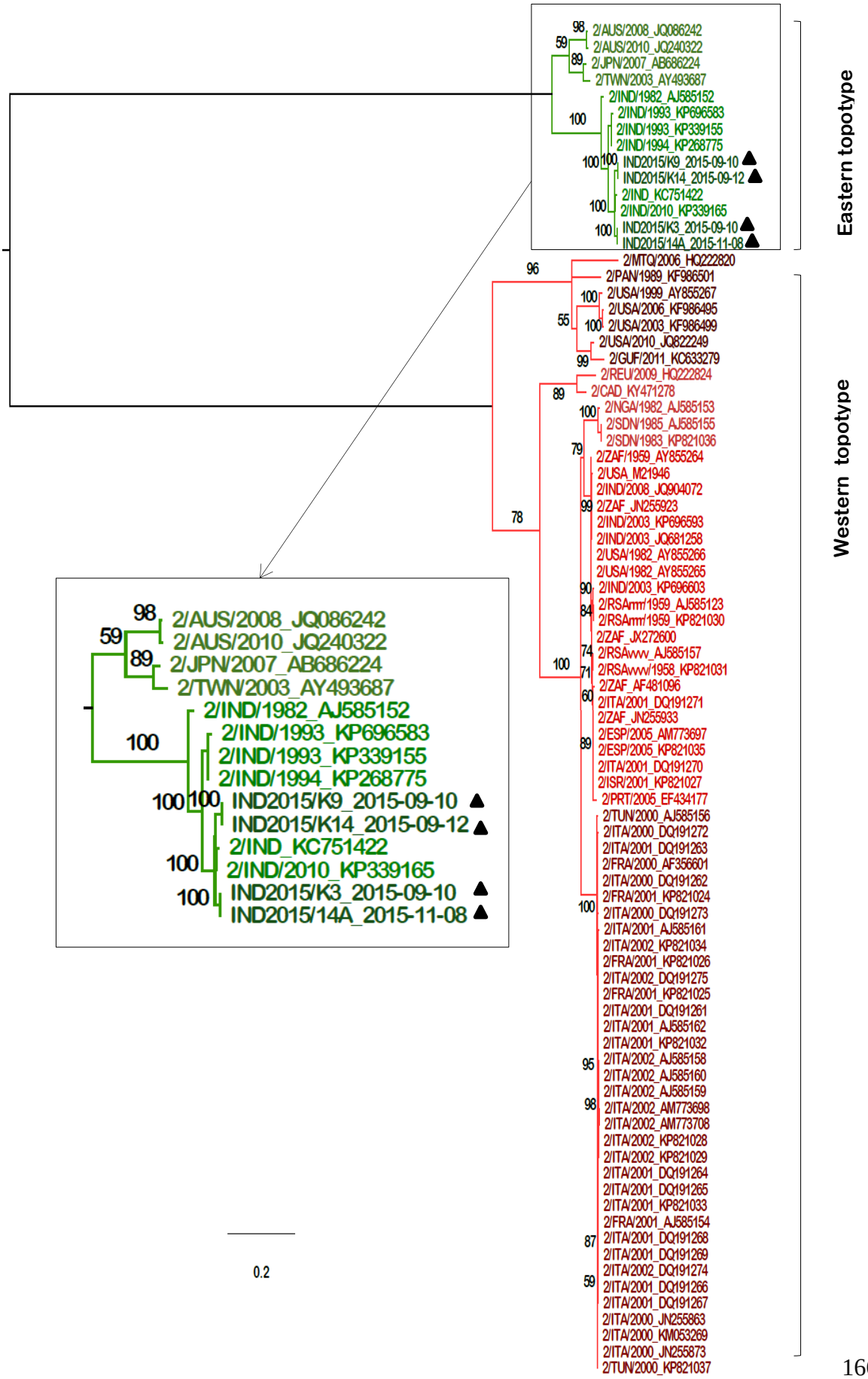


Figure 5.4 ML unrooted phylogenetic tree of Seg-2 coding region of Indian BTV-2 isolates (n=4) during 2015 (Table 5.1), reference strains of BTV-2 from around the world represented as circular (top) and rectangular (bottom) tree.

The identified serotype BTV-2 of Indian strains sequenced and analysed in this study are shown dark green coloured and marked in bold triangle. Scale bar, 0.2 nucleotide substitutions per site


Eastern topotype
Western topotype
2/AUS/2008_JQ086242
2/AUS/2010_JQ240322
2/JPN/2007_AB686224
2/TWN/2003_AY493687
2/IND/1982_AJ585152
2/IND/1993_KP696583
2/IND/1993_KP339155
2/IND/1994_KP268775
IND2015/K9_2015-09-10
IND2015/K14_2015-09-12
2/IND_KC751422
2/IND/2010_KP339165
IND2015/K3_2015-09-10
IND2015/14A_2015-11-08
2/MTQ/2006_HQ222820
2/PAN/1989_KF986501
2/USA/1999_AY855267
2/USA/2006_KF986495
2/USA/2003_KF986499
2/USA/2010_JQ822249
2/GUF/2011_KC633279
2/REU/2009_HQ222824
2/CAD_KY471278
2/NGA/1982_AJ585153
2/SDN/1985_AJ585155
2/SDN/1983_KP821036
2/ZAF/1959_AY855264
2/USA_M21946
2/IND/2008_JQ904072
2/ZAF_JN255923
2/IND/2003_KP696593
2/IND/2003_JQ681258
2/USA/1982_AY855266
2/USA/1982_AY855265
2/IND/2003_KP696603
2/RSAmm/1959_AJ585123
2/RSAmm/1959_KP821030
2/ZAF_JX272600
2/RSAwww_AJ585157
2/RSAwww/1958_KP821031
2/ZAF_AF481096
2/ITA/2001_DQ191271
2/ZAF_JN255933
2/ESP/2005_AM773697
2/ESP/2005_KP821035
2/ITA/2001_DQ191270
2/ISR/2001_KP821027
2/PRT/2005_EF434177
2/TUN/2000_AJ585156
2/ITA/2000_DQ191272
2/ITA/2001_DQ191263
2/FRA/2000_AF356601
2/ITA/2000_DQ191262
2/FRA/2001_KP821024
2/ITA/2000_DQ191273
2/ITA/2001_AJ585161
2/ITA/2002_KP821034
2/FRA/2001_KP821026
2/ITA/2002_DQ191275
2/FRA/2001_KP821025
2/ITA/2001_DQ191261
2/ITA/2001_AJ585162
2/ITA/2001_KP821032
2/ITA/2002_AJ585158
2/ITA/2002_AJ585160
2/ITA/2002_AJ585159
2/ITA/2002_AM773698
2/ITA/2002_AM773708
2/ITA/2002_KP821028
2/ITA/2002_KP821029
2/ITA/2001_DQ191264
2/ITA/2001_DQ191265
2/ITA/2001_KP821033
2/FRA/2001_AJ585154
2/ITA/2001_DQ191268
2/ITA/2001_DQ191269
2/ITA/2002_DQ191274
2/ITA/2001_DQ191266
2/ITA/2001_DQ191267
2/ITA/2000_JN255863
2/ITA/2000_KM053269
2/ITA/2000_JN255873
2/TUN/2000_KP821037
0.2

5.2.6 Phylogenetic analysis of Seg-2 from Indian BTV-4 isolates

Two Indian BTV isolates from the outbreak in Telangana during 2015 (IND2015/K4_2015-09-10 and IND2015/6PK_2015-09-07), were identified as BTV-4 by real-time RT-PCR assays targeting Seg-2. The length of Seg-2 obtained by NGS, ORF and size of VP2 protein were given in Table 5.2. The ORF of Seg-2 of these isolates was identical, indicating a common origin. Phylogenetic analyses showed that they were closely related to recent BTV-4 strains circulating in Southern India (4/IND/2013_KY947349, 4/IND/2013_KY947347, 4/IND/2007_KY947343, 4/IND/2013_KY947350, 4/IND/2013_KY947348, 4/IND/2013_KY947342, 4/IND/2013_KY947351, 4/IND/2013_KY947346, 4/IND/2012_KY947345, 4/IND/2008_KF560418 and 4/IND/2008_KY947344) with high nt and aa identity level of 98.33% to 99.69% and 98.85% to 99.79% respectively (Figure 5.5). The Indian BTV-4 isolates grouped closely with BTV-4 strain from China (4/CHN/1997_JX560414) showing overall nucleotide and amino acid identity of 91.15-91.57 % and 96.34-96.86% to the Chinese strain.

5.2.7 Phylogenetic analysis of Seg-2 / VP2 of Indian BTV-4 isolates within a global context

Phylogenetic analyses of the Seg-2 coding region of global BTV-4 strains (obtained from Gen Bank, Appendix 3) segregated them into eastern and western topotypes, that reflect the geographic origin of the virus isolates (Figure 5.5) Phylogenetic analysis of Seg-2 from the two Indian isolates (IND2015/K4_2015-09-10 and IND2015/6PK_2015-09-07) sequenced in this study showed that they group within ‘Seg-2- nucleotype A’ along with reference strains for serotype BTV-10 (BTV-4 (4/RSArrrr_KP821066), 10/RSArrrr_AJ585131), BTV-11 (11/RSArrrr_AJ585132), BTV-17 (17/RSArrrr_AJ585138), BTV-20 (20/RSArrrr_AJ585141) and BTV-24 (24/RSArrrr_AJ585145) with average nt and aa identities of 71.54% and 78.43%, 68.1% and 70.69%, 67.2% and 69.76%, 69.9% and 72.24%, 70.3% and 74.41% , 68.1% and 69.87 % respectively (Figure 5.1). However, both isolates clustered even more closely with the Chinese field strain of BTV-4 (4/CHN/1997_JX560414) with average nt identity of 91.19% and aa identity of 93.91% (Figure 5.1 and 5.5), confirming that they belong to an eastern topotypes of BTV-4.

In contrast, Seg-2 of BTV-4 isolates from Africa, Europe and America grouped separately as a western topotype of BTV-4, showing only 71.4% to 72.52 % nt and 79.5% to 80.65% aa identity with BTV-4 isolates sequenced in this study (IND2015/K4_2015-09-10 and IND2015/6PK_2015-09-07). Four subgroups were evident within the western BTV-4 topotype (Figure 5.5). One subgroup comprised of strains (4/CYP/2011_KP821042, 4/CYP/2011_KP821043, 4/CYP/2011_KP821044, 4/CYP/2011_KP821045, 4/CYP/2011_KP821046, 4/CYP/2004_KP821041, 4/CYP/1969_KP821040, 4/CYP/1969_AJ585180, 4/GRC/2012_KP821056, 4/GRC/2012_KP821057, 4/GRC/2012_KP821058, 4/GRC/2000_KP821055, 4/GRC/1999_KP821053, 4/GRC/1999_AY839947, 4/GRC/1999_KP821052, 4/GRC/1999_DQ191277, 4/GRC/2000_AJ585167, 4/GRC/2000_AY839946, 4/GRC/2000_KP821054, 4/GRC/1979_KP821051, 4/ISR/2006_KP821059, 4/ISR/2001_DQ191278, 4/ISR/2001_DQ191279, 4/TUR/1978_AJ585165, 4/TUR/1999_DQ825670) from the eastern Mediterranean region (Israel, Greece, Cyprus, Turkey) and the reference strains of BTV-4 – originally from Cyprus (4/RSArrrr_AJ585125, 4/RSArrrr_KP821066), sharing overall nucleotide and amino acid identity of 71.4% to 72.07 % and 80.13% to 80.44% with the Indian BTV-4 strains sequenced in study (IND2015/K4_2015-09-10 and IND2015/6PK_2015-09-07).

Seg-2 of the Argentinian BTV-4 isolates (4/ARG/1999_JX024950, 4/ARG/1999_JX024940, 4/ARG/1999_JX024945, 4/ARG/2002_AJ585169, 4/ARG/2009_JX024955 and 4/ARG/2010_JX024960) along with isolates from South Africa, Turkey, Italy, Egypt and vaccine strain of BTV-4 from South Africa (4/ZAF/2004_AY839948, 4/ZAF/1900_JN255943, 4/ZAF_JX272580, 4/ZAF_KM233615, 4/TUR_AJ585164 4/ITA/2002_DQ191276, 4/EGY/1977_KP821047 and 4/RSAvvvv_AJ585163) form another subgroup (64 % bootstrap support) that shares overall nt and aa identities of 71.4% to 72.52 % and 79.5% to 80.33% respectively with the Indian BTV-4 isolates (IND2015/K4_2015-09-10 and IND2015/6PK_2015-09-07).

A third BTV-4 Seg-2 subgroup was identified within the western topotype, comprising isolates (4/MAR/2009_KP821065, 4/MAR/2009_KP821062, 4/MAR/2009_KP821063, 4/MAR/2009_KP821064, 4/MAR/2004_KP821061, 4/ESP/2003_KP821067, 4/ESP/2004_KP821068, 4/ESP/2005_KP821069, 4/ESP/2010_KP821070, 4/FRA/2003_AY839945, 4/FRA/2003_KP821048, 4/FRA/2003_KP821049,

4/FRA/2003_KP821050, 4/PRT/2004_EF434176, 4/ITA/2003_JN255893, 4/ITA/2003_DQ191280, 4/ITA/2003_DQ191281 and 4/ITA/2003_JN255883) from the western Mediterranean region (Morroco, Spain, France, Portugal, Italy), supported by bootstrap values of 97%. These strains are only distantly related to the Indian BTV-4 strains IND2015/K4_2015-09-10 and IND2015/6PK_2015-09-07, sharing nt and aa identities of 72.14% to 72.34% and 80.33% to 80.65% respectively. The average nt / aa identity levels within the eastern group was 98.2% / 99.13% and 93.81% / 97.04% within the western group respectively.

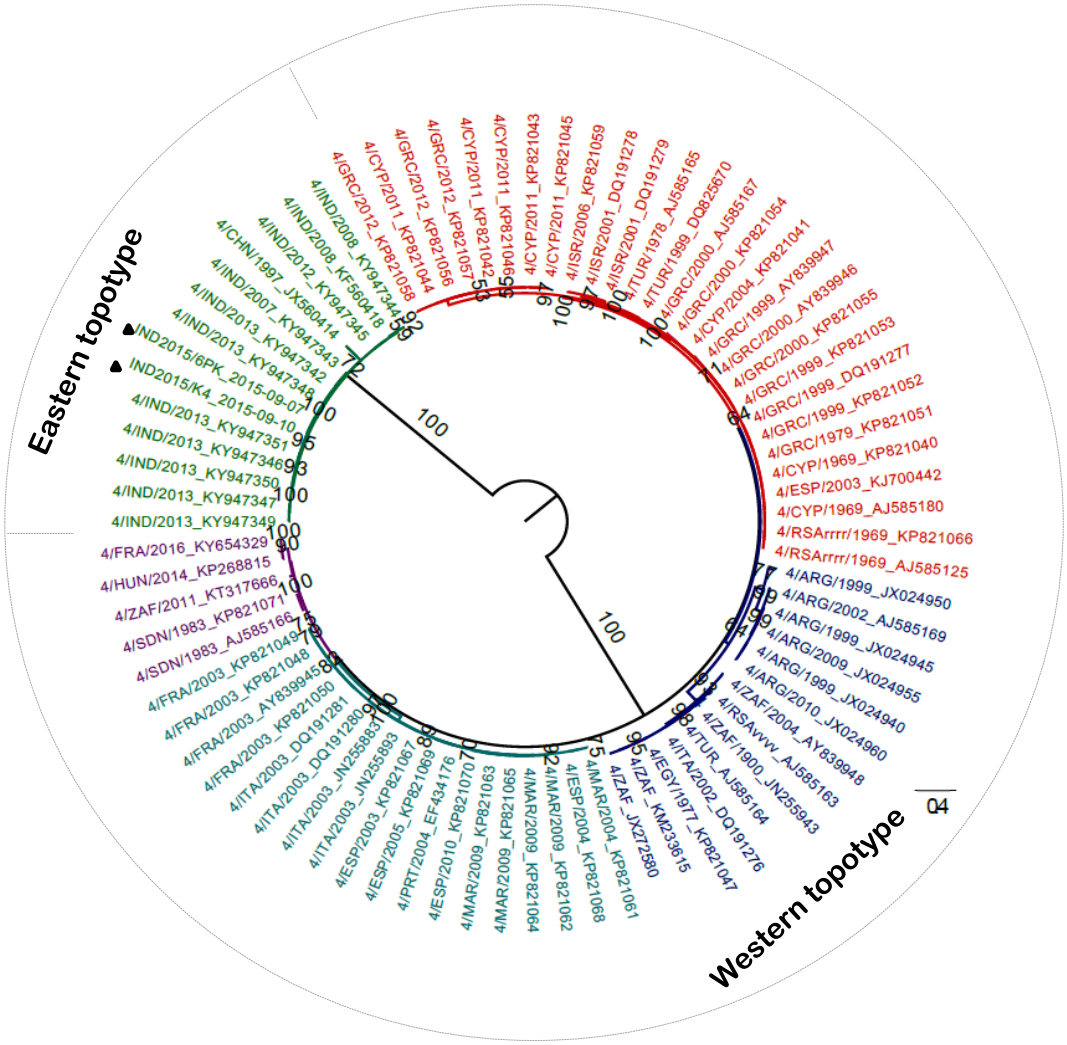
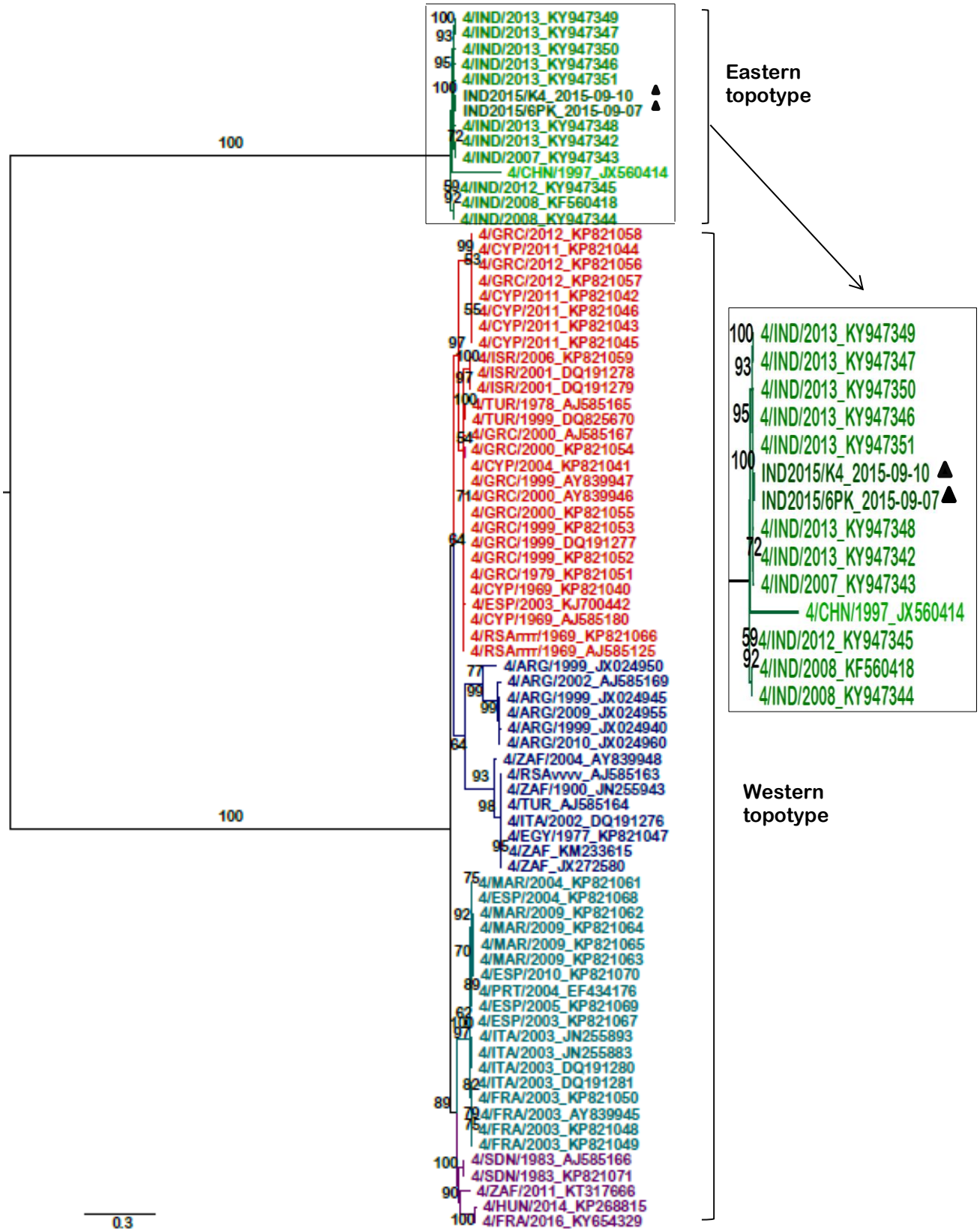


Figure 5.5 ML unrooted phylogenetic tree of coding region Seg-2/VP2 gene of Indian BTV-4 isolates (n=2) during 2014-2015 (Table 5.1) with reference strains of BTV-4 around the world represented as circular (top) and rectangular (bottom) tree.

The identified serotype BTV-4 of Indian strains sequenced and analysed in this study are shown dark green coloured and marked in bold triangle. Scale bar, 0.4 and 0.3 nucleotide substitutions per site for circular and rectangular trees respectively.



5.2.8 Phylogenetic analysis of Seg-2 from Indian BTV-5 isolates

The coding region of Seg-2 was sequenced from seventeen Indian isolates, from Tamil Nadu, Karnataka and Telangana during 2014-2015: (IND2015/471_2015-12-25, IND2015/475_2015-12-25, IND2015/476_2015-12-25, IND2015/478_2015-12-25, IND2015/491_2015-12-25, IND2015/492_2015-12-25, IND2015/404_2015-12-25, IND2015/408_2015-12-25, IND2015/486_2015-12-25, IND2015/534_2015-12-25, IND2015/547_2015-12-25, IND2015/228_2015-12-25, IND2015/CS9_2015-12-25, IND2015/V1_2015-11-26, IND2015/V2_2015-11-26, IND2015/V44_2015-11-26 and IND2014/10NLG_2014-10-03). The length of Seg-2 obtained by NGS, ORF and size of VP2 protein were given in Table 5.2.

Seg-2 of these Indian isolates clustered closely with the reference strain of BTV-5 (5/RSArrrr_KP821101), with average nt and aa identities of 94.96% and 97.77% respectively, confirming the results of serotype-specific real time RT-PCR, identifying them as isolates of BTV-5 except isolates IND2015/547_2015-12-25 and IND2015/492_2015-12-25. These two isolates IND2015/547_2015-12-25 and IND2015/492_2015-12-25 were typed wrong as BTV-10 and BTV-12 by serotype-specific real time RT-PCR.

5.2.9 Phylogenetic analysis of Seg-2 / VP2 of Indian BTV-5 isolates within a global context

A phylogenetic tree constructed on the basis of full length coding region of Seg-2 of BTV-5 sequences from different parts of the world segregated into two subclades (Figure 5.6). While seventeen BTV-5 isolates of the present study clustered within one subclade of BTV-5 together with the previously reported isolates from the China, Africa and Cameroon. The BTV-5 isolates from India, China, Cameroon and South Africa were further segregated into four subclades proposed as the distinct Indian, Chinese, and Cameroon and African subgeographical evolutionary lineages.

Phylogenetic analyses of the full ORF of Seg-2 showed that the Indian BTV-5 isolates sequenced as part of this study (IND2015/471_2015-12-25, IND2015/475_2015-12-25, IND2015/476_2015-12-25, IND2015/478_2015-12-25, IND2015/491_2015-12-25,

IND2015/492_2015-12-25, IND2015/404_2015-12-25, IND2015/408_2015-12-25, IND2015/486_2015-12-25, IND2015/534_2015-12-25, IND2015/547_2015-12-25, IND2015/228_2015-12-25, IND2015/CS9_2015-12-25, IND2015/V1_2015-11-26, IND2015/V2_2015-11-26, IND2015/V44_2015-11-26 and IND2014/10NLG_2014-10-03) grouped within the Seg-2 nucleotype E, along with the reference strains for BTV-5 (5/RSArrrr_KP821101) and BTV-9 (9/RSArrrr_KP821088) as shown in Figure 5.1. These Indian BTV-5 strains were grouped within the western topotype of BTV-5, sharing average nt/aa identity of 94.9 /97.47 % with BTV-5 reference strains from South Africa (5/RSArrrr/1953_AJ585126). These isolates also clustered with the reference strains of BTV-9 (9/RSArrrr/1942_KP821088, 9/AUS/1985_JQ086302 and IND2015/477_2015-12-25), but with lower average nt and aa identities (70.38% / 76.14%, 68.25% / 73.03 %, 68.49/73.34 respectively) than with the reference strain of BTV-5 (Figure 5.1).

The seventeen Indian BTV-5 strains clustered together, identifying as a single Indian sublineage, with overall nt and aa identity of 99.93% to 100% and 99.79% to 100%, indicating a common origin (Figure 5.6). These Indian BTV-5 strains, which were closely related to Chinese strains of BTV-5 showing average nt / aa identity of 98.71% / 98.93% respectively (suggesting a recent common ancestry), also clustered closely with a strain of BTV-5 from Cameroon (5/CMR/1982_AJ585181), showing an average nt/aa identity of 95.7% /97.26%. However, they were only distantly related to western BTV-5 strains from the French Guadeloupe Island (5/GLP/2010_HQ241072), Nigeria (5/NGA/1982_AJ585182) and USA (5/USA/2003_KX164060), with average nt/aa identities of 77.02% /85.75 %, 77.47% / 87.62 % and 77.57% /86.47 % respectively.

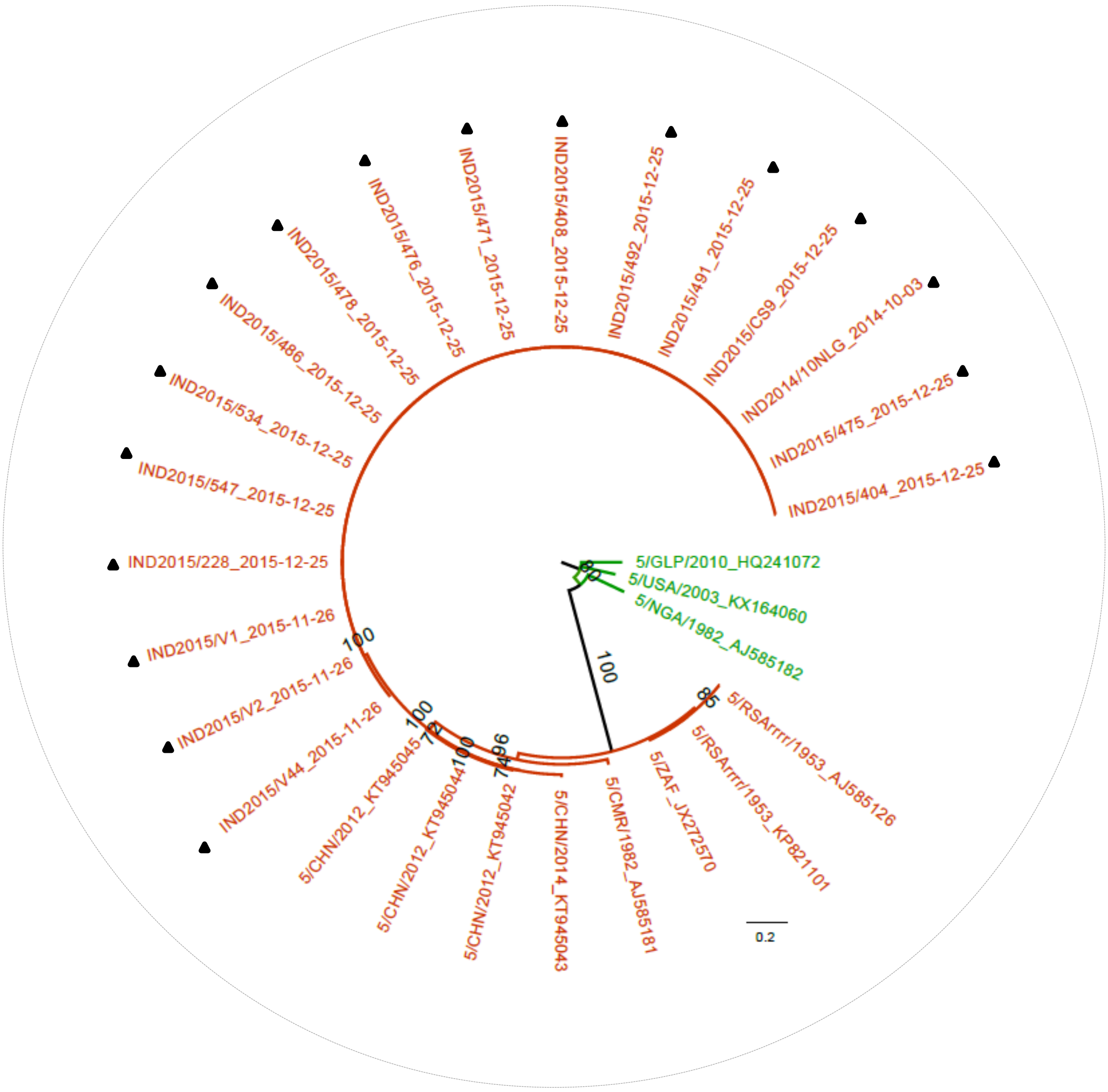
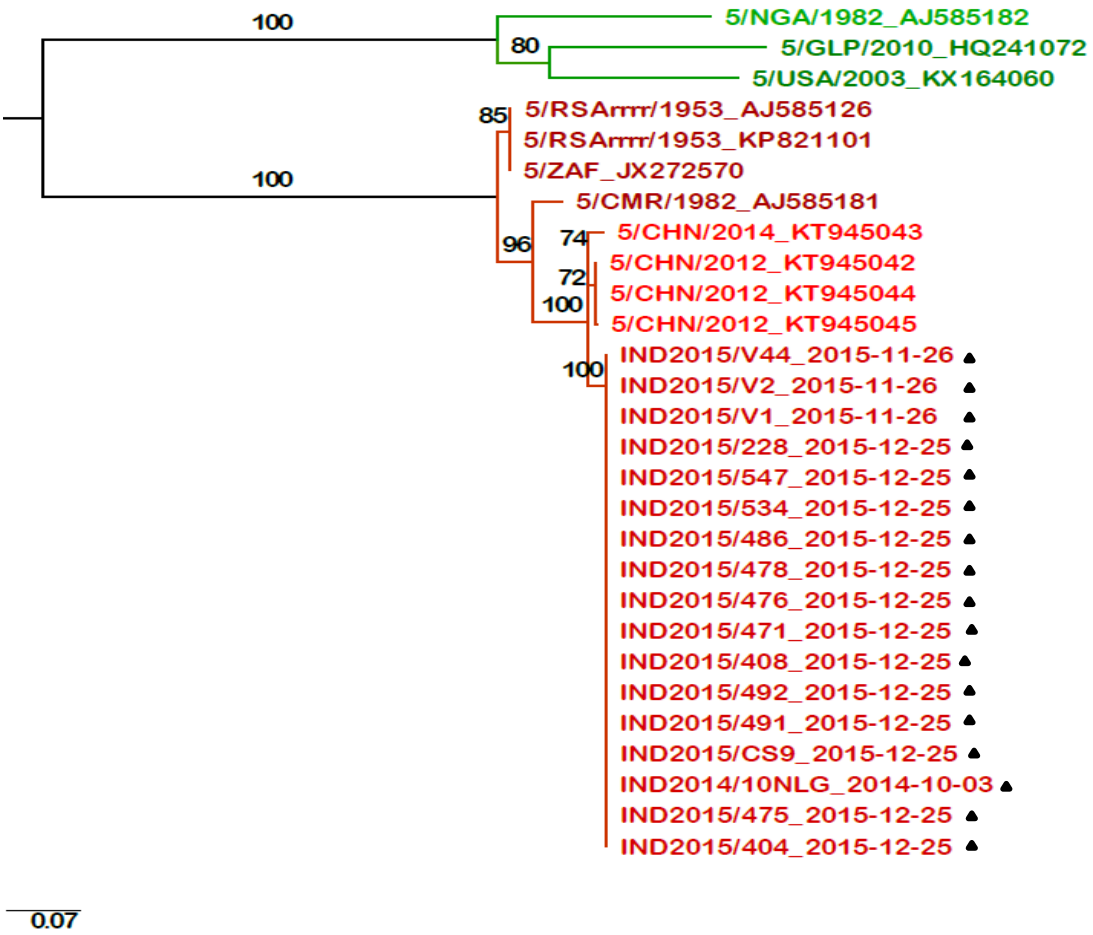


Figure 5.6 ML unrooted phylogenetic tree of coding region Seg-2/VP2 gene of Indian BTV-5 isolates (n=17) during 2014-2015 (Table 5.1) with reference and field strains of BTV-5 around the world represented as circular (top) and rectangular (bottom) tree..

The identified serotype BTV-5 of Indian strains sequenced and analysed in this study are shown maroon coloured and in bold triangle. Scale bar, 0.2 and 0.07 nucleotide substitutions per site for circular and rectangular trees.



5.2.10 Phylogenetic analysis of Seg-2 from Indian BTV-9 isolates

Seg-2 was sequenced from one Indian strain (IND2015/477_2015-12-25) isolated from Tamil Nadu State during the 2015 BTV outbreak, which was identified by RT-PCR as BTV-9. This isolate grouped within Seg-2-nucleotype E, along with the reference strains of BTV-9 (9/RSArrrr_KP821088) and BTV-5 (5/RSArrrr_KP821101). However, IND2015/477_2015-12-25 shared only 68.83% nt and 75.36% aa identity with BTV-9 (9/RSArrrr_KP821088). The length of Seg-2 obtained by NGS, ORF and size of VP2 protein were given in Table 5.2.

IND2015/477_2015-12-25 clusters closely with BTV-9 strains from Southern India, reflecting a close evolutionary relationship and indicating a recent common ancestry, sharing 97.11% to 99.09 % and 98.85% to 99.37% nt and aa identity respectively with: IND/2007_JX003688; IND/2006_JN579710; IND/2004_KP696613; IND/2005_JQ414046; IND/2005_KJ679573; IND/2003_KP696633; IND/2003_JF443155;

IND/2002_JF443156; IND/2003_JF443157; IND/2003_KP339175; IND/2003_KP339185; IND/2005_KP696642; IND/2004_KP696623 and IND/2005_KP696652, as part of an monophyletic clade of Indian BTV-9 strains (Figure 5.7).

5.2.11 Phylogenetic analysis of Seg-2 / VP2 of Indian BTV-9 isolates within a global context

Phylogenetic analysis of the full coding region of Seg-2 clearly splits global BTV-9 isolates into eastern and western topotypes that reflect the geographical origins of Seg-2 (Figure 5.7). The overall nt / aa identity within the eastern group was 98.02% /98.35%, with 95.6% / 97.31% within the western group.

IND2015/477_2015-12-25 clusters more closely with eastern field strains of BTV-9 (e.g. 9/AUS/1985_JQ086302, showing 89.37% nt and 93.72aa identity), than with the reference strain of BTV-9 from South Africa, indicating its grouping within the eastern Seg-2 topotype of BTV-9.

Four subgroups of BTV-9 Seg-2 were evident within the eastern topotypes, including isolates from Australia, Japan, India and the Mediterranean region (Figure 5.7). One subgroup contains Australian isolate 9/AUS/1985_JQ086302, sharing nt and aa identity of 89.37% and 93.72% with the Indian BTV-9 isolate (IND2015/477_2015-12-25). Japanese BTV-9 isolate (JPN/2003_AB686223) represents another subgroup (45% bootstrap support) sharing nt / aa identities of 91.04% / 93.61% respectively with the Indian BTV-9 isolate.

The third subgroup includes isolates from India (IND2015/477_2015-12-25, IND/2007_JX003688, IND/2006_JN579710, IND/2004_KP696613, IND/2005_JQ414046, IND/2005_KJ679573, IND/2003_KP696633, IND/2003_JF443155, IND/2002_JF443156, IND/2003_JF443157, IND/2003_KP339175, IND/2003_KP339185, IND/2005_KP696642, IND/2004_KP696623 and IND/2005_KP696652), collectively shares nt and aa identity of 97.11% to 97.32% and 93.61% to 99.37% with IND2015/477_2015-12-25, collectively forming an Indian sublineage within the eastern topotype (supported by bootstrap values of 94 %).

The fourth subclade within the eastern BTV-9 Seg-2 topotype, contains isolates from the Mediterranean region (Greece, Turkey, Bulgaria, Italy, Serbia, Bosnia and Hezegovina, Kosovo - supported by bootstrap values of 97%) with nt and aa identities of 96.97% to 97.34 % and 97.8 to 98.32 % with Indian strain IND2015/477_2015-12-25, indicating that these European strains had an eastern, possibly Indian origin. In contrast, Seg-2 of Africa and America isolates group separately, as a western BTV-9 topotype, showing nucleotide and aa identities of only 68.62% to 69.32 % and 73.3% to -74.97 % with Indian BTV-9 isolate IND2015/477_2015-12-25.

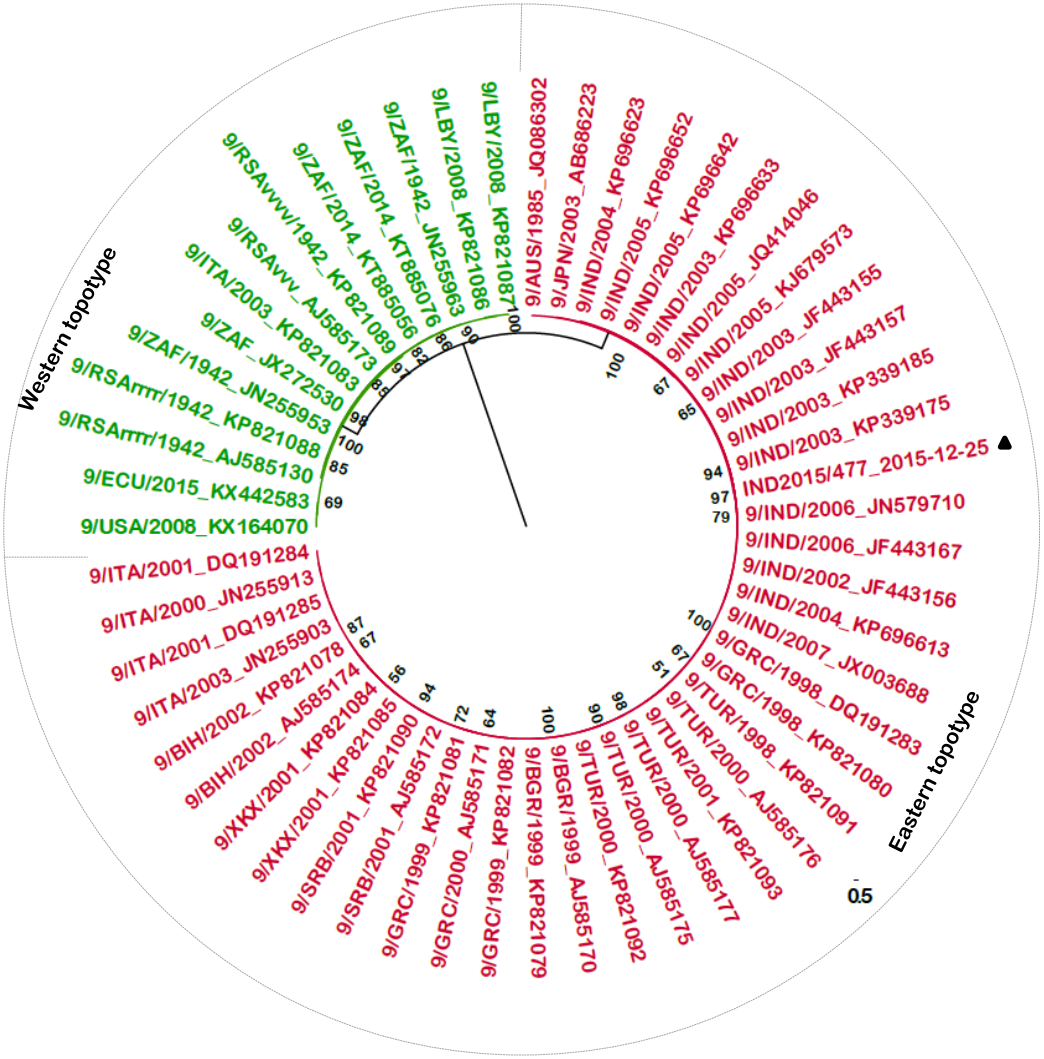
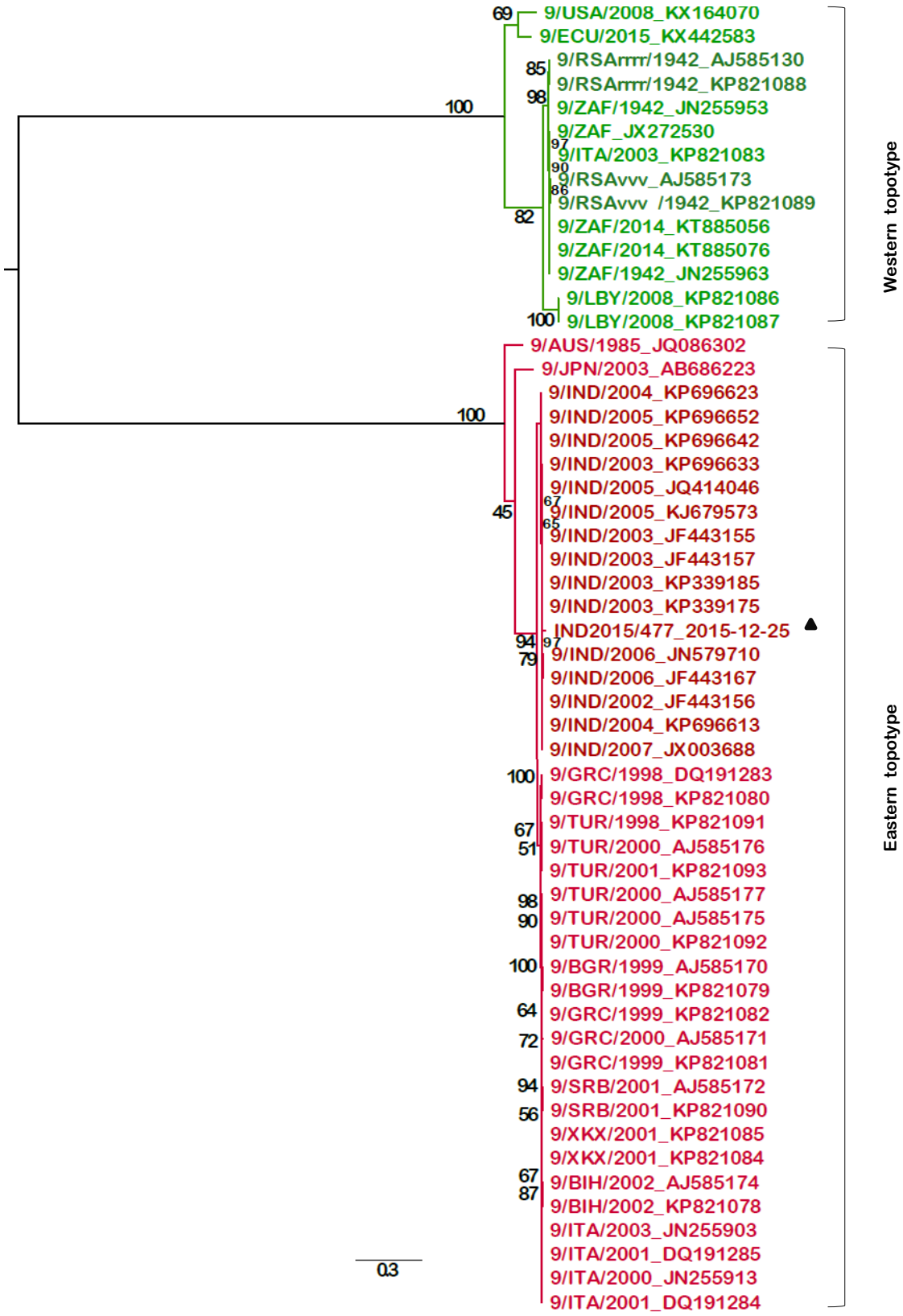


Figure 5.7 ML unrooted phylogenetic tree of coding region of Seg-2/VP2 gene of Indian BTV-9 isolate (n=1) during 2015 from Tamil Nadu, India (Table 5.1) with reference strains of BTV-9 around the world represented as circular (top) and rectangular (bottom) tree.

The identified serotype BTV-9 of Indian strain sequenced and analysed in this study was marked in a bold triangle. Scale bar, 0.5 and 0.3 nucleotide substitutions per site for circular and rectangular trees respectively.



5.2.12 Phylogenetic analysis of Seg-2 from Indian BTV-10 isolates

The full coding regions of Seg-2 from two Indian isolates (IND2014/BTV1_2014-12-06 and IND2014/BTV2_2014-12-06) from Thanjavur district of Tamil Nadu, 2014 were sequenced as part of these studies. The length of Seg-2 obtained by NGS, ORF and size of VP2 protein were given in Table 5.2. The Indian isolates (IND2014/BTV1_2014-12-06 and IND2014/BTV2_2014-12-06) clustered closely with the reference strain of BTV-10 (10/RSArrrr_AJ585131) showing average nucleotide and amino acid (nt/aa) identity of 89.34/92.78% and thus serotype was confirmed as BTV-10, consistent with the results of serotype-specific real time RT-PCR targeting Seg-2.

Phylogenetic analysis of Seg-2 showed that Indian BTV-10 isolates IND2014/BTV1_2014-12-06 and IND2014/BTV2_2014-12-06 are identical, indicating a common origin from a single epizootic. These isolates were most closely related to other BTV-10 strains from Southern India (10/IND/2004_JQ740772, 10/IND/2003_KP339245, 10/IND/2003_JF727655 and 10/IND/2004_JN704634) (Figure 5.8) sharing high nt and aa identities of 99.69% to 100 % and 99.48% to 100 % respectively, collectively grouping them within the western topotype of BTV-10 isolates (Figure 5.8).

Interestingly, the two Indian BTV-10 isolates (IND2014/BTV1_2014-12-06 and IND2014/BTV2_2014-12-06), which are from an outbreak in 2014, have identical Seg-2 sequences to an earlier Indian isolate of BTV-10 from Southern India, 2003 (10/IND/2003_KP339245), indicating continuing circulation or reintroduction of BTV-10 with the same Seg-2.

5.2.13 Phylogenetic analysis of Seg-2 / VP2 of Indian BTV-10 isolates within a global context

Indian isolates (IND2014/BTV1_2014-12-06 and IND2014/BTV2_2014-12-06) group within ‘Seg-2- nucleotype A’ along with reference strains of BTV-10 (10/RSArrrr_AJ585131), BTV-4 (4/RSArrrr/_KP821066), BTV-11 (11/RSArrrr_AJ585132), BTV-17 (17/RSArrrr_AJ585138), BTV-20 (20/RSArrrr_AJ585141) and BTV-24 (24/RSArrrr_AJ585145) (Figure 5.1).

Within this nucleotype the two Indian isolates cluster most closely with the reference strain of BTV-10 (10/RSArrrr_AJ585131), showing average nt and aa (nt/aa) identities of 89.34% / 92.78% confirming their serotype as BTV-10, consistent with results of the serotype-specific real time RT-PCR targeting Seg-2. These Indian isolates showed lower average nt and aa identities of: 67.88% / 75.93%; 67.88% / 72.76 %; 66.06% / 72.03%; 67.67%/75.03 %; and 67.25%/72.14% respectively with: BTV-24 [24/RSArrrr_AJ585145]; BTV-17 [17/RSArrrr_AJ585138]; BTV-11 [11/RSArrrr_AJ585132]; BTV-4 [4/RSArrrr_KP821066] and BTV-20 [20/RSArrrr_AJ585141], respectively.

The Indian strains of BTV-10 sequenced here, were closely clustered with five USA isolates (10/USA_M11787, 10/USA/1953_NC_006013, 10/USA_L29026, 10/USA_L29027 and 10/USA_U06786 – Figure 5.9) showing 99.48% to 99.69% nt and 99.72% to 99.9 % aa identity levels. The two Indian BTV-10 isolates (IND2014/BTV1_2014-12-06 and IND2014/BTV2_2014-12-06) were also closely related to a subgroup formed by three other USA isolates (10/USA/1980_U06780, 10/USA/1980_U06782 and 10/USA/1980_U06783) showing overall nt and aa identities of 99.16% to 99.27% and 99.48% to 99.55% respectively (Figure 5.9). Three other American BTV-10 isolates (10/USA/1990_U06781, 10/USA/1990_U06784 and 10/USA/1990_U06785) form another subgroup (100% bootstrap support) that shares overall nt and aa identities of 95.23% to 95.33 % and 95.71% to 95.82% with the Indian BTV-10 isolates (IND2014/BTV1_2014-12-06 and IND2014/BTV2_2014-12-06).

In contrast, BTV-10 isolates from South Africa and the French administrative areas of Martinique Island and Guyana in the Caribbean, were only distantly related to the Indian BTV-10 isolates (IND2014/BTV1_2014-12-06 and IND2014/BTV2_2014-12-06) showing 88.71% to 89.24 % nt and 92.47% to 92.99% aa identity.

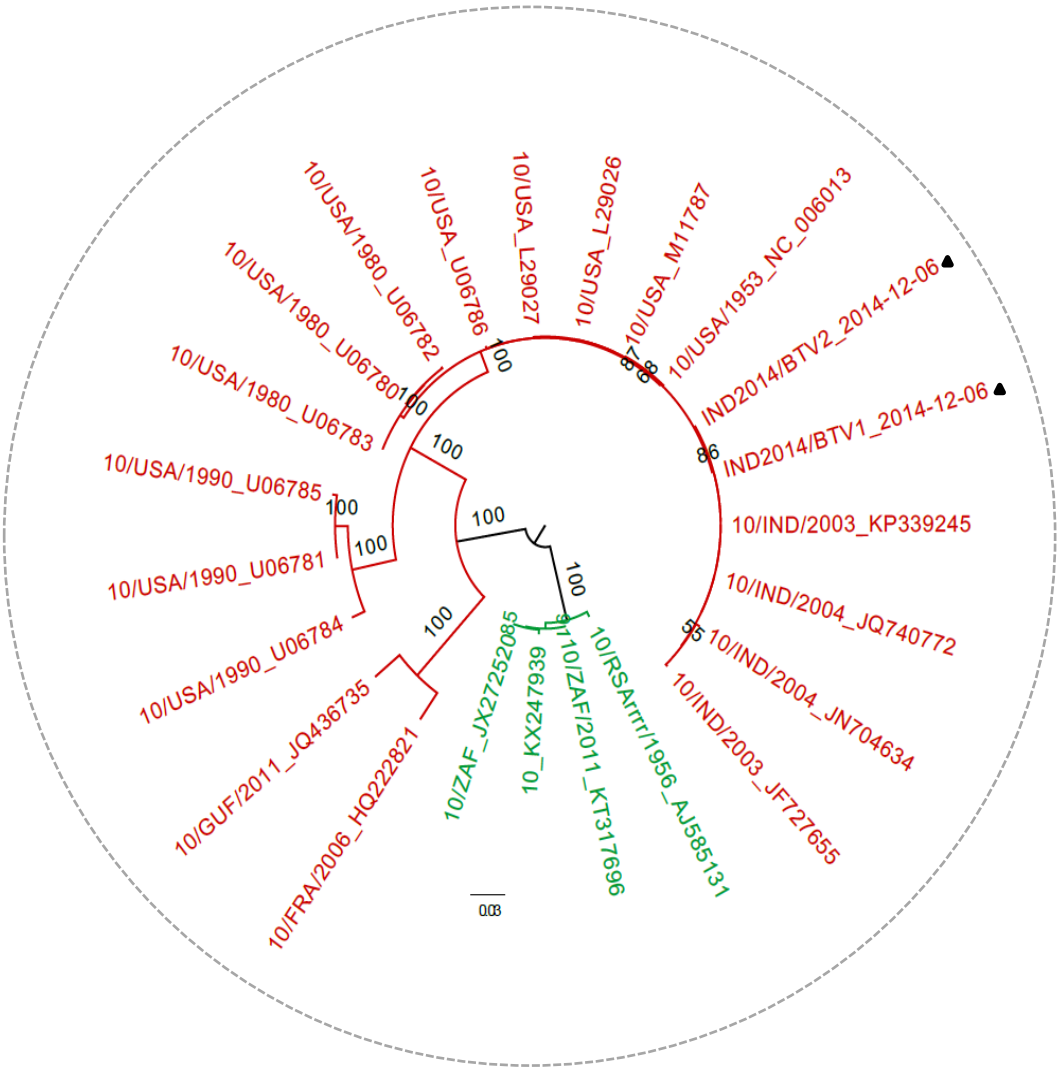
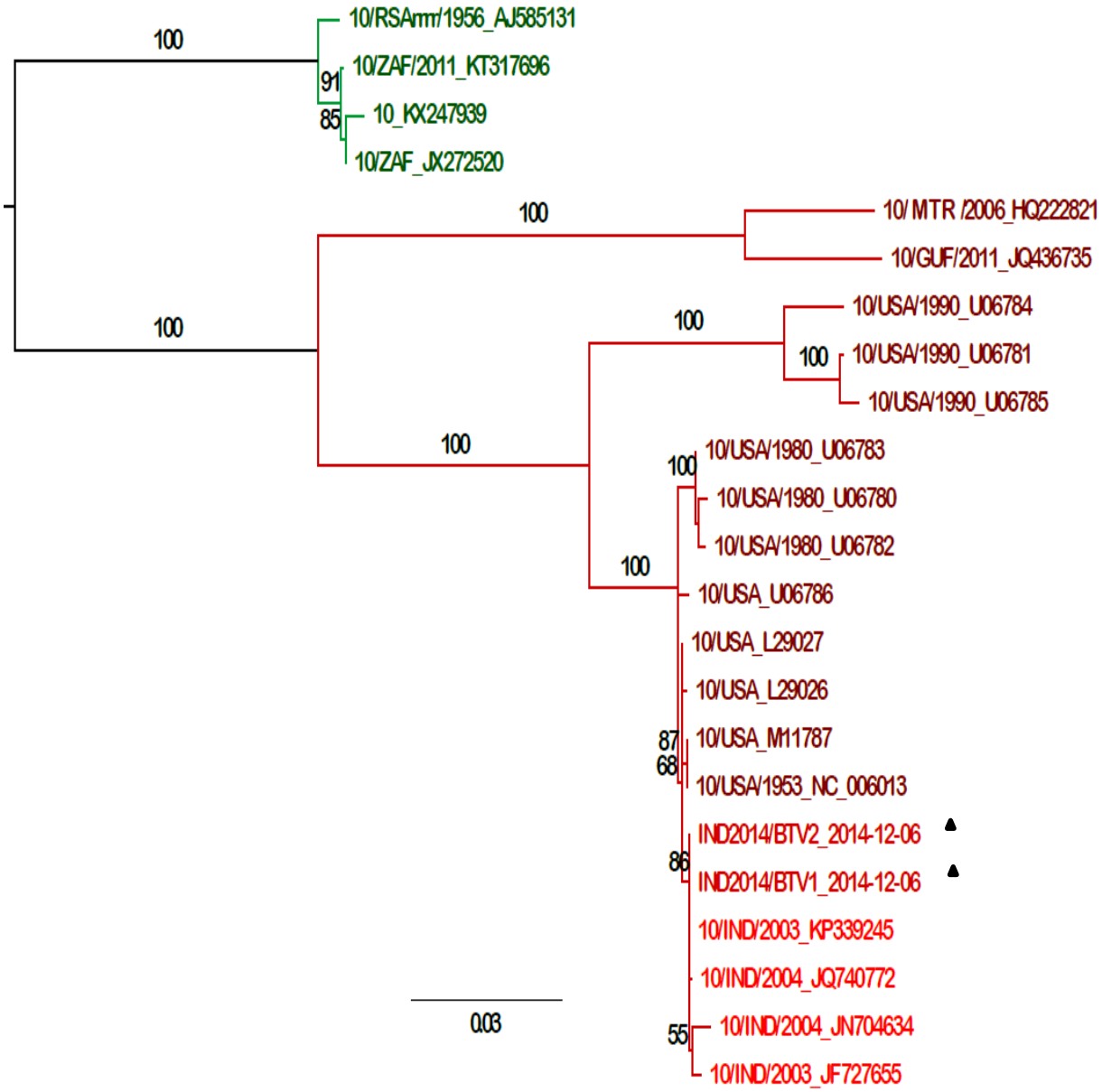


Figure 5.8 ML unrooted phylogenetic tree of coding region Seg-2/VP2 gene of Indian BTV-10 isolates (n=2) during 2014-2015 (Table 5.1) with reference strains of BTV-10 all over the world .

Trees are represented as circular (top) and rectangular (bottom) .The identified serotype BTV-10 of Indian strains sequenced and analysed in this study are shown dark red in colour andmarked with bold traingle. Scale bar, 0.03 nucleotide substitutions per site



5.2.14 Phylogenetic analysis of Seg-2 from Indian BTV-12 isolates

Seg-2 from seventeen Indian isolates that were identified as BTV-12 by RT-PCR, were sequenced as part of this study, including: IND2015/22_2015-12-18; IND2015/494_2015-12-25; IND2015/406_2015-12-25; IND2015/470_2015-12-25; IND2015/472_2015-12-25; IND2015/473_2015-12-25; IND2015/474_2015-12-25; IND2015/480_2015-12-25; IND2015/483_2015-12-25; IND2015/484_2015-12-25; IND2015/CS4_2015-12-25; IND2015/CS6_2015-12-25; IND2015/34_tissue_2015-12-2; IND2015/556_saliva_2015-12-25; IND2015/V41_2015-11-26; IND2015/V44_lamb_2015-11-26; IND2015/V44Severe_2015-11-26. The length of Seg-2 obtained by NGS, ORF and size of

VP2 protein were given in Table 5.2. These seventeen Indian isolates group with the reference strains for BTV-12 (12/RSArrrr_KP821100) and BTV-22 (22/RSArrrr_AJ585143) within Seg-2-based nucleotype G (Figure 5.1) showing nt and aa identities of 97.13% to 98 %, with the reference strain of BTV-12 (12/RSArrrr/1941_KP821100), confirming results of serotype-specific real time RT-PCR targeting Seg-2.

The Seg-2 coding region of these Indian strains showed identical nucleotide/amino acid sequences, indicating a common origin, from a single outbreak. The Indian BTV-12 strains were most closely related to other Indian strains 12/IND/2015_KX905151, 12/IND/2016_MF615238 from Haryana state and 12/IND/2011_KC662613 from Adilabad district in Telangana State, showing average nt/aa identities of 99.93% to 100%; 99.89% to 99.8%; and 99.4% to 99.6% respectively, reflecting their origin from a single virus lineage (Figure 5.9).

5.2.15 Phylogenetic analysis of Seg-2 / VP2 of Indian BTV-12 isolates within a global context

These Indian BTV-12 isolates also cluster with the reference strains of BTV-22 (22/RSArrrr_AJ585143) within nucleotype G, showing average nt and aa identities of 69.54% and 76.40% respectively. These Indian BTV-12 strains group within the western topotype of BTV-12, sharing average nt/aa identity of 97.06% to 97.58% and 97.13% to 98% with the BTV-12 reference strains from South Africa (12/RSArrrr_AJ585133 and 12/RSArrrr_KP821100) respectively.

Seg-2 of the seventeen Indian strains shared 97.16% / 98% nt/aa identities with the BTV-12 strain (12/ZAF_JX272500) from South Africa and showed 95.69/96.42 % and 96.42/96.95 % nt/aa identities to BTV-12 strains from Taiwan (12/TWN/2003_GU390659) and Japan (12/JPN/1990_AB686216) respectively. However, they showed some divergence from BTV-12 strains 12/USA/2008_KX164080, 12/USA/2012_KX164090 and 12/GUF/2011_KC633278 from USA and French Guyana respectively (87.94% / 92.53%, 88.36% / 93.79% and 87.49% / 92.74% nt/aa identity).

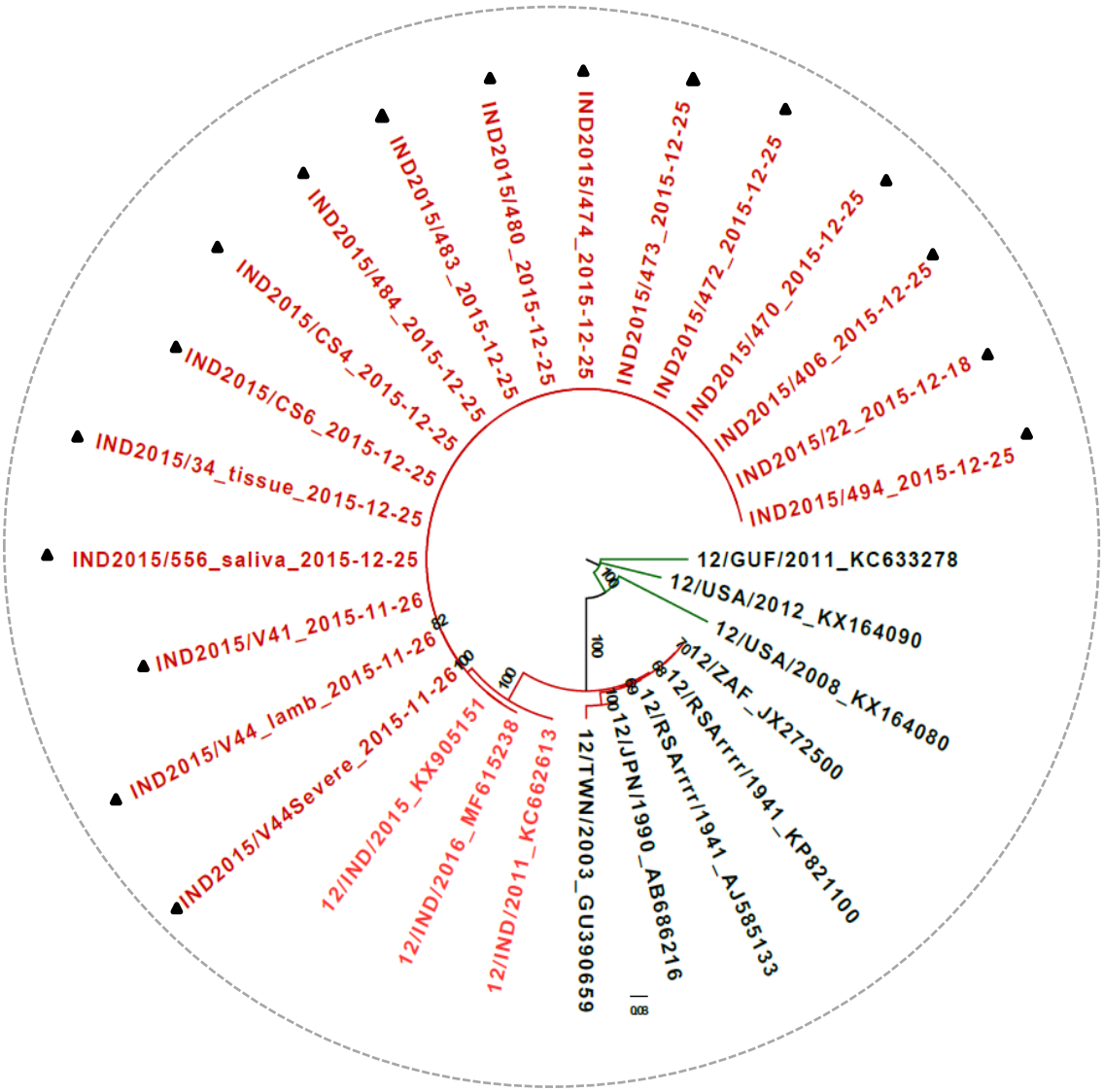
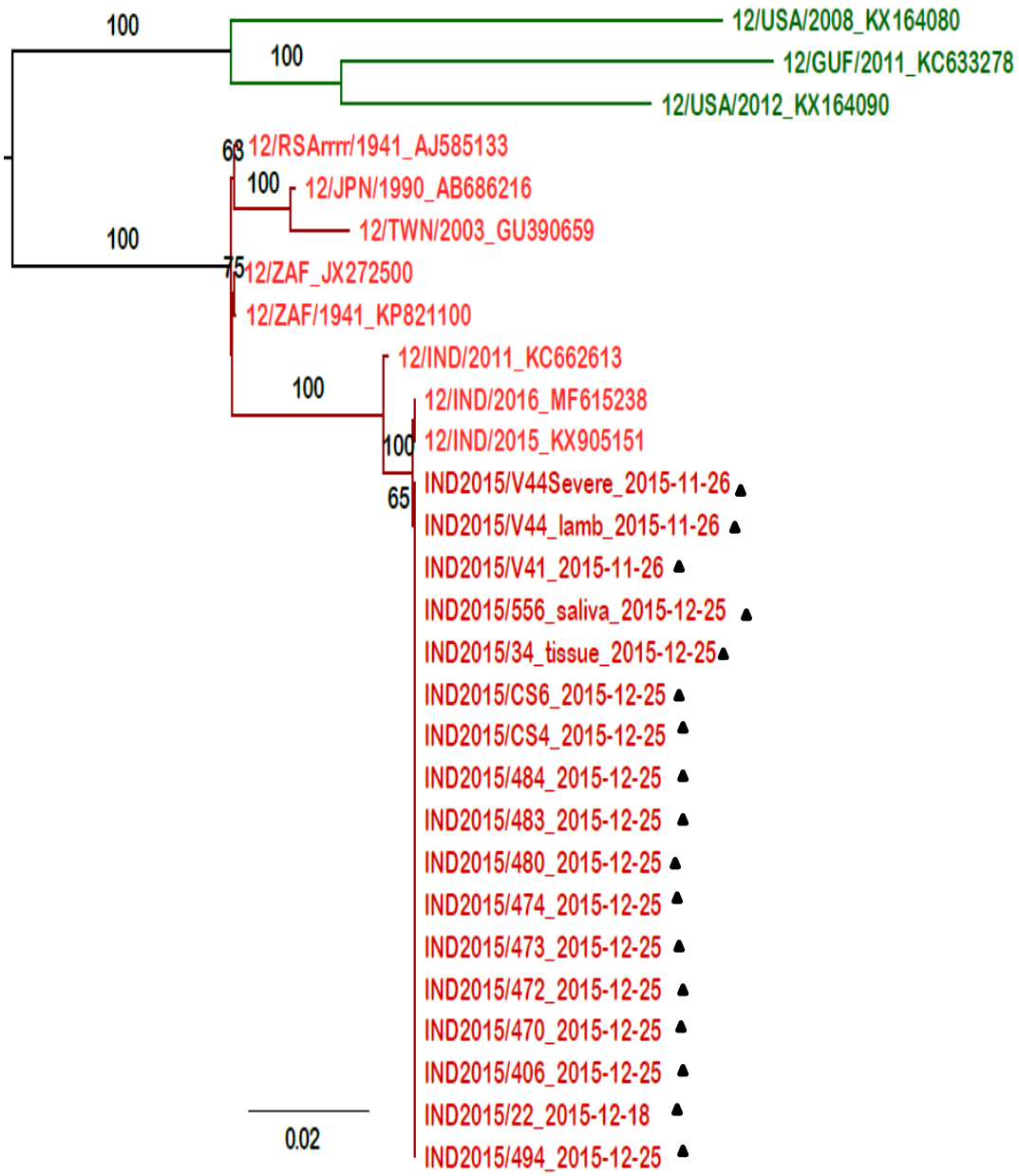


Figure 5.9 ML unrooted phylogenetic tree of coding region Seg-2/VP2 gene of Indian BTV-12 isolates (n=17) during 2014-2015 (Table 5.1) with reference strains of BTV-12 from all over the world.

Trees are represented as circular (top) and rectangular (bottom) The identified serotype BTV-12 of Indian strains sequenced and analysed in this study are shown maroon coloured and marked with bold traingle Scale bar, 0.02 nucleotide substitutions per site.



5.3 Discussion

Seven different serotypes of BTV including: BTV-1, BTV- 2, BTV- 4, BTV-5, BTV-9, BTV-10 and BTV-12, were identified by phylogenetic analysis of full ORF Seg-2 in forty six Indian BTV isolates (n=46) from BT suspected outbreaks in Southern India during 2014-2015. The majority of isolates were confirmed as BTV-5 and BTV-12, seventeen isolates each, while the remaining (twelve isolates) were identified as BTV-1 (three isolates), BTV-2 (four isolates), BTV-4 (two isolates), BTV-9 (one isolate) and BTV-10 (two isolates).

Phylogenetic analyses have previously established that Seg-2 divides into 27 distinct groups precisely representing virus serotype, with sequence identity of 68.4% to 100 % at nt and 72.6% to 100 % at aa level within each serotype(Maan et al., 2010, Maan et al., 2011b, Schulz et al., 2016) .To confirm the findings of RT-PCR typing assays, the coding region of Seg-2 from these Indian isolates was sequenced and compared to the reference strains of BTV serotypes, BTV-1 to BTV-27 available in GenBank (Figure 5.1). Seg-2 sequences of putative types BTV-28 in a Sheep pox vaccine preparation from the Middle East (Bumbarov et al., 2016), and BTV-29 in an Alpaca from South Africa (Wright, 2014, Posada, 2008) available in GenBank are not included in analysis. Three further putative types have also been detected: BTV-30, 31 and 32 from ruminants in Mongolia (EPIZONE, 2017) . In each case phylogenetic analysis revealed > 68.4 % nt sequence identity with reference strains of the corresponding serotypes, confirming the results of serotype-specific Seg-2 real time RT-PCR for serotype identification (Maan et al., 2010) except for isolates [IND2015/2017]547 tissue, [IND2015/] K13, [IND2015/271]7A and [IND2015/18] 40, the serotype detected by qPCR was different of that detected by NGS.

Sequencing studies suggest that geographical separation over an extensive period of time along with genetic drift, genetic shift and selection has contributed to genetic heterogeneity and the evolution of distinctive regional variants (eastern and western topotype or lineages) of BTV strains in different geographic regions/ecosystems. Seg-2 sequences of BTV isolates belonging to the same topotype, within each serotype, show nt / aa sequence identities of 78.2% to100% / 86.1% to 100% respectively. In contrast nt / aa identity levels of 68.4%to75.2% / 72.6%to83.2 % have been demonstrated in Seg-2 / VP2 , respectively between the major east / west topotypes of the same serotype (Maan et al., 2010).

Geographical separation has shaped BTV strains into the independently evolving eastern and western topotype over an extended period of time as represented by the evolutionary distance inferred for each genome segment (Maan et al., 2015c, Carpi et al., 2010). While the aa and nt sequences of VP2/Seg-2 show two distinct, eastern and western topotypes for serotypes BTV-1, BTV-2, BTV-3, BTV-4, BTV-9, BTV-15 and BTV-16, the remainder of the 27 BTV serotypes belong solely to either the eastern (BTV-20, BTV-21, BTV-23) or as western lineage / topotype (BTV-5, BTV-6, BTV-7, BTV-8, BTV-10, BTV-11, BTV-12, BTV-13, BTV-14, BTV-17,BTV-18, BTV-19, BTV-22, BTV-24, BTV-25, BTV-26, BTV-27). The presence of definite eastern and western topotypes of Seg-2

within BTV serotypes BTV-1, BTV-2, BTV-3, BTV-4, BTV-9, BTV-15 and BTV-16, suggests that these serotypes diverged and became genetically distinct as a first step, prior to their geographic dispersal into different regions/ ecosystems permitting them to acquire regionally unique point mutations, producing distinct topotypes within each serotype.

The serotypes that are restricted to single major topotypes, may have evolved in the same region after the early dispersal of different strains. Therefore the existence of more distinct BTV serotypes that are restricted to western ecosystems / topotypes, suggests that BTV may have originated in the west.

Seg-2 from the Indian isolates of four serotypes (**BTV-1** [IND2015/K13_2015-09-12, IND2015/40_2015-12-22 and IND2015/7A_2015-11-08]; **BTV-2** [IND2015/K3_2015-09-10, IND2015/14A_2015-11-08, IND2015/K9_2015-09-10 and IND2015/K14_2015-09-12]; **BTV-4** [IND2015/K4_2015-09-10 and IND2015/6PK_2015-09-07] and **BTV-9** [IND2015/477_2015-12-25]) all showed >87 % nucleotide and > 93 % amino acid identity with earlier defined eastern strains of the same serotype, but<75% nucleotide and < 83 % amino acid identity to 'western' topotype strains of the same BTV serotypes, identifying them as 'eastern topotype' and indicating their origin from Australasia.

In contrast Seg-2 from Indian isolates of three serotypes,**BTV-5** [IND2015/471_2015-12-25, IND2015/475_2015-12-25, IND2015/476_2015-12-25, IND2015/478_2015-12-25, IND2015/491_2015-12-25, IND2015/492_2015-12-25, IND2015/404_2015-12-25, IND2015/408_2015-12-25, IND2015/486_2015-12-25, IND2015/534_2015-12-25, IND2015/547_2015-12-25, IND2015/228_2015-12-25, IND2015/CS9_2015-12-25, IND2015/V1_2015-11-26, IND2015/V2_2015-11-26, IND2015/V44_2015-11-26 and IND2014/10NLG_2014-10-03], **BTV-10** [IND2014/BTV1_2014-12-06 and IND2014/BTV2_2014-12-06] and **BTV-12** [IND2015/22_2015-12-18; IND2015/494_2015-12-25; IND2015/406_2015-12-25; IND2015/470_2015-12-25; IND2015/472_2015-12-25; IND2015/473_2015-12-25; IND2015/474_2015-12-25; IND2015/480_2015-12-25; IND2015/483_2015-12-25; IND2015/484_2015-12-25; IND2015/CS4_2015-12-25; IND2015/CS6_2015-12-25; IND2015/34_tissue_2015-12-2; IND2015/556_saliva_2015-12-25; IND2015/V41_2015-11-26; ND2015/V44_lamb_2015-11-26; IND2015/V44Severe_2015-11-26] all showed >89 % nt and > 92 % aa identity with previously defined western strains of the same serotype, identifying them as members

of the 'western topotype' and indicating their origin from Africa or America. No BTV-5. BTV-10 and BTV-12 strains belonging to the eastern topotypes are available for Seg-2 based phylogenetic analysis in GenBank.

BTV strains belonging to different serotypes but the same "Seg-2 nucleotype" are characterised by 66.9% to 71.5% nt and 69.4% to 77.8% aa sequence identity (Maan et al., 2010, Maan et al., 2007a). Comparison of sequences of genome segment-2 from Indian isolates generated in this study, with data for Indian BTVs available from GenBank (accessed on 01.12.2017) show that the Indian viruses are collectively grouped into eight of the eleven "Seg-2 nucleotypes" A, B, C, D, E, G, H and I (Maan et al., 2007a) (Figure 5.2) and eleven serotypes namely BTV-1, 2, 3, 4, 5, 9, 10, 12, 16, 21 and 23. Phylogenetic analyses of Seg-2 of the Indian BTV strains sequenced in this study grouped them into five Seg-2 nucleotypes A, E, G, H and I (Maan et al., 2007a) along with the reference strains of the same serotype (Figure 5.1).

A high level of nucleotide sequence identity could be expected for BTV isolates originating from a single epizootic, reflecting a common lineage. BT outbreaks were reported at approximately the same time (November-December 2015) in Karnataka and Tamil Nadu, two adjacent states in southern India (Chapter 3, Figure 3.8).

Comparison of nt sequences of BTV-5 isolates from Karnataka state 2015, with BTV-5 strains isolated from Tamil Nadu 2015 and Telangana of 2014 characterised during this study, show average nt / aa sequence identities of 99.99% / 99.99 %, supporting the conclusion that they are derived from the same Seg-2 lineage and represent a single outbreak. This reflects the known interstate migration of small ruminants in India, as well as movement of adult *Culicoides*, providing transmission routes spreading the virus and disease.

The Indian BTV-5 strains cluster together identifying an Indian sublineage with Seg-2 based nt / aa sequence-identity of 99.99% / 99.99%. Evolution of a distinct Indian sub-lineage of BTV-5 Seg-2 is thought likely reflect the geographic separation of these virus strains, along with the acquisition of point mutations over a long period of time. Seg-2 of these Indian BTV-5 strains is most closely related to BTV-5 strains circulating in recent

years in mainland China, with 98.71% / 98.93% nt / aa sequence identity, indicating a recent common ancestry.

Seg-2 of the Indian and Chinese BTV-5 strains, are also collectively related to African BTV-5 strains, sharing average nt and aa identities of 94.96% and 97.77% respectively, which indicates that they share of a recent common ancestry strains and suggests the introduction of an African-lineage BTV-5 into India. The first report of BTV-5 was from South Africa (Howell and Verwoerd, 1971) and it has subsequently been isolated from Cameroon(Ekue et al., 1985b), Nigeria(ReoID, 2017), the United States of America (Johnson et al., 2007), the French administrative areas on the Caribbean-island of Guadeloupe (Legisa et al., 2014) and most recently from China (Yang et al., 2017) and India (Hemadri et al., 2016).The isolation of serotype BTV-5 has recently been reported from clinical cases of BT in sheep in Karnataka state during 2010-2011 (Hemadri et al., 2016) as well as from Telangana state during 2013 (P.P.Rao unpublished data). The presence of neutralizing antibodies to BTV-5 was previously reported in India from Tamil Nadu, Maharashtra and Gujarat state (Prasad et al., 2009). The studies reported here show that BTV-5 was involved in 2014-15 BTV outbreaks in the states of Karnataka, Telangana and Tamil Nadu in southern India, indicating the origin of these BTV-5 strains from a single virus lineage, and either the re-emergence of a pre-existing strain of Indian BTV-5, or (re-)introduction of an exotic strain. No full length Seg-2 sequence data of Indian BTV-5 isolates other than those sequenced in this study are as yet available from Genbank for phylogenetic comparison.

Following its first identification in South Africa (Gerdes, 2004), serotype BTV-12 has also been reported from other African countries such as Egypt (Barsoum, 1992), Cameroon (Ekue et al., 1985a), Kenya (Davies et al., 1992, Toye et al., 2013), Nigeria (Herniman et al., 1983) and Zimbabwe (ReoID, 2017). BTV-12 has also been reported from Central and South America, including Jamaica , Colombia, Brazil, (Homan et al., 1990, Mo et al., 1994, Clavijo et al., 2002), Asia, including Japan (Miura et al., 1982),China (Bonneau et al., 1999, Kirkland et al., 2002), Taiwan (Lee et al., 2011), Indonesia (Sendow et al., 1991), and India (Rao et al., 2015, Maan et al., 2017)and the Middle East, including Israel (Golender, 2013).

Serological evidence for the presence of BTV-12 antibodies in sheep and cattle have been documented in Ind**ia** from Haryana, Gujarat, Maharashtra, Andhra Pradesh, Karnataka and Tamil Nadu states (Prasad et al., 2009)and the first isolation of BTV-12 was reported from Andhra Pradesh in 2013a (Rao et al., 2015). Recently the circulation of BTV-12 has been reported from several states of India including the southern state of Andhra Pradesh in 2010-2013 (Rao et al., 2016b, Rao et al., 2016a) and the Northern state of Haryana in 2015-16 (Maan et al., 2017).The seventeen Indian isolates identified as BTV-12 in this study, from Karnataka and Tamil Nadu, and are nearly identical, indicating a common origin from a single epizootic. This is the first report / time that BTV serotype BTV-12 has been isolated in Tamil Nadu and Karnataka (Southern India) from BTV outbreaks in 2015. These Indian BTV-12 strains are most closely related to other recent Indian strains 12/IND/2015_KX905151, 12/IND/2016_MF615238 isolated from Haryana State in 2015-16, and recent isolates 12/IND/2011_KC662613 from the Adilabad district, Telangana State in 2013, showing average nt/aa identities of 99.93% / 100%, 99.89% / 99.8% and 99.4% / 99.6% respectively, reflecting their origin from a single Seg-2 lineage.

These Indian BTV-12 strains isolated in 2011 to 2016 (12/IND/2015_KX905151, 12/IND/2016_MF615238, 12/IND/2011_KC662613), share a high nt sequence-identity (> 99%) reflecting the circulation and maintenance of the same endemic BTV-12 strain. VP2 is responsible for essential functions of virus attachment and entry into host cells, defining serotype, leading to functional constraints that potentially reduce the level of variation in the VP2 Seg-2 (and consequently Seg-2) sequences of these Indian BTV-12 strains over the time (Bonneau et al., 1999, Legisa et al., 2013).

Cattle can act as asymptomatic viral reservoirs, potentially allowing a BTV strain to persist in the field for several years (Breard et al., 2003). Phylogenetic analyses of Seg-2 showed that BTV-12 strains from India cluster together, collectively representing an Indian lineage that is closely related to BTV-12 strains from South Africa, Taiwan and Japan, with <3.58 % nt variation overall. This suggests that all of these BTV-12 strains share a relatively recent common ancestry, suggesting that Seg-2 of the Indian strains may be derived from an African origin (with <2.84% nt variation). However Seg-2 of the Indian BTV-12 strains do diverge from the American BTV-12 strains with an average 12.07 % nucleotide diversity.

In India, BTV-4 was isolated from Andhra Pradesh and Telangana States sporadically during 2007–2012. BTV-4 was also isolated from these states in 2013 during a widespread BTV-4 outbreak (Reddy et al., 2018). Serological evidence for presence of BTV-4 antibodies in sheep has been reported from India in 1973-75 (Prasad et al., 1992, Maan et al., 2009).

BTV-4 has also been reported in Africa (Coetzee et al., 2012a, van den Bergh et al., 2016), the Mediterranean region, the Middle East, southern and central Europe (Breard et al., 2007, Gomez-Tejedor, 2004, Nomikou et al., 2009, Hornyak et al., 2015 , Savini et al., 2008, Saegerman et al., 2008), USA (Groocock and Campbell, 1982, Price and Hardy, 1954), Central and South American countries including Trinidad and Tobago, Barbados, Puerto Rico, Dominican Republic, Argentina, Brazil (Balaro and Pituco, 2014 , Lager, 2004, Legisa et al., 2014, Legisa et al., 2013, Mo et al., 1994) and Australasia, including China (Yang et al., 2012).

Two Indian isolates from sporadic outbreaks in Telangana State during 2015, were analysed in this study, showing that they share identical Seg-2 coding sequences and identifying them as BTV-4. This indicates that their Seg-2 has a common origin. These Indian BTV-4 strains share a high nucleotide identity of 98.33% to 99.69 % with recent BTV-4 strains circulating during sporadic BT outbreak in Andhra Pradesh and Telangana States in Southern India during 2007–2013, reflecting continuing circulation of the same BTV-4 Seg-2 lineage.

The Indian BTV-4 isolates cluster closely with a BTV-4 strain from China (>91% nucleotide identity), identifying their collective derivation from an eastern Seg-2 topotype, showing >91 % nt and >96 % aa identity overall. In contrast, BTV-4 isolates from Africa, the Mediterranean region and South America grouped separately, as members of a western BTV-4 Seg-2topotype, showing only 71.4% to 72.52 % nucleotide and 79.5-80.65 % amino acid identity with the eastern BTV-4 isolates sequenced in this study.

The first report of BTV-9 was from Africa (Dungu et al., 2004a) and it has subsequently been reported from Australia, South East Asia, India (Pritchard et al., 2004, Maan et al., 2009), the Mediterranean region, central Europe (Maan et al., 2009) and America (Sperlova and Zendulkova, 2011)

BTV-9 was previously reported as responsible for BT outbreaks in Andhra Pradesh during 2002-2006 (Rao et al., 2012b). The BTV-9 strain isolated during the 2015 BT outbreak in Tamil Nadu and analysed here, is most closely related and shares high nt sequence identity (> 99%) with other BTV-9 strains belonging to the eastern Seg-2 topotype / lineage of BTV-9 from Southern India during 2002-2003. This suggests that this lineage of BTV-9 Seg-2 has persisted for at least a decade in the region. However, the first time that BTV-9 was isolated from Tamil Nadu which shares borders with Telangana and Andhra Pradesh states was in 2015. This may reflect the interstate migration of ruminants and wind- driven movement of *Culicoides* spp. vectors in this region of India.

Seg-2 sequences of global isolates of BTV-9 are clearly split them into major eastern and western topotypes that reflect the geographic origin of the virus. Indian BTV-9 strains belong to the same eastern geographic group as BTV-9 from Australia, Europe and Japan but are distinct from the African/American BTV-9 strains which belong to a distinct western BTV-9 Seg-2 topotype. The Indian BTV-9 isolates show a close relationship to BTV-9 strains from the Mediterranean region (>97% nt identity), raising concerns over intercontinental livestock trade.

BTV-10 was previously isolated in India from Telangana States during 2003 (Gollapalli et al., 2012, Rao et al., 2016a) and in 2004 from Andhra Pradesh (Maan et al., 2012e). Serological evidence for the presence of BTV-10 specific antibodies in Indian ruminants was reported in 1982 (Maan et al., 2009, Choudhary, 1982, Sriguppi, 1982). BTV-10 has also been reported from America (Barber, 1979), Africa (Coetzee et al., 2012a) and the French administrative areas of Martinique and Guyane in the Caribbean (Legisa et al., 2014).

Phylogenetic analysis of Seg-2 sequences showed that the Indian BTV-10 isolates (IND2014/BTV1_2014-12-06 and IND2014/BTV2_2014-12-06) sequenced in study have identical Seg-2 coding regions, indicating their common origin. Seg-2 of these viruses is most closely related to BTV-10 strains from Southern India isolated during 2003-2004 sharing > 99% nt identity, collectively grouping them within a western / American lineage / topotype of BTV-10 (Fig. 1).

The coding region of Seg-2 from the two Indian BTV-10 isolates from the 2014 outbreak in Thanjavur district of Tamil Nadu are identical to each other and to another Indian strain of BTV-10 isolated in 2003 from Khamman district, Andhra pradesh (10/IND/2003_KP339245), indicating circulation and maintenance of the same BTV-10 Seg-2 lineage over time. The clustering of Seg-2 of Indian strains of BTV-10 with BTV-10 isolates from the USA showing >99% nt identity, strongly suggests that BTV-10 was introduced to India from the United States. Animals have been imported to India from the USA for a breed improvement programme and it is considered possible that they were carrying BTV, resulting in the introduction of this exotic BTV-10 topotype. Seg-2 of the Indian BTV-10 strains also shares >99% nucleotide identity with the live attenuated vaccine strain of BTV-10 (10/USA/1980_U06782) from the United States. This raises concerns that the western vaccine strain may have been introduction / used, either by its illegal use in India or by international trade of livestock that were (persistently) infected (Maan et al., 2015c, Maan et al., 2012e, Rao et al., 2012a). The level of viraemia in ruminants caused by the use of modified attenuated BTV vaccines can be sufficient to infect midges feeding on the vaccinated animal and therefore onward virus transmission (Batten et al., 2008, Veronesi et al., 2010). In contrast, Seg-2 of BTV-10 isolates from South Africa, Martinique and French Guyana in the Caribbean, are more distantly related to Indian BTV-10 isolates with 88.71% to 89.24 % nt identity.

BTV-1 and BTV-2 are endemic in India and are reported to be among the most common serotypes circulating in the country, especially in Northern India (Rao et al., 2016b, Biswas et al., 2015, Biswas et al., 2010, Prasad et al., 1994, Jain et al., 1986). The three Indian BTV-1 isolates (IND2015/K13_2015-09-12, IND2015/40_2015-12-22 and IND2015/7A_2015-11-08) from Telangana, Tamil Nadu and Andhra Pradesh respectively, as well as four Indian BTV-2 isolates from Telangana (IND2015/K3_2015-09-10, IND2015/K9_2015-09-10, and IND2015/K14_2015-09-12) and Andhra Pradesh (IND2015/14A_2015-11-08) from the 2015 BT outbreaks, cluster closely with the Australian BTV-1 (1/AUS/1979_JN881986) and BTV-2 strains (2/AUS/2008_JQ086242) respectively with average nt/aa identities of >87/94%, indicating in both cases that they belong to eastern topotypes.

Seg-2 of three Indian BTV-1 isolates (IND2015/K13_2015-09-12, IND2015/40_2015-12-22 and IND2015/7A_2015-11-08) from Telangana, Tamil Nadu and Andhra Pradesh

States respectively, during the 2015 BT outbreak, that were sequenced here, share high nt/aa sequence identity (> 99 %) with BTV-1 strains isolated from Southern India isolated in 2010 (1/IND/2010_KP339145, 1/IND/2010_KP339135), indicating a close ancestral relationship and a common origin. Similarly, the four Indian BTV-2 isolates during the 2015 BT outbreaks from Telangana [IND2015/K3_2015-09-10, IND2015/K9_2015-09-10, and IND2015/K14_2015-09-12] and Andhra Pradesh [IND2015/14A_2015-11-08], sequenced in this study, share > 99 % nt/aa identity to each other reflecting their common origin and represented an extension of a single outbreak. They are also closely related (> 99 % nt and aa identity) to recent BTV-2 strains isolated in southern India during 2010 (2/IND/2010_KP339165). This suggests that geographic separation and acquisition of unique point mutations over time, has led to the evolution of a distinct Indian lineages of BTV-2 Seg-2.

This is the first time that BTV-5, BTV-9 and BTV-12 have been isolated from BTV outbreaks in Tamil Nadu (Southern India) during 2015. BTV-5 was previously isolated from sheep with clinical signs of BT during 2010-2011, and has been reported recently in Karnataka state during 2013 (Hemadri et al., 2016) and from Telangana state (P.P.Rao unpublished data). However, no full length sequence of BTV-5 has as yet been reported from India. The data presented here represent the first coding region Seg-2 sequence comparison of Indian field strains of BTV-5 isolated during 2015, with other BTV-5 strains from around the world.

The full length full genome sequences (n=45) were available in Genbank database for BTV strains from India. No full Seg-2 ORF sequence data has been reported for BTV-5 from India to date. In this study, full ORF of Seg-2 from BTV serotypes BTV-1, BTV-2, BTV-4, BTV-5, BTV-9, BTV-10 and BTV-12 in 46 BTV field strains isolated during 2014-2015 from India, with well documented isolation dates, geographical location of sample collection and passage histories have been analysed. This study substantially increases number of well documented full ORF Seg-2 sequence database of BTV isolates from India. At the time of writing, forty five full length full genome sequences of BTV strains from India were available in Genbank. The forty six full ORF of Seg-2 of BTV strains sequences, from the 2014-2015 BTV outbreaks in Southern India, generated in this study increase the number of full length ORF Seg-2 sequence database of Indian BTV strains

substantially, facilitating more robust molecular epidemiological studies of the evolution, geographical lineage and spread of BTV in India.

5.4 Summary

- The data presented here for genome Seg-2 of forty six Indian isolates of BTV from outbreaks in Southern India during 2014-2015, identifies seven different BTV serotypes (BTV-1, BTV- 2, BTV- 4, BTV-5, BTV-9, BTV-10 and 12). For isolates [IND2015/2017]547 tissue, [IND2015/] K13, [IND2015/271]7A and [IND2015/18] 40, the serotype detected by qPCR was different of that detected by NGS. BTV isolates [IND2015/K13], [IND2015/18] 40 and [IND2015/271]7A and [IND2015/2017]547 tissue, the discrepancy in Ct values between the corresponding group-specific and serotype-specific qRT-PCR results was noticed. Serotype BTV-1 (which was not detected by serotype-specific qRT-PCR) was confirmed by sequence analysis of BTV isolates [IND2015/] K13, [IND2015/18] 40 and [IND2015/271]7A. Low sensitivity to Indian strains/ topotypes due to primer/probe mismatching could also result in some mixed infections being missed. The results from BTV isolate [IND2015/271]7A (which was typed by serotype-specific qRT-PCR as BTV-4, but detected as BTV-1 by sequencing) and [IND2015/] K13 and [IND2015/18] 40 (which were typed by serotype-specific qRT-PCR as BTV-2, but detected as BTV-1 by sequencing) indicate that atleast one mixed infection was missed by the serotype-specific rRT-PCR assay. Similarly, BTV isolate [IND2015/2017]547 tissue] typed as BTV-10 by qRT-PCR was confirmed BTV-12 by NGS. Sequencing of the VP2 genes of the Indian BTV isolates would allow development of better matched primers and probes and would enable further optimization of the serotype-specific rRT-PCR assays for use to identify Indian BTV serotypes.
- Phylogenetic analyses of genome segment 2 of the Indian BTV strains sequenced in this study, grouped them into five Seg-2 nucleotypes A, E, G, H and I (Maan et al., 2007a) along with the reference strains of the same serotypes.

- Genome Seg-2 from Indian isolates of four serotypes BTV-1, BTV-2, BTV-4 and BTV-9, all showed >87 % nt and > 93 % aa identity with earlier, defined eastern

strains of the same serotype, identifying them as ‘eastern topotypes’ for Seg-2 and indicating their eastern origins.

- Genome Seg-2 from Indian isolates of three serotypes, BTV-5, BTV-10 and BTV-12 all showed >89 % nucleotide and > 92 % amino acid identity with previously defined western strains of the same serotype, identifying them as ‘western topotype’/ introductions.

- Genome Seg-2 of Indian BTV-5 and BTV-12 strains share a recent common ancestry with African-lineage Seg-2 BTV-5 and BTV-12 strains, whereas Indian BTV-10 strain sequenced in study share a recent common ancestry with American BTV-10 Seg-2 lineage. However, geographical separation has allowed them to acquire unique point mutations, which over time has led to the evolution of distinct Indian sub-lineages.

- This is the first time that BTV serotypes BTV-5, BTV-9 and BTV-12 have been isolated from the BTV outbreaks in Tamil Nadu (Southern India) during 2015.

- No full ORF Seg-2 sequence data of serotype BTV-5 has been reported from India to date.

- This study increases the number of well documented full coding sequence of Seg-2 in the database for BTV isolates from India, by adding forty six full ORF Seg-2 sequences of BTV strains from India, representing a valuable addition to sequence database.

Chapter 6: General discussion and future work

6.1 Bluetongue in India

BT is one of the most important arboviral disease in tropical and subtropical countries of the world (Elbers et al., 2008b). Bluetongue was first reported in Maharashtra state of Western India, in 1964 (Sapre, 1964), with subsequent outbreaks reported in other parts of the country (Prasad et al., 1992, Sreenivasulu et al., 2004, Ranjan et al., 2015, Prasad et al., 2009). BT is now considered endemic in India, with overt clinical outbreaks reported mostly in rural mixed flocks of native sheep and goats in the Southern states (Karnataka, Tamil Nadu, Telangana and Andhra Pradesh) (Rao et al., 2016b). Since 2001, twelve serotypes (BTV-1, BTV-2, BTV-3, BTV-4, BTV-5, BTV-9, BTV-10, BTV-12, BTV-16, BTV-21, BTV-23 and BTV-24) of BTV have been identified, mainly in Southern India (Rao et al., 2016b, Hemadri et al., 2016, Krishnajyothi et al., 2016).

A pentavalent inactivated BTV vaccine (Reddy et al., 2010) was developed by the all India Network Project on Bluetongue, a partnership supported by the Indian Council of Agricultural Research (ICAR), the Government of India and the Tamil Nadu Veterinary and Animal Science University (TANUVAS). The technology was transferred to Indian Immunological Limited (IIL), Hyderabad, in February 2011 (www.icar.org.in/node/3516). The vaccine was tested from 2010 onwards in five states of Southern India, including Maharashtra (a western state), Telangana, Andhra Pradesh, Tamil Nadu and Karnataka. It was subsequently commercialized on 8 January 2015, by Indian Immunologicals Limited in Hyderabad, Telangana, as the 'Raksha Blu' vaccine (a pentavalent inactivated BTV vaccine, containing serotypes BTV-1, BTV-2, BTV-10, BTV-16 and BTV-23). Presently, this vaccine is being used in five states of Southern India (including: Maharashtra, Telangana, Andhra Pradesh, Tamil Nadu and Karnataka) on a voluntary basis but not as a mass vaccination programme.

For effective control of BTV, there is a requirement for rapid and accurate diagnosis, to support the appropriate and timely vaccination of susceptible populations. It is also important to understand the epidemiology of the disease within endemic regions, and in the

country as a whole. Routine virological surveillance is very important for identifying currently circulating BTV serotypes, to ensure the use of vaccines of the appropriate BTV serotypes and strains as part of the BT control program (Rao et al., 2016b).

The primary objectives of this project include the identification and charcaterisation of BTV strains circulating in India, to assess the efficacy of the vaccines used, and their impact on the control of homologous and heterologous BTV serotypes in vaccinated individuals and in the animal population as a whole.

6.2 Attending BT field outbreaks from Southern India

As per the official reports, no BT outbreak was identified in the unified state of Andhra Pradesh, during 2014 (AICRP, 2014). However the State of Andhra Pradesh was divided into two states, namely Telangana and Andhra Pradesh in June, 2014. In October 2014, two suspected BT outbreaks that occurred in sheep flocks that had not been vaccinated against BT, were attended as part of this project, following unofficial reports from field veterinarians, in the Warangal and Mehboobnagar districts of Telangana. No further official outbreak information was available for the present study, either from Telangana, 2014, or from the All India Coordinated Research Project on Animal Disease Monitoring and Surveillance, 2014 (AICRP, 2014).

According to the Indian Council of Agriculture Research - National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), 13 suspected BT outbreaks were reported from 7 districts of Karnataka state, from July to January 2014: including, Chikkaballapur, Koppal, Raichur, Bagalkot, Gadog, Davanagere and Tumkur. These outrbreaks affected 990 animals and caused 160 deaths in sheep during 2014 (ICAR-NIVEDI, 2015, AICRP, 2014).

Despite the official reports of 13 BT outbreaks from Karnataka state in 2014, overall the BT outbreaks information from field veterinarians was poorly communicated during 2014. This could be due to only sporadic and mild or suspected cases of BT during that year. Generally, BT outbreak reporting is poor in India as BT, even though it is a notifiable disease, and reporting only occurs when there is a severe problem in the field (ICAR-NIVEDI, 2013).

BT outbreaks were reported in Thoothukudi, Tirunelveli, Karur, Erode, Tiruppur, Chengalpattu, Salem, Dindugal, Namakkal, Sivagangai and Kancheepuram districts of Tamil Nadu, from November to January 2014-2015 during the north east monsoon (http://www.tanuvas.tn.nic.in/lab) (information provided by Pro. K.M.N. Reddy, Head of Vaccine Research Centre-Viral Vaccines, Centre for Animal Health Studies Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu). As part of this study, suspected BT cases were investigated during this period, in the Karur, Erode, Tirupur, Chengalpattu, Salem and Thoothukudi districts, with twenty five (n = 25) EDTA blood samples collected from unvaccinated flocks.

The annual report from ICAR-NIVEDI for 2010 mentioned that the incidence of bluetongue was positively associated with climatic factors, such as temperature, humidity and particularly the pattern of monsoons (ICAR-NIVEDI, 2010). Rao and colleagues also reported that in Southern India, there is a correlation between BT and climatic conditions, with outbreaks following the pattern of the monsoons, which provide conducive breeding grounds for the local *Culicoides* populations involved in BTV transmission (Rao et al., 2016a). Severe BT outbreaks were reported in Southern India during 2005, which were linked with widespread flooding caused by heavy south-west and north east monsoons (Rao et al., 2016b, Reddy et al., 2016a, ICAR-NIVEDI, 2013). Similarly, severe BT outbreaks in 1989-1990 and 1997-1998 were reported from Tamil Nadu, linked to intense north east monsoons and flooding (Reddy et al., 2016a, Ilango, 2006).

The BT season is very long in Telangana, northern Andhra Pradesh and Karnataka states, with the highest incidence starting with the south west monsoon during August and September through to the end of north east monsoon in November to December. In contrast, in the Southern interior parts of Andhra Pradesh, south east Karnataka and Tamil Nadu, the disease is observed from October to December during the north east monsoon (Sreenivasulu et al., 2004). Overall, most outbreaks occur during September to December in these four Southern states, indicating that this period of the year is conducive for *Culicoides* breeding.

There were no ‘official’ BT outbreaks in Telangana during 2014 (AICRP, 2014), which may be correlated with a weak south west monsoon that year (Bhan et al., 2015), generating an environment that was less-suitable for *Culicoides.* Although, the south west

monsoon was weak, a 'normal' north east monsoon in 2014 contributed to BT outbreaks in Tamil Nadu and the interior of Karnataka states. However, only 13 suspected BT outbreaks were officially reported from 7 districts of Karnataka state (ICAR-NIVEDI, 2015, AICRP, 2014). Thirty five BT outbreaks were reported from Andhra Pradesh in 2015, with of no official reports of BT outbreaks in 2014 (AICRP, 2014). In contrast, a strong northeast monsoon, together with cyclonic storms, caused heavy rainfall in November and December during 2015, resulting in floods in Tamil Nadu (Gaitonde and Gopichandran, 2016). The moist soil after floods, coupled with ideal wind speeds, 5 kilometer per hour may have generated a climate that was more suitable compared to 2014, for the large scale breeding of *Culicoides*, resulting in severe outbreaks of BT in the interior of Karnataka and Tamil Nadu during late 2015. The ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI) reported 56 BT outbreaks in 14 districts of Karnataka state, with 120,529 BT suspected cases and 1,4168 fatalities, during the months of November and December of 2015, (ICAR-NIVEDI, 2016, AICRP, 2016), compared to 13 BT outbreaks reported in the previous year (AICRP, 2014).

Sporadic cases of suspect BT were attended in Warangal, Karimnagar and Medak districts of Telangana state during the south west monsoon in September 2015. Severe outbreaks of BT were also attended during the north east monsoon season in: the Anantpur and Chitoor districts of southern Andhra Pradesh, during early November 2015; the Chickballapur, Devanagere, Tumkure, Kolar and Chikmagalur districts of southeatern Karnataka State, during late November 2015; and the Perambalur, Shivagangai, Pudukottai, Tirunelveli, Erode and Thoothukudi districts of Tamil Nadu, during mid-December 2015.

Studies based on ecological factors, extracting weather parameters from outbreak areas, statistical analysis involving digital maps of water bodies, rivers, soil type and land cover together with meteorological factors such as temperature (Wittmann et al., 2002), rainfall and humidity indicate that these factors can all play potentially important roles in the incidence and progression of BT outbreaks (Johnson et al., 2006, Jacquot et al., 2017). Such studies also help in the further development of a predictive model for disease outbreaks, and in planning strategies to forecast and control them (ICAR-NIVEDI, 2010).

6.3 Clinical signs in BT suspected cases

Oral lesions, congested mucosa, coronitis, pyrexia, nasal discharge and salivation were among the most frequent clinical signs observed in the affected sheep flocks during the 2014-15 BT outbreaks, in four Southern states of India (Table 3.1). A swollen cyanotic tongue, nasal discharge speckled with blood, submandibular & head oedema, oral mucosa bleeding on touch and torticollis were also observed as rare clinical symptoms. However, more severe clinical signs were noticed during the outbreaks in Karnataka and Tamil Nadu states in 2015.

This finding may reflect the involvement of more virulent strains of BTV in these outbreaks. Waldvogel and colleagues showed that BTV virulence is strain/topotype dependent, not serotype dependent (Waldvogel et al., 1986). With multiple BTV strains co-circulating in the region, it is considered likely that frequent reassortment events would have occurred, which may have generated novel genotypes/ strains with more virulent phenotypes. This hypothesis could potentially be confirmed or discounted by further phylogenetic analyses of the full genomes of the circulating viruses, to determine the parental-origins / topotypes of each genome segment.

The clinical manifestations of BTV infection are also influenced by several factors related to the age, breed, species, immune and reproductive status of the host, as well as environmental stress factors and the pathogenicity of the BTV strain involved (Mertens et al., 2009). It is therefore unsurprising that the clinical manifestations and disease severity during BT outbreaks in 2015, and observed in this study, varied from acute to subclinical forms in sheep. However, during this study symptoms were seen to the same degree in both vaccinated and non-vaccinated sheep during outbreaks in Tamil Nadu and Karnataka states in 2015. Although the goats observed during these outbreaks were mostly asymptomatic, some clinical signs including pyrexia, abortion, still-birth and ‘limping’ were observed in a few goats in Tamil Nadu, 2015.

Interestingly ten unvaccinated cattle from Thoothukudi, in a mixed infected population of mainly sheep and goat flocks, showed clinical sign of lameness and excessive salivation. Mild oral lesions and limping were also evident in three unvaccinated cattle from infected mixed flocks, again mainly of sheep and goats in Tirunelveli, Tamil Nadu state during

2015. BT is generally asymptomatic in cattle and the observation of clinical signs in these animals suggests the possible involvement of a new strain / genotype or serotype, in naïve animals (Dedolli et al., 2017).

Cattle can act as an asymptomatic reservoir, potentially allowing a BTV strain to persist in the field for several years (Breard et al., 2003). Five BTV isolates from cattle were typed as part of this study, including: [IND2015/232]556 saliva, as a mono-serotype infection; by BTV-12; [IND2015/70]532 from Thoothukudi, Tamil Nadu, 2015, as a dual infection by BTV-2 and BTV-12; [IND2015/172]471, as a mono-serotype infection by BTV-5; [IND2015/175]472 as a mono-serotype infection with BTV-12; and [IND2015/181]474 from Tirunehveli, Tamil Nadu, 2015, as a dual infection by BTV-12 and BTV-10. These serotypes, mixed strains and infections may therefore be persistent in the field providing further opportunities for multiple genome-segment reassortment-events and the emergence of novel strains / genotypes.

There are reports of clinical symptoms of BT in cattle, including viremia, anorexia, hyperemic oral and nasal mucosa, nasal discharge, excessive frothy salivation, coronitis and a decrease in milk production (Williamson et al., 2008). Clinical signs of BT can be confused with FMD and other vesicular diseases, such as those caused by such peste des petits ruminants virus (PPRV), sheeppox virus (SPPV), goatpox virus (GTPV) and Orf virus (ORFV), which all have small ruminants and cattle as natural hosts and are endemic in India. These viruses must therefore be excluded in the ‘differential diagnosis’ of BT in cattle, goats and sheep (Bexiga et al., 2007). There are also reports of concurrent infections of BT and PPR in small ruminants from India (Maan et al., 2017, Biswas et al., 2010, Mondal et al., 2013, Mondal et al., 2009). Therefore, reliable laboratory diagnostic tests play important role in diagnosis, in order to identify BTV and confirm clinical cases and as a basis for implementation of appropriate control measures, e.g vaccination.

6.4 Serogroup specific qRT-PCR to diagnose BT

Although, diagnosis based on clinical signs, remains a traditional method for the detection and monitoring of BT, this requires well experienced veterinarians for differential diagnoses (Bexiga et al., 2007). Furthermore, asymptomatic BT infection in cattle and goats can go unnoticed. Sensitive and specific qRT-PCRs for the detection and

quantitation of BTV are therefore important, especially for the positive and reliable diagnosis of BTV infections, in a situation where a number of other clinically similar infectious diseases, like FMD and PPR occur.

Chand and colleagues developed a sandwich ELISA (s-ELISA) for the detection of bluetongue virus in clinical samples (Chand et al., 2009). However, serological tests are cumbersome, time consuming and need production of reference antiserum against purified antigens. Furthermore, the use of inactivated BT vaccines, which raise antibodies to all of the viral proteins in the host, makes it difficult or impossible to 'differentiate infected from vaccinated animals' (DIVA) (Maan et al., 2015a). However, RT-PCR assays do not give significantly positive signals as a result of vaccination with the inactivated BT vaccines. They can therefore be used to detect BTV reliably in clinical samples from infected animals, even in the face of inactivated vaccination campaigns (Alpar et al., 2009). Due to the persistent viraemia induced, BTV RNA can be detected in previously infected animals, for (at least) several weeks by RT-PCR, (Mertens et al., 2009). The real-time RT-PCR assays are sensitive and specific, with the ability to quantify viral RNA templates (copy number) and importantly, avoid the need for virus isolation. However, the very high sensitivity and specificity of qRT-PCR primers and probes can also lead to false negative results due to cross contamination in the laboratory, particularly in situations where conventional RT-PCR assays have been used and have produced very large amounts of viral cDNA.

Several serogroup-specific, quantitative real-time RT-PCR (qRT PCR) assays targeting different regions of the BTV genome, such as Seg-1 (Shaw et al., 2007, Toussaint et al., 2007), Seg-5(Jimenez-Clavero et al., 2006, Wilson et al., 2004, Polci et al., 2007, Toussaint et al., 2007), Seg-9 (Maan et al., 2015a) and Seg-10 (Orru et al., 2006, Chaignat et al., 2009) are available for detection of the virus. However, the specificity and sensitivity of RT-PCR assays may be reduced by sequence variations and genomic diversity of BTV strains from different geographic origins (Batten et al., 2008), which have evolved by genetic drift, recombination and gene reassortment (Bonneau et al., 2001).

In this study, the serogroup-specific, real-time RT-PCR assay targeting Seg-9 (Maan et al., 2015a) and the Seg-10 based assay (Hofmann et al., 2008a) were used to detect BTV in clinical samples from the 2014 and 2015 outbreaks respectively. The BTV RNA detection

efficiencies and mean Ct values generated were not directly compared for Indian field samples, using these assays. However in this study, the Seg-10 based assay appeared to be much more effective at detecting diverse BTV strains than the Seg-9 based assay.

In the present study, the prevalence of BTV in 9 field samples, tested by BTV serogroup-specific qRT-PCR targeting Seg-9 was 11% (1/9) from the Warangal and Mehboobnagar, Telangana BT outbreak in 2014 and was 69.5% (16/25) in 25 samples tested by the Seg-9 based assay, from the Tamil Nadu BT outbreaks in 2015. However, BTV RNA was detected in 21 out of 23 clinical samples (91% , 21/23) (21 EDTA blood and 1 necropsy spleen) and all 60 EDTA blood samples (100%, 60/60) from Anantpur and Chitoor district of Telangana, in 2015, using the BTV serogroup-specific qRT-PCR targeting Seg-10. The prevalence of BTV in 179 field samples (all EDTA blood, except 1 necropsy spleen), tested by Seg-10 based qRT-PCR was 44.7% (80/179) from the Karnataka BT outbreak in 2015, and was 50% (75/150) in 150 samples (143 EDTA blood, 2 saliva and 4 necropsy spleen plus 1 lymph node), tested from Tamil Nadu during the 2015 BT outbreaks.

Overall, the prevalence of BTV in 447 samples collected from the four Southern states, of Telangana, Andhra Pradesh, Karnataka and Tamil Nadu during 2014-2015 outbreaks was 53% (236/447) by BTV serogroup-specific qRT-PCR. The use of the Seg-10 based assay to detect BTV in clinical samples, appears to provide a reliable, sensitive and rapid diagnostic assay to distinguish BTV infections from other clinically similar diseases and therefore to study BTV epidemiology during outbreaks in India. Our findings are in line with BTV Seg-10 based qRT-PCR amplification of BTV RNA from clinical samples carried out by Brown-Joseph and colleagues (Brown-Joseph et al., 2017).

The samples that were detected as negative, are thought to be from animals that did not have a BTV infection, in spite of presenting with BT- like symptoms. However it is also possible that other BTV strains were also circulating in the region that was not detected by the standard qRT-PCR assays. The probes used in real-time PCRs are very sensitive to probe-target mismatches (Jimenez-Clavero et al., 2006). Mutations in the virus genome within the primer or probe ‘foot-print’ can therefore result in a ‘mismatch’ in the fluorescent probe target region and BTV strains belonging to different BTV serotypes, or different topotypes, may go undetected (false-negatives). Such problems might explain the lower-sensitivity thought to have occurred with the Seg-9 specific qRT-PCR assay. This

highlights the importance of validating the assays for the regional ecosystem and viruses for which they will be used and some of the problems that can occur with differential diagnosis of BT from other endemic diseases prevalent in the region. However, the Seg-10 based assay (Hofmann et al., 2008a) appears to be able to detect all BTV strains (Hoffmann et al., 2009).

Full length sequence data for the genome segments of the BTV isolates generated in this study, will be added to databases to support the development and evaluation of alternative primers and probes required for BTV serogroup-specific and serotype-specific RT-PCR assays, to ensure their relevance for the typing and detection of Indian BTV field strains. Variations between different BTV topotypes from India make the periodic validation of group-specific quantitative real-time RT-PCR assays important for these regions, and may require the use of multiplex primer systems.

BT disease may be present as a long lived subclinical infection in cattle, along with an associated viraemia with potential to infect *Culicoides*, causing transmission of the virus and disease (Alpar et al., 2009). Cattle are also thought to be preferentially bitten by adult *Culicoides* and can serve as a reservoir host for BTV, playing a more important role in the transmission of BTV than sheep (Brewer and MacLachlan, 1994). However, BTV infections are frequently asymptomatic or inapparent in cattle and goats, with severe disease mostly restricted to sheep and some wildlife species (Verwoerd and Erasmus, 2004).

Despite their likely role in the maintenance and onward transmission of BTV, vaccination of cattle against BT is not considered essential in endemic areas of India. Although sheep and goats may be vaccinated against BTV, cattle are ignored in the ongoing pentavalent vaccination campaign in Southern India. However, cattle from Thoothukudi (n=10) and Tirunelveli (n=3), in Tamil Nadu state, 2015 were found positive by BTV serogroup-specific qRT-PCR targeting Seg-10. These results suggest that a more widespread vaccination campaign against BTV in cattle might also help to reduce the size and spread of BT outbreaks.

From 2010 onwards, a pentavalent inactivated BTV vaccine was tested in four states of Southern India before its commercialization in 2015. Presently, this vaccine against BTV-

1, BTV-2, BTV-10, BTV-16 and BTV-23, is being used on a voluntary basis although not on a massive scale. Farmers are in the practice of vaccinating sheep during October, after the rainy season has started (August-September) and after clinical signs of bluetongue disease and the outbreaks have started. During this study, it was noticed that many flocks in Tirunelveli, Tamil Nadu were vaccinated in December, 2015 after the onset of BT. Although this may help to protect the individual vaccinated animals, and none of the vaccine serotypes were detected in vaccinated animals, it may be too late to significantly reduce the scale or spread of the outbreak. Keeping these facts in view, it would be prudent to adopt a more prophylactic vaccination for BT, on a basis timed to precede the seasonal outbreaks.

The Animal Husbandry and Veterinary Science (AH & VS) departments of each state run a mandatory vaccination programme for endemic disease, including *Peste des petits ruminants* (PPR) but this does not include BT. The circulation of multiple BTV serotype in Southern India since 2001 emphasizes a need for regular virological surveillance, to provide information to support a regular re-evaluation of the serotypes and strain composition of the available vaccines.

In this study, only 3 small flocks that had been vaccinated against BT in the Chikballapur district were available for observation and sampling during the 2015 outbreak in Karnataka state. One of these flocks of sheep, in the village Vemgal, Sidlaghatta, Chikballapur district of Karnataka State, had been vaccinated with a bivalent inactivated BT vaccine containing serotypes 1 and 23. Booster dose was given after 28 days generally. The other two flocks of sheep from the village Lakkenahalli, Sidlaghatta, and from the village Genjigunte, Sidlaghatta in the Chikballapur district of Karnataka State, 2015 were vaccinated with the pentavalent inactivated BT vaccine. These sheep were all vaccinated during August with the pentavalent BTV vaccine wth a booster dose given after 28 days of primary vaccination.

To date, no study of BT suspected clinical cases that had previously vaccinated with pentavalent inactivated BTV vaccine, has been carried out in India. Six other flocks of sheep and goats were vaccinated with the pentavalent inactivated BT vaccine in Tirunelveli and one flock of sheep and goats from Erode (Tamil Nadu), highlighting the fact that vaccination was not done on a massive scale. Of the 10 samples from BT vaccinated sheep

from Karnataka, 2015, all samples were positive by serogroup-specific qRT-PCR targeting Seg-10. Of the 37 samples from BT vaccinated sheep and goats in Tamil Nadu during 2015, 25 samples were positive by serogroup-specific qRT-PCR targeting Seg-10. However none of the samples taken from vaccinated animals contained any of the vaccine serotypes, suggesting that vaccination may be effective in protecting individual animals against the homologous serotypes.

6.5 Well characterised and documented BTV strains

In the present study, BTV isolates were made using KC and BHK cells, from well documented diagnostic samples, with records of collection date, geographical location, coordinates, species and vaccination status of the individual hosts. Overall, 141 BTV isolations were made from BTV outbreaks in Southern India: 6 during 2014 from EDTA treated blood samples (5 from Tamil Nadu and 1 from Telangana); and 135 during 2015 from EDTA blood (n=126), tissues [spleen (n=6), lymph node (n=1)] or saliva (n=2).

Virus isolation represents a gold standard for the identification of BT. In India, BTV isolation is usually attempted by yolk sac (Mason et al., 1940) or intravenous inoculation (Goldsmit and Barzilai, 1985) of clinical samples into embryonated chicken eggs. This is followed by subpassaging in mammalian cells, usually BHK-21 or Vero cells (Bommineni et al., 2008, Gollapalli et al., 2012, Rao et al., 2015, Rao et al., 2012b, Susmitha et al., 2012)). However, in this study, BTV was isolated by inoculating clinical samples directly onto an insect cell line *Culicoides sonorensis* cells (KC cells) (Wechsler and McHolland, 1988) followed by adaptation to BHK-21 cells. This greatly increased the success of BTV isolation, as reported by Rao and colleagues (Rao et al., 2016a). The 141 BTV isolates made during the 2014-15 Indian BT outbreaks, were deposited with appropriate virus collection numbers in the Veterinary Biotechnology lab, LUVAS, Haryana under the Indo-UK project titled “Development of diagnostic systems, reference collections and molecular epidemiology studies for important arboviral pathogens of livestock in India". Of these, 62 BTV isolates were serotyped by qRT-PCR assay targetting Seg-2 (Maan et al., 2016) and Seg-2 sequencing of 46 BTV isolates by NGS, at the Centre for Virus Research (MRC-CVR), University of Glasgow. These well characterised and documented BTV strains may provide seed stock for relevant vaccine development, or for development and validation of diagnostic assays.

6.6 Typing of BTV isolates

The detection of 12 BTV serotypes in India since 2001 (BTV-1, BTV-2, BTV-3, BTV-4, BTV-5, BTV-9, BTV-10, BTV-12, BTV-16, BTV-21, BTV-23 and BTV-24), mainly from the peninsular region (Maan et al., 2015c), emphasizes the need for a regular re-evaluation of the serotype and strain composition of the available vaccines. It is important to know which serotypes of BTV are currently circulating in the country and region in order to select appropriate vaccine strains to control BT outbreaks, or protect individual vaccinated animals.

In this study, a combination of BTV virus isolation, followed by serotype-specific real time RT-PCR assays for Seg-2, targeting BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 (Maan et al., 2016), were used to analyse 62 isolates from BTV outbreaks in Southern India during 2014-2015. This study detected the circulation of 7 BTV serotypes in four Southern states (Telangana, Andhra Pradesh, Karnataka and Tamil Nadu) during the 2014 and 2015 BTV outbreaks, including BTV-1, BTV-2, BTV-4, BTV-5, BTV-9, BTV-10 & BTV-12, with predominance of BTV-12 (59.7%) followed by BTV-2 (30.6%), BTV-5 (29%), BTV-4 (11.3%), BTV-10 (8.1%), BTV-9 and BTV-1 each 4.8% in 62 BTV isolates typed by qRT-PCR.These results agree with the findings of several investigators who have previously described BT outbreaks involving multiple co-circulating BTV serotypes.

The involvement of multiple circulating serotypes in the same outbreak may be a common phenomenon in subtropical regions, where multiple serotypes are endemic and a congenial climate exists for the multiplication of *Culicoides* throughout the year (Rao et al., 2016a). Reddy and colleagues previously reported the occurrence of BTV-9 and BTV-10 from Andhra Pradesh in 2010 (Reddy et al., 2010). Brenner and colleagues previously reported the co-circulation of BTV-4, BTV-8, BTV-16 and BTV-24 in Israel, during 2008 (Brenner et al., 2010).

However, for isolates [IND2015/2017]547 tissue, [IND2015/] K13, [IND2015/271]7A and [IND2015/18] 40, the serotype detected by qRT-PCR (Maan et al., 2016) was different to that detected by NGS. In these BTV isolates, [IND2015/]K13, [IND2015/18] 40, [IND2015/271]7A and [IND2015/2017]547 tissue, a discrepancy was noticed in the Ct

values between the corresponding group-specific and serotype-specific qRT-PCR results. The Ct value was lower in the group specific assay than in the type specific assay in these samples which suggest that there may have been more group specific RNA than type specific RNA for a single type, and therefore, more than one serotype in the sample.

BTV-1 (which was not detected in the serotype-specific qRT-PCR assays) was identified by sequence analysis of BTV isolates [IND2015/] K13, [IND2015/18] 40 and [IND2015/271]7A. Low sensitivity to Indian strains / topotypes due to mismatches in the footprints of the either the primers or probes used in the typing assays, could result in some serotypes and mixed infections being missed. The results from BTV isolate [IND2015/271]7A (which was typed by serotype-specific qRT-PCR as BTV-4, but identified as BTV-1 by sequencing) and [IND2015/] K13 and [IND2015/18] 40 (which were typed by serotype-specific qRT-PCR as BTV-2, but identified as BTV-1 by sequencing) indicate that at least one mixed infection was missed by the serotype-specific rRT-PCR assay. Similarly, BTV isolate [IND2015/2017]547 tissue, typed as BTV-10 by qRT-PCR was identified as BTV-12 by NGS.

Significant sequence variations occurs in Seg-2 of BTV strains that are the same serotype but from different geographic origins (different topotypes) (Maan et al., 2009). Full length sequence data of the VP2 genes of the 46 Indian BTV isolates sequenced in this study will be added to databases to allow development of better matched primers and probes. They could also be used in further evaluation of alternative qRT-PCR assays required for the serotype-specific qRT-PCR assays to identify Indian BTV serotypes. These results also confirm that there is a need to re-evaluate qRT-PCR assays from time to time, to ensure their ability to detect circulating serotypes in the region. The detection of BTV strains that were ‘missed’ by qRT-PCR targeting Seg-2 (Maan et al., 2016), is in line with the findings of several other studies (Rao et al., 2016b, Krishnajyothi et al., 2016, Susmitha et al., 2012, Brown-Joseph et al., 2017).

One of the BTV field isolates, [IND2015/] 07Karn, from an unvaccinated sheep during the 2015 BT outbreak in Karnataka state, could not be typed using the serotype-specific TaqMan based qRT-PCR assay against BTV-1 to BTV-24, BTV-26, BTV-27 and BTV-29 (Maan et al., 2016). This may be due to variations in the sequence of the VP2 gene of this strain, which is currently circulating in India, leading to a mismatch in the footprints of the

either the primers or probes, compared to the sequences of the primarily western reference strains used to design the typing assays. However, it is also possible, although less likely that this virus represents an entirely novel BTV serotype, or a perhaps novel Seg-2 topotype of one of the known serotypes. A similar failure to 'type' recently occurred with the novel serotypes BTV-25 to BTV-29. However, the typing of this isolate could be achieved by next generation sequencing and phylogenetic comparison of Seg-2 to the previously characterised strains. The seg-2 has not yet been sequenced by NGS for this isolate to identify its serotype.

BTV-2, BTV-5, BTV-9, BTV-10, BTV-12 and BTV-16 (Kumar et al., 2016) were identified in 24 unvaccinated animals from Tamil Nadu state during the 2014-15 BT outbreaks. Reddy and colleagues had previously reported the isolation of BTV-1, BTV-2, BTV-16 and BTV-23 from Tamil Nadu, between 2003-2011 (Reddy et al., 2016a). However, these strains were isolated before the start of field trails of pentavalent inactivated BT vaccination at farmer's doorstep in 2010 onwards suggesting that these strains would not be affected by vaccination programme. BTV-4, BTV-5 and BTV-12 were also identified during the current project, in 11 sheep and 3 goats that had been vaccinated with the pentavalent inactivated vaccine from Tamil Nadu, 2015. The animals were vaccinated during August 2015, with booster dose of vaccine after 28 days of primary vaccination generally. Clinical samples were taken in BT outbreak, December, 2015, four months after primary vaccination.None of the vaccine serotypes (BTV-1, BTV-2, BTV-10, BTV-16 and BTV-23) were detected in the vaccinated but infected animals, even though BTV-2, BTV-10 and BTV-16 were shown to be actively circulating in the region, suggesting that the vaccination may have protected individual animals against the homologous serotypes.

In Telangana state, serotypes BTV-2, BTV-4, BTV-5, BTV-9 and BTV-12 were identified in 11 unvaccinated animals, during the 2014-2015 BT outbreaks under study. BTV-1, BTV-2, BTV-4 and BTV-12 were identified in 5 unvaccinated animals in Andhra Pradesh, during the 2015 BT outbreaks., BTV-1, BTV-2, BTV-4, BTV-9, BTV-10, BTV-12, BTV-16, BTV-21 and BTV-24 had previously been isolated, between 2002 – 2013, from Andhra Pradesh and Telangana States (Gollapalli et al., 2012, Krishnajyothi et al., 2016, Reddy et al., 2016b, Susmitha et al., 2012, Reddy et al., 2018). During 2002-2013,

serotypes BTV-1, BTV-2, BTV-4, BTV-9, BTV-10, BTV-12, BTV-16, BTV-21 and BTV-24 were isolated from Andhra Pradesh (Rao et al., 2016b).

In this study, 22 BTV isolates were derived from 11 sheep and 3 goats from Tamil Nadu, and 8 sheep from Karnataka state during 2015, all of which had been vaccinated with the pentavalent inactivated BTV vaccine (BTV-1, BTV-2, BTV-10, BTV-16 and BTV-23). The 14 BTV isolates from Tamil Nadu were typed as BTV-4, BTV-5, BTV-9 and BTV-12 (with BTV-12 in 6 isolates, BTV-5 in 4 isolates, and 4 isolates containing mixed infections: BTV-12 and BTV-4; BTV-5 and BTV-12; BTV-4 and BTV-9; BTV-4 and BTV-12). Again none of the vaccine serotypes were detected in the vaccinated but infected animals.

Similarly, The 5 BTV isolates from animals vaccinated with the pentavalent inactivated BTV vaccine and 3 from animals vaccinated with the bivalent inactivated BTV vaccine (BTV-1 and BTV-23) during BT outbreaks in Karnataka state during 2015, were typed as BTV-4, BTV-5 and BTV-12: BTV-5 in 1 isolate, BTV-12 in 3 isolates and mixed infections in 4 isolates (including 3 isolates of BTV-5 and BTV-12, and 1 isolate of BTV-4, BTV-12). Again none of the vaccine serotypes were detected in the vaccinated but infected animals, although vaccination did not appear to influence the wider circulation of these serotypes in the unvaccinated population.

A future study performed on a larger scale could help to strengthen these findings to show the efficacy of vaccination against different BTV serotypes. However, no final conclusions can currently be made because of small sample size from vaccinated animals, the relatively small scale of the vaccination programme and the short duration of the present study after the start of vaccination use.

BTV-4, BTV-5 (a rare serotype in India) and BTV-9 were identified for the first time, in Tamil Nadu, during outbreaks in 2014-2015, along with and BTV-4 identified in Karnataka state during the 2014-2015. This study also confirms the frequent occurrence of dual or triple serotype mixed infections within the same animal.

Guimaraes and colleagues have reported co-circulation of multiple serotypes involving BTV-1, BTV-4 and BTV-17 in an outbreak in Brazil during 2014, as well as triple or dual co-infections with these serotypes within the same animal (Guimaraes et al., 2017).

Recently dual infection by BTV-2 and BTV-5 has been reported in a sheep from Karnataka, in 2010 (Hemadri et al., 2016). Co-infection with multiple BTV serotypes poses the risk of the generation and emergence of novel reassortant BTV strains that could have distinct properties in terms of infectivity and pathogenicity (Maan et al., 2012d, Maan et al., 2015b, Shafiq et al., 2013).

BT outbreaks in India can be dominated by a single serotype for a number of years before its dominance changes: for example BTV-12 has been shown to circulate in Telangana and Andhra Pradesh since 2010 (Rao et al., 2016a). BTV-1 and BTV-10 in 2003, BTV-1 again from 2006-11, BTV-2 in 2007 -10, BTV-4 from 2007-13, BTV-9 from 2002-07, BTV-16 from 2007-11 (Rao et al., 2016a). Continuous surveillance is required for proper identification and characterization of BT serotypes and topotypes circulating in the endemic regions. The results presented here confirm the persistant circulation of BTV serotypes, BTV-1, BTV-2, BTV-4, BTV-5, BTV9, BTV-10 and BTV-12 in Southern India during the 2014-15 BT outbreaks. These data could be used to update and ensure the design and implementation of relevant vaccines (containing the most appropriate serotypes / strains) as part of effective control measures for BT outbreaks.

6.7 Seg-2 based phylogenetic analysis of 46 Indian BTV strains from Southern India, 2014-15

Phylogenetic analysis of the full ORF of Seg-2 identified 7 BTV serotypes: BTV-1, BTV-2, BTV- 4, BTV-5, BTV-9, BTV-10 and BTV-12 in 46 Indian BTV isolates from BT suspected outbreaks in four Southern states of India: Telangana, Andhra Pradesh, Karnataka and Tamil Nadu, during 2014-2015. The majority of BTV isolates were confirmed as BTV-5 and BTV-12, 17 isolates each, while the remaining 12 BTV isolates were identified as: 3 BTV-1, 4 BTV-2, 2 BTV-4, 1 BTV-9 and 2 BTV-10. In each case, phylogenetic analysis revealed > 68.4 % nt sequence identity with reference strains of the corresponding serotypes. These relatively high levels of sequence variation may reflect the fact that most of the reference strains for individual BTV serotypes were originally derived from South Africa and consequently belong to western topotypes, while most Indian strains belong to the major eastern topotype. It also suggests that it would be helpful to indentify specific eastern strains of each serotype that could be used as reference strains for

eastern topotypes (such as the isolates identified here). However, in most cases these sequencing and phylogenetic analysis confirmed the results of serotype-specific Seg-2 qRT-PCR used for serotype identification (Maan et al., 2016) except for isolates [IND2015/2017]547 tissue, [IND2015/]K13, [IND2015/271]7A and [IND2015/18]40, where the serotype detected by qPCR was different of that detected by NGS. The BTV isolate [IND2015/271]7A was typed by serotype-specific qRT-PCR as BTV-4, but detected as BTV-1 by sequencing. Similarly, [IND2015/] K13 and [IND2015/18] 40 were detected as BTV-1 by sequencing, although typed by serotype-specific qRT-PCR as BTV-2. Similarly, BTV isolate [IND2015/2017]547 tissue typed as BTV-10 by qRT-PCR was confirmed BTV-12 by NGS.

Seg-2 from the Indian isolates of BTV serotypes BTV-1, BTV-2, BTV-4 and BTV-9 all showed >87 % nucleotide and > 93 % amino acid identity with earlier defined eastern strains of the same serotype, identifying them as 'eastern topotype' and indicating their origin from Asia or Australasia. In contrast Seg-2 from Indian isolates of serotype, BTV-5, BTV-10 and BTV-12, all showed >89 % nt and > 92 % aa identity with previously defined western strains of the same serotype, identifying them as members of the 'western topotype' and indicating their common origin and possible introduction from from Africa or America.

Seg-2 of 3 Indian BTV-1 isolates, 1 each from Telangana, Tamil Nadu and Andhra Pradesh States respectively, during the 2015 BT outbreak, share high nt/aa sequence identity (> 99 %) with BTV-1 strains isolated from Southern India in 2010 (1/IND/2010_KP339145, 1/IND/2010_KP339135), indicating a close ancestral relationship and a recent common origin. Similarly, 4 Indian BTV-2 isolates during the 2015 BT outbreaks (3 from Telangana and 1 from Andhra Pradesh, sequenced in this study) share > 99 % nt/aa identity to each other, reflecting their common origin and represented an extension of a single outbreak. They are also closely related (> 99 % nt and aa identity) to recent BTV-2 strains isolated in southern India during 2010 (2/IND/2010_KP339165). This suggests the persistence of this virus or at least of this lineage of Seg-2 in the field for over 5 years.

Two Indian isolates from sporadic outbreaks in Telangana State during 2015 share identical Seg-2 coding sequences indicating a common origin. These Indian BTV-4 strains

also share a high nucleotide identity of 98.33% to 99.69 % with previously sequenced BTV-4 strains circulating during sporadic BT outbreak in Andhra Pradesh and Telangana States in Southern India during 2007–2013, (Reddy et al., 2018) again reflecting the continuing circulation of the same BTV-4 Seg-2 lineage.

Nucleotide sequences of BTV-5 isolates from Karnataka 2015, Tamil Nadu 2015 and Telangana 2014 generated during this study are almost identical, supporting the conclusion that they are derived from the same Seg-2 lineage and represent a single outbreak. Serological evidence for the presence of neutralizing antibodies to BTV-5 was previously reported from Tamil Nadu, Maharashtra and Gujarat state (Prasad et al., 2009). BTV-5 has recently also been reported from clinical cases of BT in sheep in Karnataka state during 2010-2011 (Hemadri et al., 2016) as well as from Telangana state during 2013 (P.P.Rao unpublished data). These studies show the origin of Seg-2 of these BTV-5 strains from a single lineage. Seg-2 of the Indian and Chinese BTV-5 strains, is also relatively closely related to African BTV-5 strains, sharing average nt and aa identities of 94.96% and 97.77% respectively, which indicates that they share of a recent common ancestry (belonging to the same topotype) and suggests the introduction of an African-lineage BTV-5 into India (or possibly *vice versa*).

BTV-9 was previously reported as responsible for BT outbreaks in Andhra Pradesh during 2002-2006 (Rao et al., 2012b). The BTV-9 strain isolated during the 2015 BT outbreak in Tamil Nadu and analysed here, is most closely related and shares high nt sequence identity (> 99%) with other BTV-9 strains belonging to the eastern Seg-2 topotype / lineage of BTV-9 from Southern India, during 2002-2003. This suggests that this lineage of BTV-9 Seg-2 has persisted for at least a decade in the region. However, the first time that BTV-9 was isolated from Tamil Nadu, which shares borders with Telangana and Andhra Pradesh states, was in 2015. This may reflect the interstate migration of ruminants or wind- driven movement of *Culicoides* spp. vectors in this region of India. The Indian BTV-9 isolates show a close relationship to BTV-9 strains from the Mediterranean region (>97% nt identity), raising concerns over intercontinental livestock trade / wider-movements of adult *Culicoides*.

The coding region of Seg-2 from the two Indian BTV-10 isolates, from the 2014 outbreak in Thanjavur district of Tamil Nadu are identical to each other and to another Indian strain

of BTV-10 isolated in 2003 from the Khamman district, Andhra Pradesh (10/IND/2003_KP339245). This indicates the circulation and maintenance of the same BTV-10 Seg-2 lineage over time. Seg-2 of these viruses is most closely related to BTV-10 strains from Southern India isolated during 2003-2004 which share > 99% nt identity, collectively grouping them within a American topotype of BTV-10. The clustering of Seg-2 of Indian strains of BTV-10 with BTV-10 isolates from the USA showing >99% nt identity, strongly suggests that this lineage of BTV-10 was introduced to India from the United States.

Animals have been imported to India from the USA for a breed improvement programmes and it is considered possible that they were carrying BTV, resulting in the introduction of this exotic BTV-10 topotype. Seg-2 of the Indian BTV-10 strains also share >99% nucleotide identity with the live attenuated vaccine strain of BTV-10 (10/USA/1980_U06782) from the United States. This raises concerns that the western vaccine strain may have been introducted by illegal use of the vaccine in India, or by international trade of livestock that were (persistently) infected with the vaccine strain (Maan et al., 2015c, Maan et al., 2012e, Rao et al., 2012a).

17 Indian BTV isolates from BTV outbreaks Karnataka and Tamil Nadu states during 2015, were identified as BTV-12 in this study, with nearly identical Seg-2 sequences, indicating a common origin from a single epizootic. This is the first time that BTV serotype BTV-12 has been isolated in Tamil Nadu and Karnataka (Southern India). These Indian BTV-12 strains are also closely related to BTV-12 isolates from Haryana State in north India during 2015-2016 as well as recent isolates 12/IND/2011_KC662613 from the Adilabad district of Telangana State in 2013, with average nt/aa identities of 99.93% / 100%, 99.89% / 99.8% and 99.4% / 99.6% respectively. This again reflects a common ancestry with a single Seg-2 lineage, suggesting the circulation and maintenance of the same endemic BTV-12 strain for several years. Phylogenetic analyses of Seg-2 showed that BTV-12 strains from India cluster together, as an Indian lineage that is closely related to BTV-12 strains from South Africa, Taiwan and Japan, with <3.58 % nt variation overall. This demonstrates that all of these BTV-12 strains belong to the same Seg-2 topotype and share a relatively recent common ancestry, suggesting that Seg-2 of the Indian strains may be derived from an African origin (with <2.84% nt variation) or *vice versa.*

This is the first time that BTV-5, BTV-9 and BTV-12 have been isolated from BTV outbreaks in Tamil Nadu during 2015. The data presented here represent the first coding region Seg-2 sequence comparison of the Indian field strains of BTV-5 isolated during 2015, with other BTV-5 strains from around the world. No full Seg-2 ORF sequence data has previousaly been reported for BTV-5 from India.

In this study, the full ORF of Seg-2 from BTV serotypes BTV-1, BTV-2, BTV-4, BTV-5, BTV-9, BTV-10 and BTV-12 in 46 BTV field strains isolated during 2014-2015 from India, along with well documented isolation dates, geographical location of sample collection and passage histories have been analysed. This study substantially increases the number of well documented full ORF Seg-2 sequences in the database of BTV isolates from India. At the time of writing, forty five full length full genome sequences of BTV strains from India were available in Genbank. The data for forty six ORF of Seg-2 from different Indian BTV strains, from the 2014-2015 BTV outbreaks in Southern India, that were generated in this study, substantially increases the number of full length Seg-2 ORF in the sequence database for Indian BTV strains. These data, aliong with the analyses of the remainder of the genomes of these viruses that is still ongoing in Glasgow will facilitate more robust molecular epidemiological studies of the evolution, reassortment, geographical lineages and spread of BTV in India.

6.8 Future work

- The full genome sequencing and analyses is still ongoing in Glasgow. Sequencing and phylogenetic analyses of the entire BTV genome has helped in understanding the origins, lineage/topotype of each genome-segment as well as the evolution and emergence of reassortant strains. The presence of multiple circulating BTV serotypes in India provides multiple opportunities for reassortment between BTV strains that could have distinct properties in terms of their infectivity and pathogenicity (Maan et al., 2012d, Maan et al., 2015b, Shafiq et al., 2013).

  Bluetongue virus evolution occurs through the a mechanism that involves circulation as a ‘quasispecies’ driven by accumulation of mutations (genetic drift), and reassortment (genetic shift), intra-segment recombination and concatermerisation (gene duplication), although the latter two mechanisms are thought to be relatively rare but significant outcomes (Carpi et al., 2010, Maan et

al., 2012c, He et al., 2010, Anthony et al., 2011, Coetzee et al., 2012b, Mohd Jaafar et al., 2014, Nomikou et al., 2015, Shafiq et al., 2013, Bonneau et al., 2001, Oberst et al., 1987, Stott et al., 1987, Shaw et al., 2013, Batten et al., 2008).

- Significant sequence variations occurs in Seg-2 of BTV strains from different geographic origins (topotypes) that belong of the same serotype (Maan et al., 2009). Therefore, close monitoring of circulating BTV strains and BTV typing by serotype-specific TaqMan based qRT-PCR assay is important to ensure that the assays used in regular virological surveillance and to assess the molecular epidemiology of BT in India, remain relevant to currently circulating strains. The full-length sequence-data of the VP2 genes from the 46 Indian BTV isolates sequenced in this study will be used for the design of better 'matched' primers and probes and will support the further evaluation of hese alternative primers and probes for use in serotype-specific qRT-PCR assays to detect, identify and distinguish Indian BTV serotypes.
- The ICAR-NIVEDI annual report 2010 mentioned that the incidence of bluetongue is positively associated with climatic factors such as temperature, humidity and particularly the pattern of monsoons (ICAR-NIVEDI, 2010). Rao and colleagues reported that in Southern India, there was a correlation between BT outbreaks and climatic conditions, with BT particularly following the pattern of monsoons (Rao et al., 2016a) which provide conducive breeding grounds for the local *Culicoides* population that is involved in BTV transmission. Studies based on ecological factors, extracting weather parameters from outbreak areas, statistical analysis involving digital maps of water bodies, rivers, soil type and land cover, together with meteorological factors such as temperature, rainfall and humidity, will further help in the development of predictive models for the disease, and in planning strategies to forecast BT outbreaks well in advance.
- In the present study, the small sample size from vaccinated animals demands a future study on a larger scale to show the efficacy of vaccination against different BTV serotypes, not only in terms of protecting individual animals against infection and clinical disease, but also in terms of its effect on the incidence and spread of the vaccine serotypes and protection of the animal population as a whole.

  The currently circulating serotypes BTV-4, BTV-5, BTV-9 and BTV-12 detected in present study are not present in the pentavalent inactivated BT vaccine and could

be included to make it more currently relevant, replacing serotypes that were not commonly found in the most recent outbreaks (such as BTV-23 & BTV-16). The current study suggests that the vaccine is effective in individual animals and if the results of virological surveillance could be linked to the composition of the vaccine (which serotypes it contains) and if it was increased in scale (possibly even including cattle), vaccination could become more effective and may even help to reduce the circulation of these serotypes in the animal population as a whole.

- Routine virological surveillance is very important for identifying currently circulating BTV serotypes to ensure the use of available vaccines of the appropriate BTV serotype and strain composition in the BT control program.
- Further extensive studies involving large numbers of clinical cases that are suspected BT are needed to fully understand the severity and clinical presentation of dual or multiple mixed BT infections.

Appendix 1 **List of primers and probes for serotype-specific qRT-PCR (Maan et al., 2016)**

£Primer name	Primer sequence 5' to 3'
BTV-1/2575-2597Fw	GTATTTCTGAYGGTATTGTYTGG
BTV-1/1599-1623Fe	GCYAAATTRCGAATCAARCATRGYG
BTV-1/2653-2633Rw	TCATCAGATACCTCGATCGCT
BTV-1/1711-1689Re	GTTARCCTCTGCAAYACAATAGG
BTV-2/1401-1421Fw	GATGAYRYAARTAYTCTGAG
BTV-2/60-81Fe	GAGCATTTGTTGAAARGTTATG
BTV-2/1528-1503Rw	GYATCYYTTTCGAARTCRATTGTRAG
BTV-2/170-148Re	GATATCRAAYGCGTACATYTCTG
BTV-3/S2/619-640Fw	GARCGGTTRTCRACGGAWGARG
BTV-3/S2/718-694Rw	TATCRTAAGCGTTATCTCCTARCYG
BTV-4/S2/2470-2488F2	GAACACGAAGATATCGCAG
BTV-4/S2/2557-2532R2	GCATARAGAAGCTARATGTATCTTCA
BTV-5/S2/08-26Fw	GCTTCTCAGGATGGATGAG
BTV-5/S2/101-79Rw	CARRTCRAYCTTAAYRTCRTAYC
BTV-6/2001-2023F	GTCGATGTYACACAGTTGATCGT
BTV-6/2112-2090R	TAGCACGTCTAATCGTTTCTATG
BTV-7/S2/1608-1631Fw	AGTATGTGAGACGTCAATCTCAGA
BTV-7/S2/1704-1682Rw	GTCTAATAGGTCCGCAGCTTTAG
BTV-8/72-93Fw	GATGGRTATGATTACATCATTG
BTV-8/159-138Rw	GAATTYCTGTYACATCGTGTCG
BTV-9/1673-1694Fw	GGTTATGCTTCAATTACGAACG
BTV-9/1706-1724Fe	GTATGATACCAGGCCAGCG
BTV-9/1779-1756Rw	GGGTCTTATGTAGGGATGTCTGTG
BTV-9/1803-1783Re	GTTCATTTTGAGGATCATCCA
BTV-10/S2/1470-1488Fw	TATTRACWACWGAACCAAACCT
BTV-10/S2/1577-1557Rw	GYGARTTRATCCRTTTGTCAT
BTV-11/S2/1510-1530Fw	GGATGCGYAYYTGAATATTAG
BTV-11/S2/1617-1596Rw	ATCTCTCCATGAGTTATTCGCA
BTV-12/S2/999-1019Fw	ATACAATTCAGGCTATCCRGA
BTV-12/S2/1136-1116Rw	CAATGATYGTTCCTCGTAAGC
BTV-13/S2/1147-1169Fw	GGTGACGTYTATTATAAATTGCG
BTV-13/S2/1225-1207Rw	GGCGATCCARATCYCGWGG
BTV-14/S2/2616-2636Fw	GCCATTGARTTTTCTGAYGAYAG
BTV-14/S2/2758-2734Rw	TCWGTATAYGCCTTAACYGCTCT
BTV-15/S2/29-47Fw	CCTGTGAGCGTGATCGAAC
BTV-15/S2/177-156Rw	CTTACACCTATGTTTCGCACTC
BTV-16/1221-1243Fw	GCGAGAGCAAGAGAAGTATATCG
BTV-16/1193-1213Fe	GACCTGAATATAAACCGCGAG
BTV-16/1337-1319Rw	GATGTTCGATACGTCTGGG
BTV-16/1320-1297Re	ATTAATCAATTCGTACTCCCAGTG
BTV-17/S2/2178-2202Fw	TGCTRAAAGAGATCAAATTTGTRCGG
BTV-17/S2/2315-2295Rw	ACTTGATCGTATCGTCAAACA
BTV-18/S2/357-381Fw	GATTATCAACCACTTAAGGTCGACG
BTV-18/S2/451-425Rw	GCTCTCTTTGCGTGTAACCTTACCGTG

BTV-19/S2/2313-2336Fw	AGTGTTGRTATCRCATAAATTACG
BTV-19/S2/2410-2379Rw	GGAAAGTYAGATGCGAAATYARRGAAGTCAAT
BTV-20/S2/1838-1856Fw	GCAATATGTCCGCATGCTG
BTV-20/S2/1928-1909Rw	GCTCCGGGCTTAATTTTTCG
BTV-21/S2/1584-1603F	GCCAGATTAAAGATAACGCA
BTV-21/S2/1686-1669R	GTAAATCGATAGGGTCCG
BTV-22/S2/1013-1032Fw	ATCTCAAGCGGTCAAACAGA
BTV-22/S2/1124-1148Rw	CCATTTCACAYGCTATTATAGTTCC
BTV-23/S2/60-81F	GCGGARYTGTTAGATGGCTATG
BTV-23/S2/148-126R	GGAATTTGWGYRACRTCATGACG
BTV-24/S2/1901-1919Fw	GAACTAYGAGAAGCTTAYR
BTV-24/S2/2016-1994Rw	GCGAAAARTCYYTCATATCTA
BTV-25/S2/2554-2571F	TTATCGGACTCGCTCGTT
BTV-25/S2/2644-2622R	GTGGATTCAACTTATTATCTCCG
BTV-26/S2/1752-1771F1	GTTATAGGCAGCAGCAATCT
BTV-26/S2/1849-1831R1	GCATATATCCCTTTCACCT
BTV-27/S2/1334-1354F	GCAAATCACAAGAAAAAGAAG
BTV-27/S2/1450-1423R	GTAACTTCAAGCTTTCCTGGCG
BTV-29/S2/1502-1522F	GAAAAGAACATCTTCAACATG
BTV-29/S2/1642-1615R	GATCTCAGTCGTGGTTACTCT
BTV-4/S2/1379-1400Fe	TTGTGTAAAGTGGATGAGGAGA
BTV-4/S2/1504-1477Re	GAAGTCTATCGTCAAAAGGTTAGGGGCT

Probe name	Probe sequence 5'to 3'
BTV-1/2604-2627Pw	CCGATCACACATCCGAACAAATGC
BTV-1/1632-1662Pe	CAACGACRGAAYGATGAYCCRATGGTGAAAC
BTV-2/1455-1428Pw	CATTCCATCCACCATCTATAATTTCCCC
BTV-2/116-139Pe	CCAAGATGGCCGACATGACGTATC
BTV-3/S2/656-688Pw	CYCCRCAGTTTCAYACAATACAGAGGAACCATC
BTV-4/S2/2502-2529P2	TACCTGTTGTGACRTCCAAGTTGGACAC
BTV-4/S2/1454-1430Pe	CCGCTCTTGATCCCACCCACCTTGA
BTV-5/S2/36-61Pw	CCGATWTTKCGRTCGAGCCAAGTTCC
BTV-6/2086-2061P	CACCTTGAYTCATCCACACTACGAAC
BTV-7/S2/1635-1660Pw	CCACAATCTAGACCCGGCAATATCGC
BTV-8/132-106Pw	CGGGCTCATCACCTTCCTCTTCAACAC
BTV-9/1703-1727Pw	CTTATATGACACTCGCCCTGCCATC
BTV-9/1735-1762Pe	CAACCCTATCAATGAGACAACGCCAGAC
BTV-10/S2/1519-1552Pw	YCTTGGYNCGCGYTCTGAATTAGTATTYCCRCCY
BTV-11/S2/1540-1573Pw	YGTGCTCCCAAGTTATTTCGATCAATGGATCTAC
BTV-12/S2/1101-1077Pw	CTCCACCATATGCGCCAACGATAGC
BTV-13/S2/1206-1175Pw	CTTATATCCCTCACGTACGCTCCAYTCATACC
BTV-14/S2/2663-2683Pw	CCGGCTTCGCGCGAGRTTYCC
BTV-15/S2/130-105Pw	CCCTCCCGATAAAGCGACCATATTCC
BTV-16/1291-1264Pw	CCTTCGTTGCTGGCTCTCCCTCTAGATC
BTV-16/1291-1264Pe	CCTTCGTTGCRGGCTCTCCTTCTAAGTC
BTV-17/S2/2224-2254Pw	CCTCCCTCTGATGTTCCTTGTTCATGATAAC
BTV-18/S2/387-414Pw	CATGTACCATCACGGATAAGCCACGCCC
BTV-19/S2/2378-2346Pw	CCAAACCTATTATARTACGCACCRAGCTCAACC

Appendix

BTV-20/S2/1876-1902Pw	CCGTAAAACCGCTTTGATGCTGATGGC
BTV-21/S2/1613-1636P	CGCTCAACGTAAAGCAGATGACCC
BTV-22/S2/1101-1077Pw	CTCCACCAGATACGCCACCGATAAC
BTV-23/S2/92-118P	CGAYGTAAGCACACGYATCGATGAACC
BTV-24/S2/1944-1973Pw	CATCAGACTTACAYGCACCCGAARATAAAY
BTV-25/S2/2576-2605P	CCCTCCCAATAACACATCCAGAGAAGTGCC
BTV-26/S2/1796-1827P1	CGAGAGGACTTCGCTATGCTAACACATTACGC
BTV-27/S2/1392-1420P	CAACGGCATGCGTGATGATATTATACGGC
BTV-29/S2/1541-1572P	CATCTCGATAACCGCAATCACCTAGTGATGCC

[£] To detect serotypes BTV-1 to BTV-27 and BTV-29.

Abbreviations: F, Forward primer; R, Reverse primer; P, Probe; w, Western; e, Eastern.

.

Appendix 2 **Details of BTV isolates from Southern India, 2014-15.**

S.No.	Virus collection number	*Ct value	Vaccination status	Place of sampling	Isolation date	Host spp	Sample
1.	[IND2015/03]2	29	UV	Kayathar,Thoothukudi, TN	20/04/2016	S	B
2.	[IND2015/06]8	30	UV	Kayathar,Thoothukudi, TN	20/04/2016	S	B
3.	[IND2015/09]12	31	UV	Kayathar,Thoothukudi, TN	20/04/2016	S	B
4.	[IND2015/12]14	30	UV	Kayathar,Thoothukudi, TN	20/04/2016	S	B
5.	[IND2015/15]22	29	V	Kayathar,Thoothukudi, TN	21/04/2016	S	B
6.	[IND2015/18]40	30	UV	Kayathar,Thoothukudi, TN	21/04/2016	G	B
7.	[IND2015/21]36	36	UV	Kayathar,Thoothukudi, TN	20/04/2016	S	B
8.	[IND2015/23]34	30	UV	Kayathar,Thoothukudi, TN	20/04/2016	S	B
9.	[IND2015/26]42	23	V	Kayathar,Thoothukudi, TN	21/04/2016	S	B
10.	[IND2015/29]52	30	V	Kayathar,Thoothukudi, TN	21/04/2016	S	B
11.	[IND2015/33]54	26	V	Kayathar,Thoothukudi, TN	21/04/2016	S	B
12.	[IND2015/36]56	30	V	Kayathar,Thoothukudi, TN	21/04/2016	S	B
13.	[IND2015/40]62	28	V	Kayathar,Thoothukudi, TN	21/04/2016	S	B
14.	[IND2015/44]68	30	UV	Kayathar,Thoothukudi, TN	21/04/2016	S	B
15.	[IND2015/47]70	31	UV	Kayathar,Thoothukudi, TN	20/04/2016	S	B
16.	[IND2015/50]72	27	UV	Kayathar,Thoothukudi, TN	21/04/2016	S	B
17.	[IND2015/53]76	30	UV	Kayathar,Thoothukudi, TN	21/04/2016	S	B
18.	[IND2015/56]80	29	UV	Kayathar,Thoothukudi, TN	21/04/2016	S	B
19.	[IND2015/59]98	31	UV	Kayathar,Thoothukudi, TN	21/04/2016	S	B
20.	[IND2015/62]100	31	UV	Kayathar,Thoothukudi, TN	20/04/2016	G	B
21.	[IND2015/64]530	31	UV	Ottadanpathi, Thoothukudi, TN	08/04/2016	C	B
22.	[IND2015/67]531	32	UV	Ottadanpathi, Thoothukudi, TN	08/04/2016	C	B
23.	[IND2015/70]532	33	UV	Ottadanpathi, Thoothukudi, TN	08/04/2016	C	B
24.	[IND2015/73]533	32	UV	Ottadanpathi, Thoothukudi, TN	20/04/2016	C	B

25.	[IND2015/76]534	33	UV	Ottadanpathi, Thoothukudi, TN	21/04/2016	G	B
26.	[IND2015/79]535	28	UV	Ottadanpathi, Thoothukudi, TN	08/04/2016	G	B
27.	[IND2015/82]536	29	UV	Ottadanpathi, Thoothukudi, TN	08/04/2016	G	B
28.	[IND2015/86]541	28	UV	Ottadanpathi, Thoothukudi, TN	20/04/2016	G	B
29.	[IND2015/89]543	31	UV	Ottadanpathi, Thoothukudi, TN	21/04/2016	S	B
30.	[IND2015/92]545	31	UV	Ottadanpathi, Thoothukudi, TN	20/04/2016	S	B
31.	[IND2015/95]548	32	UV	Ottadanpathi, Thoothukudi, TN	20/04/2016	C	B
32.	[IND2015/98]549	32	UV	Ottadanpathi, Thoothukudi, TN	20/04/2016	C	B
33.	[IND2015/101]550	29	UV	Ottadanpathi, Thoothukudi, TN	20/04/2016	C	B
34.	[IND2015/104]551	32	UV	Ottadanpathi, Thoothukudi, TN	09/04/2016	C	B
35.	[IND2015/107]552	32	UV	Ottadanpathi, Thoothukudi, TN	21/04/2016	G	B
36.	[IND2015/110]479	28	V	Tirunelveli, TN	21/04/2016	G	B
37.	[IND2015/113]482	32	V	Tirunelveli, TN	20/04/2016	S	B
38.	[IND2015/116]481	29	V	Tirunelveli, TN	21/04/2016	S	B
39.	[IND2015/118]V1	28	V	Vemgal,Sidlaghattataluk, Chikballapur,K	30/04/2016	S	B
40.	[IND2015/121]V2	28	V	Vemgal, Sidlaghattataluk, Chikballapur,K	30/04/2016	S	B
41.	[IND2015/124]V13	24	V	Vemgal, Sidlaghattataluk, Chikballapur,K	30/04/2016	S	B
42.	[IND2015/127]V43	26	V	GenjigunteSidlaghattataluk, Chikballapur, K	30/04/2016	S	B
43.	[IND2015/130]V40	26	V	Genjigunte, Sidlaghattataluk, Chikballapur K	30/04/2016	S	B
44.	[IND2015/133]V	14	V	Lakkenahalli, Sidlaghattataluk,Chikballapur,K	30/04/2016	S	B
45.	[IND2015/136]V41	21	V	Genjigunte, Sidlaghattataluk, Chikballapur,K	30/04/2016	S	B
46.	[IND2015/139]V42	14	V	Genjigunte, Sidlaghattataluk, Chikballapur,K	30/04/2016	S	B
47.	[IND2015/142]V44	28	V	Lakkenahalli, Sidlaghattataluk, Chikballapur,K	30/04/2016	S	B
48.	[IND2015/145]V44	16	V	Lakkenahalli, Sidlaghattataluk, Chikballapur,K	30/04/2016	S	B
49.	[IND2015/148]491	33	UV	Kanchipuram, TN	01/05/2016	S	B
50.	[IND2015/151]492	31	UV	Kanchipuram, TN	01/05/2016	S	B
51.	[IND2015/154]493	26	UV	Kanchipuram, TN	01/05/2016	S	B
52.	[IND2015/157]494	27	UV	Kanchipuram, TN	30/04/2016	S	B
53.	[IND2015/160]CS4	18	V	Tirunehveli, TN	30/04/2016	S	B
54.	[IND2015/163]CS6	14	V	Tirunehveli, TN	30/04/2016	S	B

55.	[IND2015/166]CS9	33	V	Tirunehveli, TN	30/04/2016	S	B
56.	[IND2015/169]470	18	V	Tirunehveli, TN	30/04/2016	S	B
57.	[IND2015/172]471	33	UV	Tirunehveli, TN	30/04/2016	C	B
58.	[IND2015/175]472	15	UV	Tirunehveli, TN	30/04/2016	S	B
59.	[IND2015/178]473	14	V	Tirunehveli, TN	30/04/2016	S	B
60.	[IND2015/181]474	18	UV	Tirunehveli, TN	30/04/2016	S	B
61.	[IND2015/184]475	36	V	Tirunehveli, TN	30/04/2016	S	B
62.	[IND2015/187]476	24	V	Tirunehveli, TN	30/04/2016	S	B
63.	[IND2015/190]477	18	V	Tirunehveli, TN	30/04/2016	S	B
64.	[IND2015/193]478	31	V	Tirunehveli, TN	30/04/2016	S	B
65.	[IND2015/196]480	20	V	Tirunehveli, TN	30/04/2016	S	B
66.	[IND2015/199]483	19	V	Tirunehveli, TN	30/04/2016	S	B
67.	[IND2015/202]484	32	V	Tirunehveli, TN	30/04/2016	S	B
68.	[IND2015/205]486	30	V	Tirunehveli, TN	30/04/2016	S	B
69.	[IND2015/208]404	36	V	Erode, TN	30/04/2016	S	B
70.	[IND2015/211]406	13	UV	Erode, TN	30/04/2016	S	B
71.	[IND2015/214]408	27	UV	Erode, TN	30/04/2016	S	B
72.	[IND2015/2017]547	35	UV	Thoothukudi, TN	30/04/2016	S	Sp
73.	[IND2015/220]228	30	UV	Tirunelveli, TN	30/04/2016	S	Sp
74.	[IND2015/223]34	15	UV	Thoothukudi,TN	30/04/2016	S	Sp
75.	[IND2015/226]PPR S	28	UV	Medak, T	30/04/2016	S	Sp
76.	[IND2015/229]555	28	UV	Thoothukudi, TN	30/04/2016	C	Sa
77.	[IND2015/232]556	30	UV	Thoothukudi, TN	30/04/2016	C	Sa
78.	[IND2015/235]BTV14	27	UV	Thoothukudi, TN	30/04/2016	S	LN
79.	[IND2015/238]6PK	10	UV	Jukal, Parkal, Warangal,T	02/05/2016	S	B
80.	[IND2015/241]K3	10	UV	Medaram, Dharmaram, Karimnagar,T	02/05/2016	S	B
81.	[IND2015/244]K4	9	UV	Mallesham, Khilavanaparthi , Karimnagar,T	02/05/2016	S	B
82.	[IND2015/247]K8	27	UV	Oongathurthi, Karimnagar,T	03/05/2016	S	B
83.	[IND2015/250]K9	10	UV	Oongathurthi, Karimnagar, T	03/05/2016	S	B
84.	[IND2015/253]K10	21	UV	Madhurampally,Pegadapally, Karimnagar, T	03/05/2016	S	B

85.	[IND2015/256]K14	11	UV	Madhurampally,Pegadapally Karimnagar, T	03/05/2016	S	B
86.	[IND2015/259]K15	26	UV	Madhurampally,Pegadapally, Karimnagar, T	03/05/2016	S	B
87.	[IND2015/262]PPR2	27	UV	Nalthur , Medak, T	03/05/2016	G	B
88.	[IND2015/265]PPR1	25	UV	Rauiapalli, Medak,T	03/05/2016	G	B
89.	[IND2015/268] 6A	28	UV	Open air jail,Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
90.	[IND2015/271] 7A	17	UV	Open air jail, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
91.	[IND2015/274] 9A	27	UV	Ammavarapalli,Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
92.	[IND2015/277]11A	28	UV	Ammavarapalli,Bukkarayasamundram, Anantpur,AP	03/05/2016	S	B
93.	[IND2015/280]12A	31	UV	Ammavarapalli,Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
94.	[IND2015/283]25A	30	UV	Ammavarapalli,Bukkarayasamundram, Anantpur,AP	03/05/2016	S	B
95.	[IND2015/286]27A	28	UV	Bomnalatapally, Bukkarayasamundram, Anantpur,AP	03/05/2016	S	B
96.	[IND2015/289]28A	26	UV	Bomnalatapally, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
97.	[IND2015/292]29A	23	UV	Bomnalatapally, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
98.	[IND2015/295]30A	28	UV	Bomnalatapally, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
99.	IND2015/298] 40A	27	UV	Bomnalatapally, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
100.	[IND2015/301]12Karn	26	UV	Gonimoradahalli,Sidlaghattataluk, Chikballapur, K	03/05/2016	S	B
101.	[IND2015/302]18Karn	28	UV	G.Kuruboahalli, Sidlaghattataluk,Chikballapur, K	03/05/2016	S	B
102.	[IND2015/307]31Karn	26	UV	VemgalSidlaghattataluk, Chikballapur, K	03/05/2016	S	B
103.	[IND2015/310]35Karn	29	UV	Genjigunte, Sidlaghattataluk, Chikballapur, K	03/05/2016	S	B
104.	[IND2015/313]40Karn	29	UV	Lakkenahalli,Sidlaghattataluk, Chikballapur, K	03/05/2016	S	B
105.	[IND2015/316]K1-K	29	UV	Vemgal,Sidlaghattataluk, Chikballapu, K	03/05/2016	S	B
106.	[IND2015/319]K2-K	26	UV	VemgalSidlaghattataluk, Chikballapur, K	03/05/2016	S	B
107.	[IND2015/322]K3-K	23	UV	Vemgal,Sidlaghattataluk, Chikballapur, K	03/05/2016	S	B
108.	[IND2015/325]K16-K	27	UV	Burudugun, chintamani, Chikballapur, K	03/05/2016	S	B
109.	[IND2015/328]K20-K	26	UV	Gangireddypalya , chintamani, Chikballapur, K	03/05/2016	S	B
110.	[IND2015/]A14	31	UV	Madhurampally,Pegadapally,Karimnagar,T	03/05/2016	S	B
111.	[IND2015/]26A	31	UV	Ammavarapalli,Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
112.	IND2015/]31A	31	UV	Bomnalatapally, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
113.	[IND2015/]32A	33	UV	Bomnalatapally, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
114.	[IND2015/]33A	31	UV	Bomnalatapally, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B

115.	[IND2015]/34A	33	UV	Bomnalatapally, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
116.	[IND2015/]35A	30	UV	Bomnalatapally, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
117.	[IND2015/]36A	30	UV	Bomnalatapally, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
118.	[IND2015/]37A	28	UV	Bomnalatapaly, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
119.	[IND2015/]38A	28	UV	Bomnalatapally, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
120.	[IND2015/]39A	32	UV	Bomnalatapally, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
121.	[IND2015/]07Karn	28	UV	Gowdanahalli, Chikballapur, K	03/05/2016	S	B
122.	[IND2015/]08Karn	33	UV	Gowdanahalli , Chikballapur, K	03/05/2016	S	B
123.	[IND2015/]32Karn	30	UV	VemgalSidlaghattataluk, Chikballapur, K	03/05/2016	S	B
124.	[IND2015/]33Karn	31	UV	VemgaSidlaghattataluk, Chikballapur, K	03/05/2016	S	B
125.	[IND2015]K1	29	UV	Medaram,Dharmaram,Karimnagar,T	03/05/2016	S	B
126.	[IND2015/]K11	30	UV	Madhurampally,Pegadapally,Karimnagar, T	03/05/2016	S	B
127.	[IND2015/]K12	30	UV	Madhurampally,Pegadapally, Karimnagar, T	03/05/2016	S	B
128.	[IND2015/]K13	28	UV	Madhurampally,Pegadapally, Karimnagar ,T	03/05/2016	S	B
129.	[IND2015/]PPR1	30	UV	Nalthur, Medak, T	03/05/2016	G	B
130.	[IND2015] 4A	30	UV	Open air jail, Bukkarayasamundram, Anantpur, AP	03/05/2016	S	B
131.	[IND2015]spleen K	28	UV	Gowdanahalli, MandikalMandal, Chikballapur, K	03/05/2016	S	Sp
132.	[IND2015]3 organ	30	UV	Thoothukudi,TN	30/04/2016	S	Sp
133.	[IND2015]19Karn	27	UV	G.Kuruboahalli, Sidlaghattataluk, Chikballapurtaluk, K	03/05/2016	S	B
134.	[IND2015]20Karn	26	UV	Chokkanahalli,Sidlaghatta,Chikballapur, K	03/05/2016	S	B
135.	[IND2015]21Karn	28	UV	Chokkanahalli,Sidlaghatta,Chikballapur, K	03/05/2016	S	B
136.	[IND2014]CHE2	28	UV	Erode,TN	02/02/2015	S	B
137.	[IND2014]CHE5	23	UV	Tirupur,TN	02/02/2015	S	B
138.	[IND2014]CHE12	30	UV	Karur,TN	02/02/2015	S	B
139.	[IND2014]CHE14	29	UV	Karur, TN	02/02/2015	S	B
140.	[IND2014]CHE3	28	UV	Karur, TN	02/02/2015	S	B
141.	[IND2014]WGL4	28	UV	Warangal, TN	30/01/2015	S	B

Appendix

*based on BTV specific qRT-PCR targeting BTV Seg- 10 (Hofmann et al; 2008) for 2015 outbreak and BTV specific qRT-PCR targeting BTV Seg-9 for 2014 outbreaks (Maan et al., 2015a)

Abbreviations: TN, Tamil Nadu; T, Telangana; K, Karnataka; AP, Andhra Pradesh; V, Vaccinated; UV, Unvaccinated; S, Sheep; G, Goat; C, Cattle; B, Blood; Sp, Spleen; LN, Lymph node; Sa, Saliva

Appendix 3 **Detail of Seg-2 sequences of BTV reference strains available from GenBank.**

Seg-2 sequences of reference strains of BTV serotypes BTV-1 to BTV-27 available from GenBank

Serotype	Country code	Year of sampling	GenBank accession number
BTV-1	AUS	1979	JN881986
BTV-1	RSArrrr	-	FJ969720
BTV-2	AUS	2008	JQ086242
BTV-2	RSArrrr	1959	KP821030
BTV-3	RSArrrr	1943	KP821039
BTV-4	RSArrrr	1969	KP821066
BTV-4	CHN	1997	JX560414
BTV-5	RSArrrr	1953	KP821101
BTV-6	RSArrrr	-	AJ585127
BTV-7	RSArrrr	-	AJ585128
BTV-8	RSArrrr	1937	KP821076
BTV-9	AUS	1985	JQ086302
BTV-9	RSArrrr	1942	KP821088
BTV-10	RSArrrr	-	AJ585131
BTV-11	RSArrrr	-	AJ585132
BTV-12	RSArrrr	1941	KP821100
BTV-13	RSArrrr	-	AJ585134
BTV-14	RSArrrr	1959	KP820983
BTV-15	RSArrrr	1960	KP820984
BTV-16	RSArrrr	-	AJ585149
BTV-17	RSArrrr	-	AJ585138
BTV-18	RSArrrr	-	AJ585139
BTV-19	RSArrrr	-	AJ585140
BTV-20	RSArrrr	-	AJ585141
BTV-21	RSArrrr	-	AJ585142
BTV-22	RSArrrr	-	AJ585143
BTV-23	RSArrrr	-	AJ585144

BTV-24	RSArrrr	-	AJ585145
BTV-25	CHE	2007	EU839840
BTV-26	KWT	2010	HM590642
BTV-27	FRA	-	KM200718

The country codes were Australia (AUS), Kuwait (KWT), Switzerland (CHE), France: Corsica (FRA), China (CHN) and India (IND). RSArrrr stood for a reference strain (rrrr) originated in Republic of South Africa (RSA).

Seg-2 sequences of reference Indian strains of BTV serotypes available from GenBank

Serotype	Country Code	State	Year of sampling	GenBank Accession no.
BTV-1	IND	Thoothukudi, Tamil Nadu	2003	KP696563
BTV-1	IND	Haryana	1999	AJ585114
BTV-1	IND	Chennai	2001	AJ585115
BTV-1	IND	Avikanagar, Rajasthan	1992	AJ585111
BTV-1	IND	-	1992	AJ585112
BTV-1	IND	-	1988	AJ585113
BTV-1	IND	Khammam, Andhra Pradesh	2008	JX399149
BTV-1	IND	Andhra Pradesh	2008	KF664124
BTV-1	IND	Karimnagar, Telangana	2010	KP339135
BTV-1	IND	-	1985	KP696508
BTV-1	IND	Coimbatore, Tamil Nadu	2003	KP696553
BTV-1	IND	Gujarat	2007	KF664114
BTV-1	IND	-	2003	KP696573
BTV-1	IND	-	2008	KU234258
BTV-2	IND	-	1982	AJ585152
BTV-2	IND	-	1994	KP268775
BTV-2	IND	Chittoor, Andhra Pradesh	1993	KP339155
BTV-2	IND	South India	1993	KP696563

Appendix

BTV-3	IND	-	2003	JQ771814
BTV-4	IND	Andhra Pradesh	2008	KF560418
BTV-9	IND	Andhra Pradesh	2006	JF443167
BTV-9	IND	Andhra Pradesh	2007	JX003688
BTV-9	IND	-	2003	KP339176
BTV-9	IND	Mahboobnagar, Andhra Pradesh	2003	KP339185
BTV-9	IND	Hyderabad	2005	KP696633
BTV-9	IND	Andhra Pradesh	2004	KP696613
BTV-9	IND	Hyderabad	2005	KP696633
BTV-9	IND	Hyderabad	2004	KP696623
BTV-9	IND	Hyderabad	2005	KP696642
BTV-10	IND	Khammam, Andhra Pradesh	2003	KP339245
BTV-12	IND	Andhra Pradesh	2011	KC662613
BTV-12	IND	Pataudi, Gurugram, Haryana	2016	MF615238
BTV-16	IND	Karur, Tamil Nadu	2014	KX302635
BTV-16	IND	Adilabad, Telangana	2011	KP339215
BTV-16	IND	Mahboobnagar, Telangana	2010	KP339205
BTV-16	IND	Mahboobnagar, Telangana	2010	KP339195
BTV-16	IND	Nellore, Andhra Pradesh	2010	KP339225
BTV-16	IND	Chennai	2010	JQ924821
BTV-16	PAK	West Pakistan	1960	JX272460
BTV-16	IND	Andhra Pradesh	2008	KF664104
BTV-16	IND	Gujarat	2011	KF664134
BTV-21	IND	South India	2006	KP339235
BTV-23	IND	Bangalore	1997	AJ585189
BTV-23	IND	Maharastra	1988	JQ771824

The country code: India (IND)

Seg-2 sequences of reference Indian strains of BTV-1 available from GenBank

Serotype	Country code	Year of sampling	Genbank accession number
BTV-1	ZAF	-	X55800
BTV-1	ZAF	-	JX272380
BTV-1	RSAvvvv	1958	KP821017
BTV-1	RSAvvvv	-	AJ585110
BTV-1	SDN	1987	AJ585117
BTV-1	SDN	1987	KP821022
BTV-1	RSArrrr	1958	AJ585122
BTV-1	CHN	1997	JN848760
BTV-1	ZAF	-	FJ969720
BTV-1	ZAF	-	JX680458
BTV-1	NGA	1982	AJ585118
BTV-1	CMR	1982	AJ585119
BTV-1	CMR	1982	KP820998
BTV-1	LBY	2007	KP821008
BTV-1	MAR	2007	KP821011
BTV-1	TUN	2011	KJ577105
BTV-1	ITA	2010	KJ577115
BTV-1	DZA	2008	KP820997
BTV-1	ITA	2013	KJ577125
BTV-1	ITA	2013	KJ661730
BTV-1	ITA	2013	KJ019206
BTV-1	ITA	2012	KJ577095
BTV-1	DZA	2006	KP820996
BTV-1	MAR	2006	EU625362
BTV-1	DZA	2006	EU625361
BTV-1	DZA	2006	KP820995
BTV-1	MAR	2006	KP821009
BTV-1	MAR	2009	KP821016

Serotype	Country code	Year of sampling	GenBank accession number
BTV-1	AUS	2010	KM099538
BTV-1	AUS	2010	KM099539
BTV-1	AUS	1994	KM099528
BTV-1	AUS	1993	KM099527
BTV-1	AUS	1997	KM099530
BTV-1	AUS	1996	KM099529
BTV-1	AUS	1999	KM099532
BTV-1	AUS	1999	KM099531
BTV-1	AUS	2002	KM099533
BTV-1	AUS	2004	KM099535
BTV-1	AUS	2005	KM099536
BTV-1	AUS	2002	KM099534
BTV-1	AUS	2008	KM099537
BTV-1	CHN	1979	KC879616
BTV-1	MYS	1987	AJ585116
BTV-1	KOR	2010	KC153300
BTV-1	GRC	2001	KP821006
BTV-1	GRC	2001	KP821007
BTV-1	GRC	2001	JN635334
BTV-1	GRC	2001	AJ585121
BTV-1	GRC	2001	KP821005
BTV-1	IND	1988	AJ585113
BTV-1	IND	1992	AJ585112
BTV-1	IND	2008	KF563945
BTV-1	IND	2007	KF563935
BTV-1	IND	2007	KF563933
BTV-1	IND	2007	KF563934
BTV-1	IND	2008	KF664124

Appendix

BTV-1	MAR	2009	KP821014	BTV-1	IND	2008	JX399149
BTV-1	MAR	2009	KP821015	BTV-1	IND	-	KC751420
BTV-1	ITA	2006	KJ736002	BTV-1	IND	2010	KP339145
BTV-1	TUN	2007	KP821023	BTV-1	IND	-	KC751421
BTV-1	MAR	2007	KP821012	BTV-1	IND	2010	KP339135
BTV-1	MAR	2007	KP821010	BTV-1	IND	1992	AJ585111
BTV-1	MAR	2007	KP821013	BTV-1	IND	1994	KF563930
BTV-1	ESP	2007	KP821018	BTV-1	IND	2003	KP696553
BTV-1	GIB	2007	KP821004	BTV-1	IND	1985	KP696508
BTV-1	PRT	2007	EU498674	BTV-1	IND	1985	KF563929
BTV-1	ESP	2007	KP821019	BTV-1	IND	1999	AJ585114
BTV-1	FRA	2008	KP821001	BTV-1	IND	2001	AJ585115
BTV-1	FRA	2008	KP821000	BTV-1	IND	2003	KP696563
BTV-1	FRA	2008	KP820999	BTV-1	IND	2007	KF563938
BTV-1	FRA	2008	KP821002	BTV-1	IND	2007	JX101695
BTV-1	FRA	2008	JX861499	BTV-1	IND	2003	KF563932
BTV-1	FRA	2007	FJ437557	BTV-1	IND	2011	KF563946
BTV-1	FRA	2007	JX861489	BTV-1	IND	2007	JX101694
BTV-1	ESP	2009	KP821021	BTV-1	IND	2008	KF563940
BTV-1	FRA	2008	KP821003	BTV-1	IND	2008	HM014236
BTV-1	ESP	2008	KP821020	BTV-1	IND	2008	KF563939
BTV-1	USA	2010	KX164020	BTV-1	IND	1999	KF563931
BTV-1	GUF	2015	KY049854	BTV-1	IND	2003	KP696573
BTV-1	GUF	2011	JQ436736	BTV-1	IND	2008	KF563941
BTV-1	GUF	2011	KY049844	BTV-1	IND	2007	JN558348
BTV-1	AUS	1981	KM099523	BTV-1	IND	2007	KF563936
BTV-1	AUS	1986	KM099524	BTV-1	IND	2008	KU234258
BTV-1	AUS	1988	KM099525	BTV-1	IND	2008	KF563944
BTV-1	AUS	1979	M21844	BTV-1	IND	2008	KF563943
BTV-1	AUS	1981	AJ585178	BTV-1	IND	2007	KF664114
BTV-1	AUS	1979	JN881986	BTV-1	IND	2011	KP735614
BTV-1	AUS	-	AJ585120	BTV-1	IND	2008	KF563942
BTV-1	AUS	-	X06464	BTV-1	IND	2007	JN558349
BTV-1	AUS	1989	KM099526	BTV-1	IND	2007	KF563937

Appendix

The country codes were France:Corsica (FRA), South Africa (ZAF), China (CHN), Greece(GRC), Spain (ESP), Italy (ITA), Morroco (MAR), Portugal (PRT), Sudan (SDN), India (IND), Nigeria (NGA), Cameroon (CMR), Libya (LBY), Tunisia (TUN), Algeria (DZA), Gibraltar (GIB), Malaysia (MYS), South Korea (KOR), Australia (AUS), Greece (GRC) , French-Guiana (GUF) and United States of America (USA). RSArrrr and RSAvvvv stood for a reference strain (rrrr) and vaccine strain (vvvv) originated in Republic of South Africa (RSA).

Seg-2 sequences of reference Indian strains of BTV-2 available from GenBank

Serotype	Country code	Year of sampling	GenBank accession number	Serotype	Country code	Year of sampling	GenBank accession number
BTV-2	AUS	2008	JQ086242	BTV-2	USA	1982	AY855266
BTV-2	AUS	2010	JQ240322	BTV-2	USA	1982	AY855265
BTV-2	JPN	2007	AB686224	BTV-2	IND	2003	KP696603
BTV-2	TWN	2003	AY493687	BTV-2	RSArrrr	1959	AJ585123
BTV-2	IND	1982	AJ585152	BTV-2	RSArrrr	1959	KP821030
BTV-2	IND	1993	KP696583	BTV-2	ZAF	-	JX272600
BTV-2	IND	1993	KP339155	BTV-2	RSAvvvv	-	AJ585157
BTV-2	IND	1994	KP268775	BTV-2	RSAvvvv	1958	KP821031
BTV-2	IND	-	KC751422	BTV-2	ZAF	-	AF481096
BTV-2	IND	2010	KP339165	BTV-2	ITA	2001	DQ191271
BTV-2	MTQ	2006	HQ222820	BTV-2	ZAF	-	JN255933
BTV-2	PAN	1989	KF986501	BTV-2	ESP	2005	AM773697
BTV-2	USA	1999	AY855267	BTV-2	ESP	2005	KP821035
BTV-2	USA	2006	KF986495	BTV-2	ITA	2001	DQ191270
BTV-2	USA	2003	KF986499	BTV-2	ISR	2001	KP821027
BTV-2	USA	2010	JQ822249	BTV-2	PRT	2005	EF434177
BTV-2	GUF	2011	KC633279	BTV-2	TUN	2000	AJ585156
BTV-2	REU	2009	HQ222824	BTV-2	ITA	2000	DQ191272
BTV-2	CAD	-	KY471278	BTV-2	ITA	2001	DQ191263
BTV-2	NGA	1982	AJ585153	BTV-2	FRA	2000	AF356601
BTV-2	SDN	1985	AJ585155	BTV-2	ITA	2000	DQ191262
BTV-2	SDN	1983	KP821036	BTV-2	FRA	2001	KP821024

BTV-2	ZAF	1959	AY855264	BTV-2	ITA	2000	DQ191273
BTV-2	USA	-	M21946	BTV-2	ITA	2001	AJ585161
BTV-2	IND	2008	JQ904072	BTV-2	ITA	2002	KP821034
BTV-2	IND	2003	KP696593	BTV-2	FRA	2001	KP821026
BTV-2	IND	2003	JQ681258	BTV-2	ITA	2002	DQ191275
BTV-2	ITA	2001	AJ585162	BTV-2	FRA	2001	KP821025
BTV-2	ITA	2001	KP821032	BTV-2	ITA	2001	DQ191261
BTV-2	ITA	2002	AJ585158	BTV-2	FRA	2001	AJ585154
BTV-2	ITA	2002	AJ585160	BTV-2	ITA	2001	DQ191268
BTV-2	ITA	2002	AJ585159	BTV-2	ITA	2001	DQ191269
BTV-2	ITA	2002	AM773698	BTV-2	ITA	2002	DQ191274
BTV-2	ITA	2002	AM773708	BTV-2	ITA	2001	DQ191266
BTV-2	ITA	2002	KP821028	BTV-2	ITA	2001	DQ191267
BTV-2	ITA	2002	KP821029	BTV-2	ITA	2000	JN255863
BTV-2	ITA	2001	DQ191264	BTV-2	ITA	2000	KM053269
BTV-2	ITA	2001	DQ191265	BTV-2	ITA	2000	JN255873
BTV-2	ITA	2001	KP821033	BTV-2	TUN	2000	KP821037

The country codes are France : Corsica (FRA), South Africa (ZAF), China (CHN), Greece(GRC), Cyprus (CYP), Israel (ISR), Turkey (TUR), Spain (ESP), Argentina (ARG), Italy (ITA), Egypt (EGY), Morroco (MAR), Portugal (PRT), Sudan (SDN), Hungary (HUN) and India (IND). RSArrrr and RSAvvvv stood for a reference and vaccine strain respectively, originated in Republic of South Africa (RSA).

Seg-2 sequences of reference Indian strains of BTV-4 available from GenBank

Serotype	Country code	Year of sampling	Gen Bank accession number	Serotype	Country code	Year of sampling	GenBank accession number
BTV-4	IND	2013	KY947349	BTV-4	RSArrrr	1969	KP821066
BTV-4	IND	2013	KY947347	BTV-4	RSArrrr	1969	AJ585125
BTV-4	IND	2013	KY947350	BTV-4	ARG	1999	JX024950
BTV-4	IND	2013	KY947346	BTV-4	ARG	2002	AJ585169
BTV-4	IND	2013	KY947351	BTV-4	ARG	1999	JX024945

BTV-4	IND	2013	KY947348	BTV-4	ARG	2009	JX024955
BTV-4	IND	2013	KY947342	BTV-4	ARG	1999	JX024940
BTV-4	IND	2007	KY947343	BTV-4	ARG	2010	JX024960
BTV-4	CHN	1997	JX560414	BTV-4	ZAF	2004	AY839948
BTV-4	IND	2012	KY974345	BTV-4	RSArrrr	-	AJ585163
BTV-4	IND	2008	KF560418	BTV-4	ZAF	1900	JN255943
BTV-4	IND	2008	KY947344	BTV-4	TUR	-	AJ585164
BTV-4	GRC	2012	KP821058	BTV-4	ITA	2002	DQ191276
BTV-4	CYP	2011	KP821044	BTV-4	EGY	1977	KP821047
BTV-4	GRC	2012	KP821056	BTV-4	ZAF	-	KM233615
BTV-4	GRC	2012	KP821057	BTV-4	ZAF	-	JX272580
BTV-4	CYP	2011	KP821042	BTV-4	MAR	2004	KP821061
BTV-4	CYP	2011	KP821046	BTV-4	ESP	2004	KP821068
BTV-4	CYP	2011	KP821045	BTV-4	MAR	2009	KP821064
BTV-4	ISR	2006	KP821059	BTV-4	MAR	2009	KP821065
BTV-4	ISR	2001	DQ191278	BTV-4	MAR	2009	KP821063
BTV-4	ISR	2001	DQ191279	BTV-4	ESP	2010	KP821070
BTV-4	TUR	1978	AJ585165	BTV-4	PRT	2004	EF434176
BTV-4	TUR	1999	DQ825670	BTV-4	ESP	2005	KP821069
BTV-4	GRC	2000	AJ585167	BTV-4	ESP	2003	KP821067
BTV-4	GRC	2000	KP821054	BTV-4	ITA	2003	JN255893
BTV-4	CYP	2004	KP821041	BTV-4	ITA	2003	JN255883
BTV-4	GRC	1999	AY839947	BTV-4	ITA	2003	DQ191280
BTV-4	GRC	2000	AY839946	BTV-4	ITA	2003	DQ191281
BTV-4	GRC	2000	KP821055	BTV-4	FRA	2003	KP821050
BTV-4	GRC	1999	KP821053	BTV-4	FRA	2003	AY839945
BTV-4	GRC	1999	DQ191277	BTV-4	FRA	2003	KP821048
BTV-4	GRC	1999	KP821052	BTV-4	FRA	2003	KP821049
BTV-4	GRC	1979	KP821051	BTV-4	SDN	1983	AJ585166
BTV-4	CYP	1969	KP821040	BTV-4	SDN	1983	KP821071
BTV-4	ESP	2003	KJ700442	BTV-4	ZAF	2011	KT317666
BTV-4	CYP	1969	AJ585180	BTV-4	HUN	2014	KP268815
				BTV-4	FRA	2016	KY654329

Appendix

The country codes were France : Corsica (FRA), South Africa (ZAF), China (CHN), Greece(GRC), Cyprus (CYP), Israel (ISR), Turkey (TUR), Spain (ESP), Argentina (ARG), Italy (ITA), Egypt (EGY), Morroco (MAR), Portugal (PRT), Sudan (SDN), Hungary (HUN) and India (IND). RSArrrr stood for a reference strain (rrrr) originated in Republic of South Africa (RSA).

Seg-2 sequences of reference Indian strains of BTV-5 available from GenBank

Serotype	Country code	Year of sampling	GenBank accession number
BTV-5	NGA	1982	AJ585182
BTV-5	GLP	2010	HQ241072
BTV-5	USA	2003	KX164060
BTV-5	RSArrrr	1953	AJ585126
BTV-5	RSArrrr	1953	KP821101
BTV-5	ZAF	-	JX272570
BTV-5	CMR	1982	AJ585181
BTV-5	CHN	2014	KT945043
BTV-5	CHN	2012	KT945042
BTV-5	CHN	2012	KT945044
BTV-5	CHN	2012	KT945045

The country codes were Nigeria (NGA), French Guadeloupe Island (GLP), United States of America (USA), South Africa (ZAF), Cameroon (CMR), China (CHN) and India (IND). RSArrrr stood for a reference strain (rrrr) originated in Republic of South Africa (RSA).

Seg-2 sequences of reference Indian strains of BTV-9 available from GenBank

Serotype	Country code	Year of sampling	GenBank accession number	Serotype	Country code	Year of sampling	GenBank accession number
BTV-9	RSArrrr	1942	KP821088	BTV-9	ZAF	2014	KT885056
BTV-9	RSArrrr	1942	AJ585130	BTV-9	RSAvvvv	1942	KP821089

Appendix

BTV-9	ECU	2015	KX442583	BTV-9	RSAvvv	-	AJ585173
BTV-9	USA	2008	KX164070	BTV-9	ITA	2003	KP821083
BTV-9	ITA	2001	DQ191284	BTV-9	ZAF	-	JX272530
BTV-9	ITA	2000	JN255913	BTV-9	ZAF	1942	JN255953
BTV-9	ITA	2001	DQ191285	BTV-9	AUS	1985	JQ086302
BTV-9	ITA	2003	JN255903	BTV-9	JPN	2003	AB686223
BTV-9	BIH	2002	KP821078	BTV-9	IND	2004	KP696623
BTV-9	BIH	2002	AJ585174	BTV-9	IND	2005	KP696652
BTV-9	XKX	2001	KP821084	BTV-9	IND	2005	KP696642
BTV-9	XKX	2001	KP821085	BTV-9	IND	2003	KP696633
BTV-9	SRB	2001	KP821090	BTV-9	IND	2005	JQ414046
BTV-9	SRB	2001	AJ585172	BTV-9	IND	2005	KJ679573
BTV-9	GRC	1999	KP821081	BTV-9	IND	2003	JF443155
BTV-9	GRC	2000	AJ585171	BTV-9	IND	2003	JF443157
BTV-9	GRC	1999	KP821082	BTV-9	IND	2003	KP339185
BTV-9	LBY	2008	KP821087	BTV-9	IND	2003	KP339175
BTV-9	LBY	2008	KP821086	BTV-9	IND	2006	JN579710
BTV-9	ZAF	1942	JN255963	BTV-9	IND	2006	JF443167
BTV-9	ZAF	2014	KT885076	BTV-9	IND	2002	JF443156
BTV-9	GRC	1998	DQ191283	BTV-9	IND	2007	JX003688
BTV-9	GRC	1998	KP821080	BTV-9	IND	2004	KP696613
BTV-9	TUR	1998	KP821091	BTV-9	TUR	2000	KP821092
BTV-9	TUR	2000	AJ585176	BTV-9	BGR	1999	AJ585170
BTV-9	TUR	2001	KP821093	BTV-9	BGR	1999	KP821079
BTV-9	TUR	2000	AJ585177	BTV-9	TUR	2000	AJ585175

The country codes were United States of America (USA), Euadore (ECU), South Africa (ZAF), Libya (LBY), Australia (AUS), Japan (JPN) ,India (IND), Greece (GRC), Turkey (TUR), Bulgaria (BGR), Serbia (SRB), Bosnia & Hezegovina (BIH) and Kosovo (XKX). RSArrrr and RSAvvvv stood for a reference and vaccine strain respectively from South Africa.

Appendix

Seg-2 sequences of reference Indian strains of BTV-10 available from GenBank

Serotype	Country code	Year of sampling	GenBank accession number
BTV-10	RSArrrr	1956	AJ585131
BTV-10	ZAF	2011	KT317696
BTV-10	-	-	KX247939
BTV-10	ZAF	-	JX272520
BTV-10	MTR	2006	HQ222821
BTV-10	GUF	2011	JQ436735
BTV-10	USA	1990	U06784
BTV-10	USA	1990	U06781
BTV-10	USA	1990	U06785
BTV-10	USA	1980	U06783
BTV-10	USA	1980	U06780
BTV-10	USA	1980	U06782
BTV-10	USA	-	U06786
BTV-10	USA	-	L29027
BTV-10	USA	-	L29026
BTV-10	USA	-	M11787
BTV-10	USA	1953	NC006013
BTV-10	IND	2003	KP339245
BTV-10	IND	2004	JQ740772
BTV-10	IND	2004	JN704634
BTV-10	IND	2003	JF727655

The country codes as per ISO 3166-1 alpha-3 are France: Guyane (GUF), France: Martinique Island (MTQ), United States of America (USA), South Africa (ZAF) and India (IND). RSArrrr stands for a reference strain (rrrr) originated in Republic of South Africa (RSA).

Appendix

Seg-2 sequences of reference Indian strains of BTV-12 available from GenBank

Serotype	Country code	Year of sampling	GenBank accession number
BTV-12	USA	2008	KX164080
BTV-12	GUF	2011	KC633278
BTV-12	USA	2012	KX164090
BTV-12	RSArrrr	1941	AJ585133
BTV-12	JPN	1990	AB686216
BTV-12	TWN	2003	GU390659
BTV-12	ZAF	-	JX272500
BTV-12	ZAF	1941	KP821100
BTV-12	IND	2011	KC662613
BTV-12	IND	2016	MF615238
BTV-12	IND	2015	KX905151

The country codes were France Guyane (GUF), United States of America (USA), South Africa (ZAF), Japan (JPN), Taiwan (TWN) and India (IND). RSArrrr stood for a reference strain (rrah) originated in Republic of South Africa (RSA).

Appendix 4 **Percentage of nucleotide identity in Seg-2 between BTV strains sequenced in this study and global reference strains of BTV.**

Percentage of nucleotide identity in Seg-2 between Indian BTV-1 strains sequenced in this study and global reference eastern BTV-1 strains

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	1/AUS/1979_M21844	100	99.38	99.38	99.34	94.73	94.94	94.91	95.05	95.15	95.15	95.39	95.05	95.15	95.36	95.32	86.8	86.87	86.87
2	1/AUS/1981_AJ585178	99.38	100	100	99.97	95.15	95.36	95.32	95.39	95.56	95.56	95.81	95.46	95.56	95.77	95.74	87.21	87.28	87.28
3	1/AUS/1979_JN881986	99.38	100	100	99.97	95.15	95.36	95.32	95.39	95.56	95.56	95.81	95.46	95.56	95.77	95.74	87.21	87.28	87.28
4	1/AUS_X06464	99.34	99.97	99.97	100	95.11	95.32	95.29	95.36	95.53	95.53	95.77	95.43	95.53	95.74	95.7	87.18	87.25	87.25
5	1/AUS/2010_KM099538	94.73	95.15	95.15	95.11	100	98.54	98.51	98.72	98.75	98.75	98.93	98.65	98.82	99.03	99	86.66	86.8	86.8
6	1/AUS/2010_KM099539	94.94	95.36	95.36	95.32	98.54	100	98.65	98.86	98.96	98.96	99.06	98.93	99.03	99.24	99.2	87.21	87.35	87.35
7	1/AUS/2008_KM099537	94.91	95.32	95.32	95.29	98.51	98.65	100	99.38	99.55	99.48	98.96	99.31	99.34	99.41	99.38	87.01	87.14	87.14
8	1/AUS/2005_KM099536	95.05	95.39	95.39	95.36	98.72	98.86	99.38	100	99.69	99.69	99.17	99.51	99.55	99.62	99.58	86.8	86.94	86.94
9	1/AUS/2002_KM099534	95.15	95.56	95.56	95.53	98.75	98.96	99.55	99.69	100	99.79	99.27	99.62	99.65	99.72	99.69	87.04	87.18	87.18
10	1/AUS/2004_KM099535	95.15	95.56	95.56	95.53	98.75	98.96	99.48	99.69	99.79	100	99.27	99.62	99.65	99.72	99.69	86.9	87.04	87.04
11	1/AUS/1989_KM099526	95.39	95.81	95.81	95.77	98.93	99.06	98.96	99.17	99.27	99.27	100	99.17	99.34	99.55	99.51	87.08	87.21	87.21
12	1/AUS/2002_KM099533	95.05	95.46	95.46	95.43	98.65	98.93	99.31	99.51	99.62	99.62	99.17	100	99.55	99.62	99.58	87.08	87.21	87.21
13	1/AUS/1999_KM099531	95.15	95.56	95.56	95.53	98.82	99.03	99.34	99.55	99.65	99.65	99.34	99.55	100	99.79	99.76	87.04	87.18	87.18
14	1/AUS/1996_KM099529	95.36	95.77	95.77	95.74	99.03	99.24	99.41	99.62	99.72	99.72	99.55	99.62	99.79	100	99.97	87.11	87.25	87.25
15	1/AUS/1997_KM099530	95.32	95.74	95.74	95.7	99	99.2	99.38	99.58	99.69	99.69	99.51	99.58	99.76	99.97	100	87.14	87.28	87.28
16	IND2015/7A_2015-11-	86.8	87.21	87.21	87.18	86.66	87.21	87.01	86.8	87.04	86.9	87.08	87.08	87.04	87.11	87.14	100	99.17	99.17
17	IND2015/K13_2015-09	86.87	87.28	87.28	87.25	86.8	87.35	87.14	86.94	87.18	87.04	87.21	87.21	87.18	87.25	87.28	99.17	100	100
18	IND2015/40_2015-12-	86.87	87.28	87.28	87.25	86.8	87.35	87.14	86.94	87.18	87.04	87.21	87.21	87.18	87.25	87.28	99.17	100	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-1 strains sequenced in this study and global reference eastern BTV-1 strains

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1/MYS/1987_AJ585116	100	93.94	92	90.51	90.51	90.82	90.92	90.92	91.2	90.58	90.89	90.89	91.03	91.03	91.09	91.09	90.96	90.82	90.89	91.06	91.23	91.65	91.37	92.07	91.82	91.89	92.03
1/KOR/2010_KC153300	93.94	100	92.13	90.33	90.33	90.58	90.61	90.61	90.71	90.51	90.75	90.75	90.89	90.89	90.96	90.96	90.54	90.54	90.61	90.71	90.82	91.2	90.89	91.58	91.27	91.34	91.41
1/CHN/1979_KC879616	92	92.13	100	91.16	91.16	91.41	91.58	91.58	91.41	91.09	91.09	91.09	91.3	91.3	91.37	91.37	91.16	91.23	91.23	91.48	91.51	91.93	91.65	92.38	92.07	92.2	92.27
1/GRC/2001_AJ585121	90.51	90.33	91.16	100	100	99.48	99.58	99.58	94.91	94.42	94.32	94.32	94.53	94.53	94.53	94.53	94.28	94.32	94.42	94.59	94.87	95.29	95.25	95.5	95.46	95.53	95.67
1/GRC/2001_KP821005	90.51	90.33	91.16	100	100	99.48	99.58	99.58	94.91	94.42	94.32	94.32	94.53	94.53	94.53	94.53	94.28	94.32	94.42	94.59	94.87	95.29	95.25	95.5	95.46	95.53	95.67
1/GRC/2001_JN635334	90.82	90.58	91.41	99.48	99.48	100	99.83	99.83	95.15	94.66	94.56	94.56	94.77	94.77	94.77	94.77	94.53	94.56	94.66	94.84	95.08	95.5	95.39	95.74	95.7	95.77	95.91
1/GRC/2001_KP821006	90.92	90.61	91.58	99.58	99.58	99.83	100	100	95.25	94.7	94.66	94.66	94.87	94.87	94.87	94.87	94.63	94.66	94.77	94.94	95.25	95.67	95.56	95.91	95.88	95.95	96.08
1/GRC/2001_KP821007	90.92	90.61	91.58	99.58	99.58	99.83	100	100	95.25	94.7	94.66	94.66	94.87	94.87	94.87	94.87	94.63	94.66	94.77	94.94	95.25	95.67	95.56	95.91	95.88	95.95	96.08
1/IND/2007_KF563935	91.2	90.71	91.41	94.91	94.91	95.15	95.25	95.25	100	98.13	98.48	98.48	98.61	98.61	98.68	98.68	98.68	98.72	98.68	99.06	97.54	97.96	98.37	98.16	98.27	98.27	98.41
IND2015/7A_2015-11-	90.58	90.51	91.09	94.42	94.42	94.66	94.7	94.7	98.13	100	99.17	99.17	99.38	99.38	99.31	99.31	98.61	98.72	98.75	99	96.29	96.71	97.16	96.99	96.95	96.95	97.09
IND2015/K13_2015-09	90.89	90.75	91.09	94.32	94.32	94.56	94.66	94.66	98.48	99.17	100	100	99.65	99.65	99.72	99.72	98.96	99.06	99.1	99.34	96.6	97.02	97.44	97.3	97.26	97.26	97.4
IND2015/40_2015-12-	90.89	90.75	91.09	94.32	94.32	94.56	94.66	94.66	98.48	99.17	100	100	99.65	99.65	99.72	99.72	98.96	99.06	99.1	99.34	96.6	97.02	97.44	97.3	97.26	97.26	97.4
1/IND_KC751420	91.03	90.89	91.3	94.53	94.53	94.77	94.87	94.87	98.61	99.38	99.65	99.65	100	100	99.79	99.79	99.1	99.2	99.24	99.48	96.74	97.16	97.57	97.44	97.4	97.4	97.54
1/IND/2010_KP339145	91.03	90.89	91.3	94.53	94.53	94.77	94.87	94.87	98.61	99.38	99.65	99.65	100	100	99.79	99.79	99.1	99.2	99.24	99.48	96.74	97.16	97.57	97.44	97.4	97.4	97.54
1/IND_KC751421	91.09	90.96	91.37	94.53	94.53	94.77	94.87	94.87	98.68	99.31	99.72	99.72	99.79	99.79	100	100	99.17	99.27	99.31	99.55	96.81	97.23	97.64	97.51	97.47	97.47	97.61
1/IND/2010_KP339135	91.09	90.96	91.37	94.53	94.53	94.77	94.87	94.87	98.68	99.31	99.72	99.72	99.79	99.79	100	100	99.17	99.27	99.31	99.55	96.81	97.23	97.64	97.51	97.47	97.47	97.61
1/IND/2007_KF563934	90.96	90.54	91.16	94.28	94.28	94.53	94.63	94.63	98.68	98.61	98.96	98.96	99.1	99.1	99.17	99.17	100	99.13	99.17	99.48	96.71	97.12	97.61	97.4	97.37	97.37	97.51
1/IND/2007_KF563933	90.82	90.54	91.23	94.32	94.32	94.56	94.66	94.66	98.72	98.72	99.06	99.06	99.2	99.2	99.27	99.27	99.13	100	99.27	99.51	96.95	97.26	97.71	97.51	97.54	97.47	97.61
1/IND/2008_JX399149	90.89	90.61	91.23	94.42	94.42	94.66	94.77	94.77	98.68	98.75	99.1	99.1	99.24	99.24	99.31	99.31	99.17	99.27	100	99.55	96.78	97.19	97.61	97.47	97.44	97.44	97.57
1/IND/2008_KF664124	91.06	90.71	91.48	94.59	94.59	94.84	94.94	94.94	99.06	99	99.34	99.34	99.48	99.48	99.55	99.55	99.48	99.51	99.55	100	97.02	97.44	97.85	97.71	97.68	97.68	97.82
1/IND/2007_JN558348	91.23	90.82	91.51	94.87	94.87	95.08	95.25	95.25	97.54	96.29	96.6	96.6	96.74	96.74	96.81	96.81	96.71	96.95	96.78	97.02	100	99.58	98.2	98.54	98.72	98.79	98.86
1/IND/2007_KF563936	91.65	91.2	91.93	95.29	95.29	95.5	95.67	95.67	97.96	96.71	97.02	97.02	97.16	97.16	97.23	97.23	97.12	97.26	97.19	97.44	99.58	100	98.61	98.96	99.13	99.2	99.27
1/IND/2008_KF563945	91.37	90.89	91.65	95.25	95.25	95.39	95.56	95.56	98.37	97.16	97.44	97.44	97.57	97.57	97.64	97.64	97.61	97.71	97.61	97.85	98.2	98.61	100	98.61	98.79	98.79	98.86
1/IND/1992_AJ585112	92.07	91.58	92.38	95.5	95.5	95.74	95.91	95.91	98.16	96.99	97.3	97.3	97.44	97.44	97.51	97.51	97.4	97.51	97.47	97.71	98.54	98.96	98.61	100	99.13	99.2	99.34
1/IND/1994_KF563930	91.82	91.27	92.07	95.46	95.46	95.7	95.88	95.88	98.27	96.95	97.26	97.26	97.4	97.4	97.47	97.47	97.37	97.54	97.44	97.68	98.72	99.13	98.79	99.13	100	99.38	99.51
1/IND/1985_KF563929	91.89	91.34	92.2	95.53	95.53	95.77	95.95	95.95	98.27	96.95	97.26	97.26	97.4	97.4	97.47	97.47	97.37	97.47	97.44	97.68	98.79	99.2	98.79	99.2	99.38	100	99.58
1/IND/1985_KP696508	92.03	91.41	92.27	95.67	95.67	95.91	96.08	96.08	98.41	97.09	97.4	97.4	97.54	97.54	97.61	97.61	97.51	97.61	97.57	97.82	98.86	99.27	98.86	99.34	99.51	99.58	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-1 strains sequenced in this study and global reference western BTV-1 strains

		1	2	3	4	5	6	7	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	IND2015/7A_2015-11-	100	99.17	99.17	74.64	74.81	74.91	74.74	74.98	74.95	74.95	75.02	74.88	74.98	74.98	74.98	74.91	74.91	74.81	74.98	74.88	75.02	74.57	74.95	75.36	74.5	74.71	74.74	73.7	73.6
2	IND2015/K13_2015-09	99.17	100	100	74.67	74.64	74.74	74.57	74.81	74.77	74.77	74.84	74.71	74.81	74.81	74.81	74.74	74.74	74.57	74.81	74.71	74.84	74.53	75.05	75.26	74.53	74.67	74.71	73.8	73.56
3	IND2015/40_2015-12-	99.17	100	100	74.67	74.64	74.74	74.57	74.81	74.77	74.77	74.84	74.71	74.81	74.81	74.81	74.74	74.74	74.57	74.81	74.71	74.84	74.53	75.05	75.26	74.53	74.67	74.71	73.8	73.56
4	1/LBY/2007_KP821008	74.64	74.67	74.67	100	97.05	97.09	96.95	97.19	97.16	97.16	97.26	97.09	97.3	97.3	97.23	97.26	97.3	97.12	97.16	97.05	94.14	94.94	95.7	94.59	93.9	95.05	95.08	87.01	85.83
5	1/MAR/2009_KP821014	74.81	74.64	74.64	97.05	100	99.41	99.27	99.51	99.48	99.48	99.51	99.55	99.62	99.62	99.62	99.72	99.69	99.38	99.48	99.38	94.18	94.98	95.7	94.7	93.97	95.05	95.08	87.04	86.14
6	1/ITA/2010_KJ577115	74.91	74.74	74.74	97.09	99.41	100	99.45	99.69	99.65	99.72	99.62	99.45	99.72	99.65	99.58	99.62	99.65	99.55	99.72	99.62	94.21	95.01	95.77	94.7	94.04	95.08	95.11	87.25	86.14
7	1/FRA/2008_KP821001	74.74	74.57	74.57	96.95	99.27	99.45	100	99.69	99.79	99.65	99.48	99.31	99.58	99.51	99.45	99.48	99.51	99.27	99.38	99.27	94.14	94.87	95.56	94.63	93.94	94.98	95.01	86.97	86
9	1/ESP/2008_KP821020	74.98	74.81	74.81	97.19	99.51	99.69	99.69	100	99.9	99.9	99.72	99.55	99.83	99.76	99.69	99.72	99.76	99.51	99.62	99.51	94.32	95.11	95.81	94.87	94.14	95.18	95.22	87.14	86.17
10	1/FRA/2008_JX861499	74.95	74.77	74.77	97.16	99.48	99.65	99.79	99.9	100	99.86	99.69	99.51	99.79	99.72	99.65	99.69	99.72	99.48	99.58	99.48	94.35	95.08	95.77	94.84	94.11	95.15	95.18	87.18	86.21
11	1/FRA/2007_FJ437557	74.95	74.77	74.77	97.16	99.48	99.72	99.65	99.9	99.86	100	99.69	99.51	99.79	99.72	99.65	99.69	99.72	99.48	99.58	99.48	94.28	95.08	95.77	94.84	94.11	95.15	95.18	87.11	86.14
12	1/GIB/2007_KP821004	75.02	74.84	74.84	97.26	99.51	99.62	99.48	99.72	99.69	99.69	100	99.55	99.83	99.76	99.69	99.72	99.76	99.51	99.62	99.51	94.39	95.18	95.88	94.94	94.21	95.25	95.29	87.14	86.11
13	1/MAR/2006_EU625362	74.88	74.71	74.71	97.09	99.55	99.45	99.31	99.55	99.51	99.51	99.55	100	99.65	99.65	99.79	99.83	99.72	99.41	99.51	99.41	94.21	95.08	95.74	94.77	94.01	95.08	95.11	87.11	86.14
14	1/PRT/2007_EU498674	74.98	74.81	74.81	97.3	99.62	99.72	99.58	99.83	99.79	99.79	99.83	99.65	100	99.86	99.79	99.83	99.86	99.62	99.72	99.62	94.42	95.22	95.91	94.91	94.25	95.29	95.32	87.25	86.21
15	1/MAR/2007_KP821010	74.98	74.81	74.81	97.3	99.62	99.65	99.51	99.76	99.72	99.72	99.76	99.65	99.86	100	99.79	99.83	99.86	99.62	99.72	99.62	94.42	95.22	95.88	94.91	94.21	95.29	95.32	87.18	86.28
16	1/DZA/2006_EU625361	74.98	74.81	74.81	97.23	99.62	99.58	99.45	99.69	99.65	99.65	99.69	99.79	99.79	99.79	100	99.83	99.86	99.55	99.65	99.55	94.42	95.15	95.81	94.98	94.21	95.29	95.32	87.18	86.21
17	1/MAR/2006_KP821009	74.91	74.74	74.74	97.26	99.72	99.62	99.48	99.72	99.69	99.69	99.72	99.83	99.83	99.83	99.83	100	99.9	99.58	99.69	99.58	94.39	95.25	95.91	94.87	94.18	95.25	95.29	87.21	86.24
18	1/ITA/2006_KJ736002	74.91	74.74	74.74	97.3	99.69	99.65	99.51	99.76	99.72	99.72	99.76	99.72	99.86	99.86	99.86	99.9	100	99.62	99.72	99.62	94.42	95.22	95.88	94.91	94.21	95.29	95.32	87.25	86.28
19	1/TUN/2011_KJ577105	74.81	74.57	74.57	97.12	99.38	99.55	99.27	99.51	99.48	99.48	99.51	99.41	99.62	99.62	99.55	99.58	99.62	100	99.69	99.51	94.25	95.05	95.77	94.73	94.04	95.11	95.15	87.08	86.17
20	1/DZA/2008_KP820997	74.98	74.81	74.81	97.16	99.48	99.72	99.38	99.62	99.58	99.58	99.62	99.51	99.72	99.72	99.65	99.69	99.72	99.69	100	99.76	94.28	95.08	95.74	94.77	94.07	95.15	95.18	87.25	86.14
21	1/ITA/2013_KJ661730	74.88	74.71	74.71	97.05	99.38	99.62	99.27	99.51	99.48	99.48	99.51	99.41	99.62	99.62	99.55	99.58	99.62	99.51	99.76	100	94.25	95.05	95.7	94.73	94.04	95.11	95.15	87.21	86.11
22	1/ZAF_JX272380	75.02	74.84	74.84	94.14	94.18	94.21	94.14	94.32	94.35	94.28	94.39	94.21	94.42	94.42	94.42	94.39	94.42	94.25	94.28	94.25	100	95.46	96.08	95.88	96.12	96.15	96.19	88.22	87.28
23	1/NGA/1982_AJ585118	74.57	74.53	74.53	94.94	94.98	95.01	94.87	95.11	95.08	95.08	95.18	95.08	95.22	95.22	95.15	95.25	95.22	95.05	95.08	95.05	95.46	100	97.89	95.63	95.95	95.88	95.91	87.04	86.31
24	1/CMR/1982_KP820998	74.95	75.05	75.05	95.7	95.7	95.77	95.56	95.81	95.77	95.77	95.88	95.74	95.91	95.88	95.81	95.91	95.88	95.77	95.74	95.7	96.08	97.89	100	96.22	95.77	96.5	96.53	87.6	86.87
25	1/SDN/1987_KP821022	75.36	75.26	75.26	94.59	94.7	94.7	94.63	94.87	94.84	94.84	94.94	94.77	94.91	94.91	94.98	94.87	94.91	94.73	94.77	94.73	95.88	95.63	96.22	100	95.91	97.05	97.09	87.6	86.8
26	1/RSArrrr/1958_AJ58	74.5	74.53	74.53	93.9	93.97	94.04	93.94	94.14	94.11	94.11	94.21	94.01	94.25	94.21	94.21	94.18	94.21	94.04	94.07	94.04	96.12	95.95	95.77	95.91	100	98.06	98.09	87.11	86.11
27	1/ZAF_FJ969720	74.71	74.67	74.67	95.05	95.05	95.08	94.98	95.18	95.15	95.15	95.25	95.08	95.29	95.29	95.29	95.25	95.29	95.11	95.15	95.11	96.15	95.88	96.5	97.05	98.06	100	99.97	87.98	86.76
28	1/CHN/1997_JN848760	74.74	74.71	74.71	95.08	95.08	95.11	95.01	95.22	95.18	95.18	95.29	95.11	95.32	95.32	95.32	95.29	95.32	95.15	95.18	95.15	96.19	95.91	96.53	97.09	98.09	99.97	100	88.01	86.8
29	1/USA/2010_KX164020	73.7	73.8	73.8	87.01	87.04	87.25	86.97	87.14	87.18	87.11	87.14	87.11	87.25	87.18	87.18	87.21	87.25	87.08	87.25	87.21	88.22	87.04	87.6	87.6	87.11	87.98	88.01	100	90.75
30	1/GUF/2011_JQ436736	73.6	73.56	73.56	85.83	86.14	86.14	86	86.17	86.21	86.14	86.11	86.14	86.21	86.28	86.21	86.24	86.28	86.17	86.14	86.11	87.28	86.31	86.87	86.8	86.11	86.76	86.8	90.75	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-2 strains sequenced in this study and global reference eastern BTV-2 strains

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	IND2015/K9_2015-09-10	100	100	99.07	99.07	99.24	99.45	97.27	97.61	98.06	98.1	88.85	88.89	89.03	88.82
2	IND2015/K14_2015-09-12	100	100	99.07	99.07	99.24	99.45	97.27	97.61	98.06	98.1	88.85	88.89	89.03	88.82
3	IND2015/K3_2015-09-10	99.07	99.07	100	100	99.27	99.48	97.44	97.72	98.17	98.2	88.85	88.89	88.99	88.72
4	IND2015/14A_2015-11-08	99.07	99.07	100	100	99.27	99.48	97.44	97.72	98.17	98.2	88.85	88.89	88.99	88.72
5	2/IND_KC751422	99.24	99.24	99.27	99.27	100	99.79	97.47	97.89	98.34	98.37	88.85	88.89	89.13	88.85
6	2/IND/2010_KP339165	99.45	99.45	99.48	99.48	99.79	100	97.61	98.03	98.48	98.51	88.96	88.99	89.24	88.96
7	2/IND/1982_AJ585152	97.27	97.27	97.44	97.44	97.47	97.61	100	97.72	98.17	98.2	90.03	90	90	89.65
8	2/IND/1993_KP696583	97.61	97.61	97.72	97.72	97.89	98.03	97.72	100	99.41	99.45	88.82	88.85	88.82	88.54
9	2/IND/1994_KP268775	98.06	98.06	98.17	98.17	98.34	98.48	98.17	99.41	100	99.97	89.24	89.27	89.24	88.96
10	2/IND/1993_KP339155	98.1	98.1	98.2	98.2	98.37	98.51	98.2	99.45	99.97	100	89.27	89.3	89.27	88.99
11	2/AUS/2008_JQ086242	88.85	88.85	88.85	88.85	88.85	88.96	90.03	88.82	89.24	89.27	100	99.69	95.22	94.77
12	2/AUS/2010_JQ240322	88.89	88.89	88.89	88.89	88.89	88.99	90	88.85	89.27	89.3	99.69	100	95.12	94.67
13	2/JPN/2007_AB686224	89.03	89.03	88.99	88.99	89.13	89.24	90	88.82	89.24	89.27	95.22	95.12	100	98.55
14	2/TWN/2003_AY493687	88.82	88.82	88.72	88.72	88.85	88.96	89.65	88.54	88.96	88.99	94.77	94.67	98.55	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-2 strains sequenced in this study and global reference western BTV-2 strains

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	IND2015/K9_2015-09-10	100	100	99.07	99.07	71.89	71.75	72	72.07	72	71.1	71.17	71.48	71.37	71.37	71.51	71.48	71.55	71.58	71.55	71.55	71.55	71.55	71.55
2	IND2015/K14_2015-09-12	100	100	99.07	99.07	71.89	71.75	72	72.07	72	71.1	71.17	71.48	71.37	71.37	71.51	71.48	71.55	71.58	71.55	71.55	71.55	71.55	71.55
3	IND2015/K3_2015-09-10	99.07	99.07	100	100	71.96	71.86	72.1	72.17	72.1	71.34	71.41	71.55	71.44	71.44	71.41	71.37	71.44	71.48	71.44	71.44	71.44	71.44	71.44
4	IND2015/14A_2015-11-08	99.07	99.07	100	100	71.96	71.86	72.1	72.17	72.1	71.34	71.41	71.55	71.44	71.44	71.41	71.37	71.44	71.48	71.44	71.44	71.44	71.44	71.44
5	2/MTQ/2006_HQ222820	71.89	71.89	71.96	71.96	100	91.03	91.03	90.9	91	91.93	91.03	81.9	82.04	82.04	81.59	81.52	81.55	81.62	81.55	81.59	81.59	81.59	81.59
6	2/PAN/1989_KF986501	71.75	71.75	71.86	71.86	91.03	100	92.94	92.7	92.73	93.35	92.18	82.62	82.69	82.69	82.31	82.24	82.28	82.35	82.28	82.31	82.31	82.31	82.31
7	2/USA/1999_AY855267	72	72	72.1	72.1	91.03	92.94	100	99.03	99.07	94.39	93.18	82.76	83	83	82.73	82.73	82.76	82.83	82.76	82.8	82.8	82.8	82.8
8	2/USA/2006_KF986495	72.07	72.07	72.17	72.17	90.9	92.7	99.03	100	99.83	94.36	93.22	82.52	82.76	82.76	82.76	82.76	82.8	82.87	82.8	82.83	82.83	82.83	82.83
9	2/USA/2003_KF986499	72	72	72.1	72.1	91	92.73	99.07	99.83	100	94.25	93.04	82.42	82.66	82.66	82.66	82.66	82.69	82.76	82.69	82.73	82.73	82.73	82.73
10	2/USA/2010_JQ822249	71.1	71.1	71.34	71.34	91.93	93.35	94.39	94.36	94.25	100	97.68	82.76	82.94	82.94	82.55	82.49	82.52	82.59	82.52	82.55	82.55	82.55	82.55
11	2/GUF/2011_KC633279	71.17	71.17	71.41	71.41	91.03	92.18	93.18	93.22	93.04	97.68	100	83.07	83.25	83.25	82.69	82.62	82.66	82.73	82.66	82.69	82.69	82.69	82.69
12	2/NGA/1982_AJ585153	71.48	71.48	71.55	71.55	81.9	82.62	82.76	82.52	82.42	82.76	83.07	100	99.34	99.34	95.43	95.4	95.43	95.43	95.43	95.47	95.47	95.47	95.47
13	2/SDN/1985_AJ585155	71.37	71.37	71.44	71.44	82.04	82.69	83	82.76	82.66	82.94	83.25	99.34	100	100	95.19	95.15	95.19	95.19	95.19	95.22	95.22	95.22	95.22
14	2/SDN/1983_KP821036	71.37	71.37	71.44	71.44	82.04	82.69	83	82.76	82.66	82.94	83.25	99.34	100	100	95.19	95.15	95.19	95.19	95.19	95.22	95.22	95.22	95.22
15	2/TUN/2000_AJ585156	71.51	71.51	71.41	71.41	81.59	82.31	82.73	82.76	82.66	82.55	82.69	95.43	95.19	95.19	100	99.83	99.86	99.86	99.86	99.9	99.9	99.9	99.9
16	2/ITA/2000_JN255873	71.48	71.48	71.37	71.37	81.52	82.24	82.73	82.76	82.66	82.49	82.62	95.4	95.15	95.15	99.83	100	99.9	99.9	99.9	99.93	99.93	99.93	99.93
17	2/ITA/2001_DQ191263	71.55	71.55	71.44	71.44	81.55	82.28	82.76	82.8	82.69	82.52	82.66	95.43	95.19	95.19	99.86	99.9	100	99.93	99.93	99.97	99.97	99.97	99.97
18	2/ITA/2000_DQ191262	71.58	71.58	71.48	71.48	81.62	82.35	82.83	82.87	82.76	82.59	82.73	95.43	95.19	95.19	99.86	99.9	99.93	100	99.93	99.97	99.97	99.97	99.97
19	2/FRA/2001_KP821024	71.55	71.55	71.44	71.44	81.55	82.28	82.76	82.8	82.69	82.52	82.66	95.43	95.19	95.19	99.86	99.9	99.93	99.93	100	99.97	99.97	99.97	99.97
20	2/FRA/2000_AF356601	71.55	71.55	71.44	71.44	81.59	82.31	82.8	82.83	82.73	82.55	82.69	95.47	95.22	95.22	99.9	99.93	99.97	99.97	99.97	100	100	100	100
21	2/ITA/2000_JN255863	71.55	71.55	71.44	71.44	81.59	82.31	82.8	82.83	82.73	82.55	82.69	95.47	95.22	95.22	99.9	99.93	99.97	99.97	99.97	100	100	100	100
22	2/ITA/2000_KM053269	71.55	71.55	71.44	71.44	81.59	82.31	82.8	82.83	82.73	82.55	82.69	95.47	95.22	95.22	99.9	99.93	99.97	99.97	99.97	100	100	100	100
23	2/TUN/2000_KP821037	71.55	71.55	71.44	71.44	81.59	82.31	82.8	82.83	82.73	82.55	82.69	95.47	95.22	95.22	99.9	99.93	99.97	99.97	99.97	100	100	100	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-2 strains sequenced in this study and global reference western BTV-2 strains

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	IND2015/K9_2015-09-10	100	100	99.07	99.07	71.44	71.48	71.48	71.48	71.48	71.34	71.62	71.62	71.55	71.69	71.72	71.82	71.79	71.82
2	IND2015/K14_2015-09-12	100	100	99.07	99.07	71.44	71.48	71.48	71.48	71.48	71.34	71.62	71.62	71.55	71.69	71.72	71.82	71.79	71.82
3	IND2015/K3_2015-09-10	99.07	99.07	100	100	71.34	71.37	71.37	71.37	71.37	71.24	71.51	71.51	71.44	71.58	71.62	71.72	71.69	71.72
4	IND2015/14A_2015-11-08	99.07	99.07	100	100	71.34	71.37	71.37	71.37	71.37	71.24	71.51	71.51	71.44	71.58	71.62	71.72	71.69	71.72
5	2/ITA/2001_AJ585161	71.44	71.44	71.34	71.34	100	99.83	99.83	99.83	99.83	95.74	96.16	96.16	96.09	96.23	96.26	96.37	96.37	96.4
6	2/ITA/2001_DQ191261	71.48	71.48	71.37	71.37	99.83	100	100	100	100	95.64	96.05	96.05	95.98	96.12	96.16	96.26	96.26	96.3
7	2/FRA/2001_KP821025	71.48	71.48	71.37	71.37	99.83	100	100	100	100	95.64	96.05	96.05	95.98	96.12	96.16	96.26	96.26	96.3
8	2/FRA/2001_KP821026	71.48	71.48	71.37	71.37	99.83	100	100	100	100	95.64	96.05	96.05	95.98	96.12	96.16	96.26	96.26	96.3
9	2/ITA/2002_KP821034	71.48	71.48	71.37	71.37	99.83	100	100	100	100	95.64	96.05	96.05	95.98	96.12	96.16	96.26	96.26	96.3
10	2/PRT/2005_EF434177	71.34	71.34	71.24	71.24	95.74	95.64	95.64	95.64	95.64	100	99	99	98.89	99.03	99.1	99.17	99.17	99.2
11	2/RSArrrr/1959_AJ585123	71.62	71.62	71.51	71.51	96.16	96.05	96.05	96.05	96.05	99	100	100	99.79	99.52	99.69	99.65	99.65	99.69
12	2/RSArrrr/1959_KP821030	71.62	71.62	71.51	71.51	96.16	96.05	96.05	96.05	96.05	99	100	100	99.79	99.52	99.69	99.65	99.65	99.69
13	2/IND/2003_KP696603	71.55	71.55	71.44	71.44	96.09	95.98	95.98	95.98	95.98	98.89	99.79	99.79	100	99.58	99.62	99.65	99.65	99.69
14	2/USA_M21946	71.69	71.69	71.58	71.58	96.23	96.12	96.12	96.12	96.12	99.03	99.52	99.52	99.58	100	99.76	99.79	99.79	99.83
15	2/IND/2003_KP696593	71.72	71.72	71.62	71.62	96.26	96.16	96.16	96.16	96.16	99.1	99.69	99.69	99.62	99.76	100	99.83	99.83	99.86
16	2/USA/1982_AY855266	71.82	71.82	71.72	71.72	96.37	96.26	96.26	96.26	96.26	99.17	99.65	99.65	99.65	99.79	99.83	100	99.93	99.97
17	2/USA/1982_AY855265	71.79	71.79	71.69	71.69	96.37	96.26	96.26	96.26	96.26	99.17	99.65	99.65	99.65	99.79	99.83	99.93	100	99.97
18	2/ZAF/1959_AY855264	71.82	71.82	71.72	71.72	96.4	96.3	96.3	96.3	96.3	99.2	99.69	99.69	99.69	99.83	99.86	99.97	99.97	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-2 strains sequenced in this study and global reference western BTV-2 strains

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	IND2015/K9_2015-09-10	100	100	99.07	99.07	71.86	71.82	71.75	71.79	71.72	71.82	71.75	71.82	71.79	71.79	72.79	72.17
2	IND2015/K14_2015-09-12	100	100	99.07	99.07	71.86	71.82	71.75	71.79	71.72	71.82	71.75	71.82	71.79	71.79	72.79	72.17
3	IND2015/K3_2015-09-10	99.07	99.07	100	100	71.75	71.72	71.65	71.69	71.62	71.72	71.65	71.72	71.69	71.69	72.86	72.1
4	IND2015/14A_2015-11-08	99.07	99.07	100	100	71.75	71.72	71.65	71.69	71.62	71.72	71.65	71.72	71.69	71.69	72.86	72.1
5	2/ZAF_JX272600	71.86	71.86	71.75	71.75	100	99.97	99.9	99.93	99.58	99.76	99.79	99.79	99.83	99.83	88.72	89.27
6	2/ZAF_JN255923	71.82	71.82	71.72	71.72	99.97	100	99.93	99.97	99.55	99.72	99.76	99.76	99.79	99.79	88.68	89.24
7	2/IND/2003_JQ681258	71.75	71.75	71.65	71.65	99.9	99.93	100	99.97	99.48	99.65	99.69	99.69	99.72	99.72	88.61	89.17
8	2/IND/2008_JQ904072	71.79	71.79	71.69	71.69	99.93	99.97	99.97	100	99.52	99.69	99.72	99.72	99.76	99.76	88.65	89.2
9	2/ZAF_AF481096	71.72	71.72	71.62	71.62	99.58	99.55	99.48	99.52	100	99.69	99.72	99.72	99.76	99.76	88.58	89.2
10	2/ESP/2005_KP821035	71.82	71.82	71.72	71.72	99.76	99.72	99.65	99.69	99.69	100	99.9	99.9	99.93	99.93	88.68	89.24
11	2/ITA/2001_DQ191271	71.75	71.75	71.65	71.65	99.79	99.76	99.69	99.72	99.72	99.9	100	99.93	99.97	99.97	88.72	89.27
12	2/ITA/2001_DQ191270	71.82	71.82	71.72	71.72	99.79	99.76	99.69	99.72	99.72	99.9	99.93	100	99.97	99.97	88.72	89.27
13	2/ZAF_JN255933	71.79	71.79	71.69	71.69	99.83	99.79	99.72	99.76	99.76	99.93	99.97	99.97	100	100	88.75	89.3
14	2/ISR/2001_KP821027	71.79	71.79	71.69	71.69	99.83	99.79	99.72	99.76	99.76	99.93	99.97	99.97	100	100	88.75	89.3
15	2/REU/2009_HQ222824	72.79	72.79	72.86	72.86	88.72	88.68	88.61	88.65	88.58	88.68	88.72	88.72	88.75	88.75	100	96.16
16	2/CAD_KY471278	72.17	72.17	72.1	72.1	89.27	89.24	89.17	89.2	89.2	89.24	89.27	89.27	89.3	89.3	96.16	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-4 strains sequenced in this study and global reference eastern BTV-4 strains.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	4/CHN/1997_JX560414	100	91.4	91.36	91.19	91.19	91.29	91.15	91.43	91.33	91.33	91.36	91.47	91.57	91.54
2	4/IND/2013_KY947347	91.4	100	99.83	99.3	99.3	99.37	99.34	99.41	99.44	99.44	99.55	98.85	98.47	98.64
3	4/IND/2013_KY947349	91.36	99.83	100	99.27	99.27	99.34	99.3	99.37	99.41	99.41	99.51	98.75	98.43	98.61
4	IND2015/K4_2015-09-10	91.19	99.3	99.27	100	100	99.51	99.48	99.55	99.58	99.58	99.69	98.64	98.33	98.5
5	IND2015/6PK_2015-09-07	91.19	99.3	99.27	100	100	99.51	99.48	99.55	99.58	99.58	99.69	98.64	98.33	98.5
6	4/IND/2013_KY947350	91.29	99.37	99.34	99.51	99.51	100	99.55	99.62	99.65	99.65	99.76	98.71	98.4	98.57
7	4/IND/2007_KY947343	91.15	99.34	99.3	99.48	99.48	99.55	100	99.58	99.62	99.76	99.72	98.68	98.43	98.61
8	4/IND/2013_KY947348	91.43	99.41	99.37	99.55	99.55	99.62	99.58	100	99.69	99.76	99.79	98.75	98.43	98.61
9	4/IND/2013_KY947351	91.33	99.44	99.41	99.58	99.58	99.65	99.62	99.69	100	99.72	99.83	98.78	98.47	98.64
10	4/IND/2013_KY947342	91.33	99.44	99.41	99.58	99.58	99.65	99.76	99.76	99.72	100	99.83	98.78	98.54	98.71
11	4/IND/2013_KY947346	91.36	99.55	99.51	99.69	99.69	99.76	99.72	99.79	99.83	99.83	100	98.89	98.57	98.75
12	4/IND/2012_KY947345	91.47	98.85	98.75	98.64	98.64	98.71	98.68	98.75	98.78	98.78	98.89	100	98.92	99.09
13	4/IND/2008_KF560418	91.57	98.47	98.43	98.33	98.33	98.4	98.43	98.43	98.47	98.54	98.57	98.92	100	99.83
14	4/IND/2008_KY947344	91.54	98.64	98.61	98.5	98.5	98.57	98.61	98.61	98.64	98.71	98.75	99.09	99.83	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-4 strains sequenced in this study and global reference western BTV-4 strains.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
IND2015/K4_2015-09-10	100	100	99.51	99.48	99.55	99.58	99.58	99.69	98.64	98.33	98.5	71.4	71.82	71.68	71.79	71.79	71.89	72.41	72.48	72.52	72.38	72.45	72.48	72.48	72.45
IND2015/6PK_2015-09-07	100	100	99.51	99.48	99.55	99.58	99.58	99.69	98.64	98.33	98.5	71.4	71.82	71.68	71.79	71.79	71.89	72.41	72.48	72.52	72.38	72.45	72.48	72.48	72.45
4/ARG/1999_JX024950	71.4	71.4	71.44	71.44	71.44	71.37	71.44	71.44	71.72	71.47	71.58	100	94.5	94.98	94.81	94.85	94.81	90.28	90.04	90.11	89.9	89.93	90	90	89.97
4/ARG/2002_AJ585169	71.82	71.82	71.86	71.79	71.93	71.79	71.86	71.86	72	71.47	71.58	94.5	100	98.78	98.89	98.85	98.89	89.69	89.52	89.59	89.38	89.41	89.48	89.48	89.45
4/ARG/1999_JX024940	71.68	71.68	71.72	71.65	71.79	71.65	71.72	71.72	71.86	71.33	71.44	94.98	98.78	100	99.69	99.65	99.62	89.45	89.24	89.31	89.1	89.13	89.2	89.2	89.17
4/ARG/2010_JX024960	71.79	71.79	71.82	71.75	71.89	71.75	71.82	71.82	71.96	71.44	71.54	94.81	98.89	99.69	100	99.76	99.79	89.52	89.31	89.38	89.17	89.2	89.27	89.27	89.24
4/ARG/1999_JX024945	71.79	71.79	71.82	71.75	71.89	71.75	71.82	71.82	71.96	71.44	71.54	94.85	98.85	99.65	99.76	100	99.83	89.48	89.24	89.31	89.1	89.13	89.2	89.2	89.17
4/ARG/2009_JX024955	71.89	71.89	71.93	71.86	72	71.86	71.93	71.93	72.07	71.54	71.65	94.81	98.89	99.62	99.79	99.83	100	89.48	89.27	89.34	89.13	89.17	89.24	89.24	89.2
4/ZAF/2004_AY839948	72.41	72.41	72.45	72.34	72.45	72.34	72.38	72.38	72.52	72.1	72.27	90.28	89.69	89.45	89.52	89.48	89.48	100	98.47	98.68	98.68	98.71	98.78	98.78	98.75
4/ZAF_KM233615	72.48	72.48	72.45	72.41	72.52	72.38	72.45	72.45	72.52	72.17	72.34	90.04	89.52	89.24	89.31	89.24	89.27	98.47	100	99.79	99.58	99.65	99.69	99.69	99.65
4/ZAF_JX272580	72.52	72.52	72.48	72.45	72.55	72.41	72.48	72.48	72.55	72.2	72.38	90.11	89.59	89.31	89.38	89.31	89.34	98.68	99.79	100	99.79	99.83	99.9	99.9	99.86
4/EGY/1977_KP821047	72.38	72.38	72.34	72.31	72.41	72.27	72.34	72.34	72.41	72.07	72.24	89.9	89.38	89.1	89.17	89.1	89.13	98.68	99.58	99.79	100	99.83	99.9	99.9	99.86
4/TUR_AJ585164	72.45	72.45	72.41	72.38	72.48	72.34	72.41	72.41	72.48	72.14	72.31	89.93	89.41	89.13	89.2	89.13	89.17	98.71	99.65	99.83	99.83	100	99.93	99.93	99.9
4/RSAvvvv_AJ585163	72.48	72.48	72.45	72.41	72.52	72.38	72.45	72.45	72.52	72.17	72.34	90	89.48	89.2	89.27	89.2	89.24	98.78	99.69	99.9	99.9	99.93	100	100	99.97
4/ZAF/1900_JN255943	72.48	72.48	72.45	72.41	72.52	72.38	72.45	72.45	72.52	72.17	72.34	90	89.48	89.2	89.27	89.2	89.24	98.78	99.69	99.9	99.9	99.93	100	100	99.97
4/ITA/2002_DQ191276	72.45	72.45	72.41	72.38	72.48	72.41	72.41	72.41	72.48	72.14	72.31	89.97	89.45	89.17	89.24	89.17	89.2	98.75	99.65	99.86	99.86	99.9	99.97	99.97	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-4 strains sequenced in this study and global reference western BTV-4 strains

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1	IND2015/K4_2015-09-10	100	100	71.6	71.7	71.7	71.7	71.7	71.7	71.7	71.7	72.1	72.0	71.9	71.7	71.7	71.4	71.5	71.5	71.5	71.5	71.5	71.5	71.5	71.5	71.4	71.4	71.5	71.5	71.5
2	IND2015/6PK_2015-09-07	100	100	71.6	71.7	71.7	71.7	71.7	71.7	71.7	71.7	72.1	72.0	71.9	71.7	71.7	71.4	71.5	71.5	71.5	71.5	71.5	71.5	71.5	71.5	71.4	71.4	71.5	71.5	71.5
3	4/CYP/2011_KP821042	71.6	71.6	100	99.9	99.9	99.9	99.9	99.9	99.9	99.9	95.0	95.3	95.3	96.1	96.1	96.1	96.3	96.3	96.3	96.3	96.3	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2
4	4/GRC/2012_KP821056	71.7	71.7	99.9	100	100	99.9	99.9	99.9	99.9	100	95.1	95.3	95.3	96.1	96.1	96.1	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.2	96.2	96.2	96.2	96.3
5	4/GRC/2012_KP821057	71.7	71.7	99.9	100	100	99.9	99.9	99.9	99.9	100	95.1	95.3	95.3	96.1	96.1	96.1	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.2	96.2	96.2	96.2	96.3
6	4/CYP/2011_KP821043	71.7	71.7	99.9	99.9	99.9	100	100	100	99.9	100	95.1	95.3	95.3	96.1	96.1	96.1	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.2	96.2	96.2	96.2	96.3
7	4/CYP/2011_KP821045	71.7	71.7	99.9	99.9	99.9	100	100	100	99.9	100	95.1	95.3	95.3	96.1	96.1	96.1	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.2	96.2	96.2	96.2	96.3
8	4/CYP/2011_KP821046	71.7	71.7	99.9	99.9	99.9	100	100	100	99.9	100	95.1	95.3	95.3	96.1	96.1	96.1	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.2	96.2	96.2	96.2	96.3
9	4/GRC/2012_KP821058	71.7	71.7	99.9	99.9	99.9	99.9	99.9	99.9	100	100	95.1	95.3	95.4	96.1	96.1	96.2	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.2	96.2	96.3	96.3	96.3
10	4/CYP/2011_KP821044	71.7	71.7	99.9	100	100	100	100	100	100	100	95.1	95.3	95.4	96.1	96.1	96.2	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.2	96.2	96.3	96.3	96.3
11	4/ISR/2006_KP821059	72.1	72.1	95.0	95.1	95.1	95.1	95.1	95.1	95.1	95.1	100	99.6	99.7	98.4	98.4	98.0	98.3	98.3	98.3	98.3	98.3	98.2	98.2	98.2	98.2	98.2	98.3	98.2	98.2
12	4/ISR/2001_DQ191278	72.0	72.0	95.3	95.3	95.3	95.3	95.3	95.3	95.3	95.3	99.6	100	99.8	98.6	98.6	98.2	98.4	98.4	98.4	98.4	98.4	98.4	98.4	98.3	98.3	98.3	98.4	98.4	98.4
13	4/ISR/2001_DQ191279	71.9	71.9	95.3	95.3	95.3	95.3	95.3	95.3	95.4	95.4	99.7	99.8	100	98.6	98.6	98.2	98.5	98.5	98.5	98.5	98.5	98.4	98.4	98.4	98.4	98.4	98.5	98.4	98.4
14	4/TUR/1978_AJ585165	71.7	71.7	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	98.4	98.6	98.6	100	100	99.3	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.4	99.5	99.4	99.4	99.4	99.5
15	4/TUR/1999_DQ825670	71.7	71.7	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	98.4	98.6	98.6	100	100	99.3	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.4	99.5	99.4	99.4	99.4	99.5
16	4/ESP/2003_KJ700442	71.4	71.4	96.1	96.1	96.1	96.1	96.1	96.1	96.2	96.2	98.0	98.2	98.2	99.3	99.3	100	99.8	99.8	99.8	99.8	99.8	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7
17	4/RSArrrr/1969_AJ58512	71.5	71.5	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.3	98.3	98.4	98.5	99.5	99.5	99.8	100	100	100	100	100	100	100	99.9	99.9	99.9	99.9	99.9	100
18	4/CYP/1969_AJ585180	71.5	71.5	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.3	98.3	98.4	98.5	99.5	99.5	99.8	100	100	100	100	100	100	100	99.9	99.9	99.9	99.9	99.9	100
19	4/CYP/1969_KP821040	71.5	71.5	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.3	98.3	98.4	98.5	99.5	99.5	99.8	100	100	100	100	100	100	100	99.9	99.9	99.9	99.9	99.9	100
20	4/GRC/1979_KP821051	71.5	71.5	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.3	98.3	98.4	98.5	99.5	99.5	99.8	100	100	100	100	100	100	100	99.9	99.9	99.9	99.9	99.9	100
21	4/RSArrrr/1969_KP82106	71.5	71.5	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.3	98.3	98.4	98.5	99.5	99.5	99.8	100	100	100	100	100	100	100	99.9	99.9	99.9	99.9	99.9	100
22	4/GRC/1999_DQ191277	71.5	71.5	96.2	96.3	96.3	96.3	96.3	96.3	96.3	96.3	98.2	98.4	98.4	99.5	99.5	99.7	100	100	100	100	100	100	100	99.9	99.9	99.9	99.9	99.9	99.9
23	4/GRC/1999_KP821052	71.5	71.5	96.2	96.3	96.3	96.3	96.3	96.3	96.3	96.3	98.2	98.4	98.4	99.5	99.5	99.7	100	100	100	100	100	100	100	99.9	99.9	99.9	99.9	99.9	99.9
24	4/GRC/2000_KP821055	71.5	71.5	96.2	96.3	96.3	96.3	96.3	96.3	96.3	96.3	98.2	98.3	98.4	99.4	99.4	99.7	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100	99.9	99.9	99.9	99.9	99.9
25	4/GRC/2000_AJ585167	71.4	71.4	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	98.2	98.3	98.4	99.5	99.5	99.7	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100	99.9	99.9	99.9	99.9
26	4/GRC/1999_KP821053	71.4	71.4	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	98.2	98.3	98.4	99.4	99.4	99.7	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100	99.9	99.9	99.9
27	4/CYP/2004_KP821041	71.5	71.5	96.2	96.2	96.2	96.2	96.2	96.2	96.3	96.3	98.3	98.4	98.5	99.4	99.4	99.7	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100	99.9	100
28	4/GRC/2000_KP821054	71.5	71.5	96.2	96.2	96.2	96.2	96.2	96.2	96.3	96.3	98.2	98.4	98.4	99.4	99.4	99.7	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100	100
29	4/GRC/2000_AY839946	71.5	71.5	96.2	96.3	96.3	96.3	96.3	96.3	96.3	96.3	98.2	98.4	98.4	99.5	99.5	99.7	100.0	100.0	100.0	100.0	100.0	99.9	99.9	99.9	99.9	99.9	100	100	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-4 strains sequenced in this study and global reference western BTV-4 strains

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	IND2015/K4_2015-09-10	100	100	72.3	72.2	72.3	72.3	72.3	72.3	72.3	72.2	72.3	72.3	72.3	72.2	72.3	72.2	72.2	72.2	72.1	72.3	72.2	72.2	72.3	72.3	72.5
2	IND2015/6PK_2015-09-07	100	100	72.3	72.2	72.3	72.3	72.3	72.3	72.3	72.2	72.3	72.3	72.3	72.2	72.3	72.2	72.2	72.2	72.1	72.3	72.2	72.2	72.3	72.3	72.5
3	4/HUN/2014_KP268815	72.3	72.3	100	99.8	94.0	94.2	94.3	94.3	94.3	94.3	94.3	94.4	94.4	94.5	94.5	94.5	94.4	94.4	94.5	94.6	94.5	94.6	95.7	95.7	95.6
4	4/FRA-COR/2016_KY65432	72.2	72.2	99.8	100	94.0	94.2	94.3	94.3	94.3	94.3	94.3	94.4	94.4	94.5	94.5	94.5	94.4	94.4	94.5	94.6	94.5	94.6	95.7	95.7	95.7
5	4/MAR/2009_KP821065	72.3	72.3	94.0	94.0	100	99.6	99.8	99.8	99.7	98.8	99.0	99.2	99.2	99.4	99.3	99.4	99.4	99.2	99.1	99.3	99.3	99.2	95.9	95.9	95.0
6	4/ESP/2010_KP821070	72.3	72.3	94.2	94.2	99.6	100	99.9	99.9	99.9	98.9	99.2	99.3	99.3	99.5	99.5	99.5	99.5	99.3	99.3	99.4	99.4	99.4	96.0	96.0	95.1
7	4/MAR/2009_KP821062	72.3	72.3	94.3	94.3	99.8	99.9	100	100	100	99.0	99.3	99.4	99.4	99.6	99.6	99.6	99.6	99.4	99.4	99.6	99.5	99.5	96.1	96.1	95.2
8	4/MAR/2009_KP821063	72.3	72.3	94.3	94.3	99.8	99.9	100	100	100	99.0	99.3	99.4	99.4	99.6	99.6	99.6	99.6	99.4	99.4	99.6	99.5	99.5	96.1	96.1	95.2
9	4/MAR/2009_KP821064	72.3	72.3	94.3	94.3	99.7	99.9	100	100	100	99.0	99.3	99.4	99.4	99.6	99.6	99.6	99.6	99.4	99.4	99.6	99.5	99.5	96.1	96.1	95.2
10	4/FRA-COR/2003_AY83994	72.2	72.2	94.3	94.3	98.8	98.9	99.0	99.0	99.0	100	99.1	99.3	99.3	99.2	99.2	99.3	99.3	99.6	99.5	99.4	99.5	99.5	96.1	96.1	95.2
11	4/ITA/2003_JN255893	72.3	72.3	94.3	94.3	99.0	99.2	99.3	99.3	99.3	99.1	100	99.9	99.9	99.4	99.4	99.5	99.5	99.5	99.5	99.7	99.6	99.6	96.1	96.1	95.2
12	4/ITA/2003_DQ191280	72.3	72.3	94.4	94.4	99.2	99.3	99.4	99.4	99.4	99.3	99.9	100	100	99.6	99.6	99.7	99.7	99.7	99.6	99.9	99.8	99.7	96.2	96.2	95.3
13	4/ITA/2003_JN255883	72.3	72.3	94.4	94.4	99.2	99.3	99.4	99.4	99.4	99.3	99.9	100	100	99.6	99.6	99.7	99.7	99.7	99.6	99.9	99.8	99.7	96.2	96.2	95.3
14	4/PRT/2004_EF434176	72.2	72.2	94.5	94.5	99.4	99.5	99.6	99.6	99.6	99.2	99.4	99.6	99.6	100	100	99.8	99.8	99.6	99.6	99.7	99.7	99.7	96.2	96.2	95.3
15	4/ESP/2005_KP821069	72.3	72.3	94.5	94.5	99.3	99.5	99.6	99.6	99.6	99.2	99.4	99.6	99.6	100	100	99.8	99.8	99.6	99.5	99.7	99.7	99.6	96.2	96.2	95.3
16	4/MAR/2004_KP821061	72.2	72.2	94.5	94.5	99.4	99.5	99.6	99.6	99.6	99.3	99.5	99.7	99.7	99.8	99.8	100	99.9	99.7	99.6	99.8	99.8	99.7	96.2	96.2	95.4
17	4/ESP/2004_KP821068	72.2	72.2	94.4	94.4	99.4	99.5	99.6	99.6	99.6	99.3	99.5	99.7	99.7	99.8	99.8	99.9	100	99.7	99.6	99.8	99.8	99.7	96.2	96.2	95.3
18	4/FRA-COR/2003_KP82104	72.2	72.2	94.4	94.4	99.2	99.3	99.4	99.4	99.4	99.6	99.5	99.7	99.7	99.6	99.6	99.7	99.7	100	100	99.8	99.9	99.9	96.3	96.3	95.3
19	4/FRA-COR/2003_KP82104	72.1	72.1	94.5	94.5	99.1	99.3	99.4	99.4	99.4	99.5	99.5	99.6	99.6	99.6	99.5	99.6	99.6	100	100	99.8	99.9	99.8	96.3	96.3	95.3
20	4/ESP/2003_KP821067	72.3	72.3	94.6	94.6	99.3	99.4	99.6	99.6	99.6	99.4	99.7	99.9	99.9	99.7	99.7	99.8	99.8	99.8	99.8	100	99.9	99.9	96.4	96.4	95.5
21	4/ITA/2003_DQ191281	72.2	72.2	94.5	94.5	99.3	99.4	99.5	99.5	99.5	99.5	99.6	99.8	99.8	99.7	99.7	99.8	99.8	99.9	99.9	99.9	100	100	96.4	96.4	95.4
22	4/FRA-COR/2003_KP82105	72.2	72.2	94.6	94.6	99.2	99.4	99.5	99.5	99.5	99.5	99.6	99.7	99.7	99.7	99.6	99.7	99.7	99.9	99.8	99.9	100	100	96.4	96.4	95.4
23	4/SDN/1983_AJ585166	72.3	72.3	95.7	95.7	95.9	96.0	96.1	96.1	96.1	96.1	96.1	96.2	96.2	96.2	96.2	96.2	96.2	96.3	96.3	96.4	96.4	96.4	100	100	96.7
24	4/SDN/1983_KP821071	72.3	72.3	95.7	95.7	95.9	96.0	96.1	96.1	96.1	96.1	96.1	96.2	96.2	96.2	96.2	96.2	96.2	96.3	96.3	96.4	96.4	96.4	100	100	96.7
25	4/ZAF/2011_KT317666	72.5	72.5	95.6	95.7	95.0	95.1	95.2	95.2	95.2	95.2	95.2	95.3	95.3	95.3	95.3	95.4	95.3	95.3	95.3	95.5	95.4	95.4	96.7	96.7	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-5 strains sequenced in this study and global reference western BTV-5 strains

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	IND2015/404_2015-12-25	100	99.93	99.93	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	98.43	98.74	98.78	98.78	95.68	94.87	94.94	94.94	77.44	76.99	77.55
2	IND2015/475_2015-12-25	99.93	100	99.93	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	98.43	98.74	98.78	98.78	95.68	94.87	94.94	94.94	77.44	76.99	77.55
3	IND2015/CS9_2015-12-25	99.93	99.93	100	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	99.97	98.43	98.74	98.78	98.78	95.68	94.87	94.94	94.94	77.44	77.02	77.55
4	IND2015/491_2015-12-25	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
5	IND2015/492_2015-12-25	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
6	IND2015/408_2015-12-25	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
7	IND2015/471_2015-12-25	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
8	IND2015/476_2015-12-25	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
9	IND2015/478_2015-12-25	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
10	IND2015/486_2015-12-25	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
11	IND2015/534_2015-12-25	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
12	IND2015/547_2015-12-25	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
13	IND2015/228_2015-12-25	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
14	IND2015/V1_2015-11-26	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
15	IND2015/V2_2015-11-26	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
16	IND2015/V44_2015-11-26	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
17	IND2014/10NLG_2014-	99.97	99.97	99.97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	98.47	98.78	98.81	98.81	95.71	94.91	94.98	94.98	77.48	77.02	77.58
18	5/CHN/2014_KT945043	98.43	98.43	98.43	98.47	98.47	98.47	98.47	98.47	98.47	98.47	98.47	98.47	98.47	98.47	98.47	98.47	98.47	100	98.99	99.02	99.02	95.85	95.05	95.12	95.12	77.34	76.85	77.86
19	5/CHN/2012_KT945045	98.74	98.74	98.74	98.78	98.78	98.78	98.78	98.78	98.78	98.78	98.78	98.78	98.78	98.78	98.78	98.78	98.78	98.99	100	99.86	99.9	96.16	95.5	95.57	95.57	77.27	76.85	77.75
20	5/CHN/2012_KT945042	98.78	98.78	98.78	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	99.02	99.86	100	99.9	96.13	95.4	95.47	95.47	77.23	76.74	77.72
21	5/CHN/2012_KT945044	98.78	98.78	98.78	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	98.81	99.02	99.9	99.9	100	96.13	95.4	95.47	95.47	77.23	76.74	77.72
22	5/CMR/1982_AJ585181	95.68	95.68	95.68	95.71	95.71	95.71	95.71	95.71	95.71	95.71	95.71	95.71	95.71	95.71	95.71	95.71	95.71	95.85	96.16	96.13	96.13	100	96.69	96.76	96.76	77.55	77.16	78.31
23	5/RSArrrr/1953_AJ585126	94.87	94.87	94.87	94.91	94.91	94.91	94.91	94.91	94.91	94.91	94.91	94.91	94.91	94.91	94.91	94.91	94.91	95.05	95.5	95.4	95.4	96.69	100	99.93	99.93	77.65	77.16	78.17
24	5/ZAF_JX272570	94.94	94.94	94.94	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	95.12	95.57	95.47	95.47	96.76	99.93	100	100	77.68	77.2	78.21
25	5/RSArrrr/1953_KP821101	94.94	94.94	94.94	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	94.98	95.12	95.57	95.47	95.47	96.76	99.93	100	100	77.68	77.2	78.21
26	5/NGA/1982_AJ585182	77.44	77.44	77.44	77.48	77.48	77.48	77.48	77.48	77.48	77.48	77.48	77.48	77.48	77.48	77.48	77.48	77.48	77.34	77.27	77.23	77.23	77.55	77.65	77.68	77.68	100	85.22	85.77
27	5/GLP/2010_HQ241072	76.99	76.99	77.02	77.02	77.02	77.02	77.02	77.02	77.02	77.02	77.02	77.02	77.02	77.02	77.02	77.02	77.02	76.85	76.85	76.74	76.74	77.16	77.16	77.2	77.2	85.22	100	86.72
28	5/USA/2003_KX164060	77.55	77.55	77.55	77.58	77.58	77.58	77.58	77.58	77.58	77.58	77.58	77.58	77.58	77.58	77.58	77.58	77.58	77.86	77.75	77.72	77.72	78.31	78.17	78.21	78.21	85.77	86.72	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-9 strains sequenced in this study and global reference eastern BTV-9 strains

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	9/AUS/1985_JQ086302	100	90.7	89.4	89.6	89.7	89.7	89.6	89.6	89.8	89.8	89.8	89.8	89.8	89.8	89.5	89.6	89.6	89.5	89.5	89.6	89.7	89.8	89.6	89.6	89.6	89.6	89.6	89.6	89.6	89.6	89.8
2	9/JPN/2003_AB686223	90.7	100	91.0	91.4	91.5	91.5	91.4	91.5	91.6	91.6	91.6	91.6	91.6	91.6	91.0	91.1	91.1	91.1	91.1	91.1	91.3	91.3	91.2	91.1	91.1	91.2	91.2	91.3	91.3	91.3	91.4
3	IND2015/477_2015-12	89.4	91.0	100	99.0	99.1	98.9	98.8	98.9	99.0	99.0	99.0	99.1	99.1	99.1	97.0	97.1	97.1	97.0	97.0	97.1	97.2	97.3	97.1	97.1	97.1	97.1	97.1	97.2	97.2	97.2	97.3
4	9/IND/2006_JN579710	89.6	91.4	99.0	100	100	99.4	99.3	99.4	100	100	100	100	100	100	97.6	97.7	97.7	97.6	97.6	97.7	97.8	97.8	97.7	97.7	97.7	97.7	97.7	97.8	97.8	97.8	97.9
5	9/IND/2006_JF443167	89.7	91.5	99.1	100	100	100	99.4	100	100	100	100	100	100	100	97.7	97.8	97.8	97.7	97.7	97.8	97.9	97.9	97.8	97.8	97.8	97.8	97.8	97.9	97.9	97.9	98.0
6	9/IND/2004_KP696613	89.7	91.5	98.9	99.4	100	100	100	100	100	100	100	100	100	100	97.8	97.9	97.9	97.9	97.9	97.9	98.0	98.1	97.9	97.9	97.9	97.9	98.0	98.0	98.0	98.0	98.1
7	9/IND/2005_JQ414046	89.6	91.4	98.8	99.3	99.4	100	100	100	100	100	100	100	100	100	97.8	97.9	97.9	97.9	97.9	97.9	98.1	98.1	97.9	98.0	98.0	98.0	98.1	98.1	98.1	98.1	98.2
8	9/IND/2005_KJ679573	89.6	91.5	98.9	99.4	100	100	100	100	100	100	100	100	100	100	97.7	97.8	97.8	97.9	97.9	97.8	98.0	98.0	97.9	97.9	97.9	97.9	98.0	98.0	98.0	98.0	98.1
9	9/IND/2003_KP696633	89.8	91.6	99.0	100	100	100	100	100	100	100	100	100	100	100	97.8	97.9	97.9	98.0	98.0	97.9	98.1	98.1	98.0	98.0	98.0	98.1	98.1	98.1	98.1	98.1	98.2
10	9/IND/2003_JF443155	89.8	91.6	99.0	100	100	100	100	100	100	100	100	100	100	100	97.9	98.0	98.0	97.9	97.9	98.0	98.1	98.2	98.0	98.0	98.0	98.0	98.1	98.1	98.1	98.1	98.2
11	9/IND/2003_JF443157	89.8	91.6	99.0	100	100	100	100	100	100	100	100	100	100	100	97.9	98.0	98.0	97.9	97.9	98.0	98.1	98.2	98.0	98.0	98.0	98.0	98.1	98.1	98.1	98.1	98.2
12	9/IND/2002_JF443156	89.8	91.6	99.1	100	100	100	100	100	100	100	100	100	100	100	97.9	98.0	98.0	98.0	98.0	98.0	98.2	98.2	98.1	98.0	98.0	98.1	98.1	98.2	98.2	98.2	98.3
13	9/IND/2003_KP339175	89.8	91.6	99.1	100	100	100	100	100	100	100	100	100	100	100	97.9	98.0	98.0	98.0	98.0	98.0	98.2	98.2	98.1	98.0	98.0	98.1	98.1	98.2	98.2	98.2	98.3
14	9/IND/2003_KP339185	89.8	91.6	99.1	100	100	100	100	100	100	100	100	100	100	100	97.9	98.0	98.0	98.0	98.0	98.0	98.2	98.2	98.1	98.0	98.0	98.1	98.1	98.2	98.2	98.2	98.3
15	9/GRC/2000_AJ585171	89.5	91.0	97.0	97.6	97.7	97.8	97.8	97.7	97.8	97.9	97.9	97.9	97.9	97.9	100	100	100	100	100	100	100	100	100	100	100	100	100	99.3	99.3	99.4	100
16	9/BGR/1999_AJ585170	89.6	91.1	97.1	97.7	97.8	97.9	97.9	97.8	97.9	98.0	98.0	98.0	98.0	98.0	100	100	100	100	100	100	100	100	100	100	100	100	100	99.4	99.4	100	100
17	9/BGR/1999_KP821079	89.6	91.1	97.1	97.7	97.8	97.9	97.9	97.8	97.9	98.0	98.0	98.0	98.0	98.0	100	100	100	100	100	100	100	100	100	100	100	100	100	99.4	99.4	100	100
18	9/SRB/2001_AJ585172	89.5	91.1	97.0	97.6	97.7	97.9	97.9	97.9	98.0	97.9	97.9	98.0	98.0	98.0	100	100	100	100	100	100	100	100	100	100	100	100	100	99.3	99.3	100	100
19	9/SRB/2001_KP821090	89.5	91.1	97.0	97.6	97.7	97.9	97.9	97.9	98.0	97.9	97.9	98.0	98.0	98.0	100	100	100	100	100	100	100	100	100	100	100	100	100	99.3	99.3	100	100
20	9/ITA/2000_JN255913	89.6	91.1	97.1	97.7	97.8	97.9	97.9	97.8	97.9	98.0	98.0	98.0	98.0	98.0	100	100	100	100	100	100	100	100	100	100	100	100	100	99.4	99.4	100	100
21	9/GRC/1999_KP821081	89.7	91.3	97.2	97.8	97.9	98.0	98.1	98.0	98.1	98.1	98.1	98.2	98.2	98.2	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
22	9/GRC/1999_KP821082	89.8	91.3	97.3	97.8	97.9	98.1	98.1	98.0	98.1	98.2	98.2	98.2	98.2	98.2	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
23	9/ITA/2001_DQ191285	89.6	91.2	97.1	97.7	97.8	97.9	97.9	97.9	98.0	98.0	98.0	98.1	98.1	98.1	100	100	100	100	100	100	100	100	100	100	100	100	100	99.4	99.4	100	100
24	9/BIH/2002_AJ585174	89.6	91.1	97.1	97.7	97.8	97.9	98.0	97.9	98.0	98.0	98.0	98.0	98.0	98.0	100	100	100	100	100	100	100	100	100	100	100	100	100	99.4	99.4	100	100
25	9/BIH/2002_KP821078	89.6	91.1	97.1	97.7	97.8	97.9	98.0	97.9	98.0	98.0	98.0	98.0	98.0	98.0	100	100	100	100	100	100	100	100	100	100	100	100	100	99.4	99.4	100	100
26	9/XKX/2001_KP821085	89.6	91.2	97.1	97.7	97.8	97.9	98.0	97.9	98.1	98.0	98.0	98.1	98.1	98.1	100	100	100	100	100	100	100	100	100	100	100	100	100	99.4	99.4	100	100
27	9/XKX/2001_KP821084	89.6	91.2	97.1	97.7	97.8	98.0	98.1	98.0	98.1	98.1	98.1	98.1	98.1	98.1	100	100	100	100	100	100	100	100	100	100	100	100	100	99.4	99.4	100	100
28	9/GRC/1998_DQ191283	89.6	91.3	97.2	97.8	97.9	98.0	98.1	98.0	98.1	98.1	98.1	98.2	98.2	98.2	99.3	99.4	99.4	99.3	99.3	99.4	100	100	99.4	99.4	99.4	99.4	99.4	100	100	100	100
29	9/GRC/1998_KP821080	89.6	91.3	97.2	97.8	97.9	98.0	98.1	98.0	98.1	98.1	98.1	98.2	98.2	98.2	99.3	99.4	99.4	99.3	99.3	99.4	100	100	99.4	99.4	99.4	99.4	99.4	100	100	100	100
30	9/TUR/2000_AJ585176	89.6	91.3	97.2	97.8	97.9	98.0	98.1	98.0	98.1	98.1	98.1	98.2	98.2	98.2	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
31	9/TUR/1998_KP821091	89.8	91.4	97.3	97.9	98.0	98.1	98.2	98.1	98.2	98.2	98.2	98.3	98.3	98.3	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-9 strains sequenced in this study and global reference western BTV-9 strains

	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16
IND2015/477_2015-12-25	100	69.04	69	68.62	68.65	68.65	68.83	68.83	68.83	68.76	68.69	68.69	68.69	68.79	69.32
9/LBY/2008_KP821086	69.04	100	99.86	95.19	95.15	95.15	95.36	95.36	95.43	95.33	95.26	95.26	95.26	85.77	85.91
9/LBY/2008_KP821087	69	99.86	100	95.12	95.08	95.08	95.29	95.29	95.36	95.26	95.19	95.19	95.19	85.77	85.84
9/ITA/2003_KP821083	68.62	95.19	95.12	100	99.9	99.9	99.65	99.65	99.72	99.83	99.9	99.9	99.9	86.26	87.03
9/RSAvvv_AJ585173	68.65	95.15	95.08	99.9	100	100	99.62	99.62	99.69	99.79	99.86	99.86	99.86	86.23	87.03
9/RSAvvvv/1942_KP82108	68.65	95.15	95.08	99.9	100	100	99.62	99.62	99.69	99.79	99.86	99.86	99.86	86.23	87.03
9/RSArrrr/1942_AJ58513	68.83	95.36	95.29	99.65	99.62	99.62	100	100	99.93	99.83	99.76	99.76	99.76	86.47	87.24
9/RSArrrr/1942_KP82108	68.83	95.36	95.29	99.65	99.62	99.62	100	100	99.93	99.83	99.76	99.76	99.76	86.47	87.24
9/ZAF/1942_JN255953	68.83	95.43	95.36	99.72	99.69	99.69	99.93	99.93	100	99.9	99.83	99.83	99.83	86.54	87.31
9/ZAF_JX272530	68.76	95.33	95.26	99.83	99.79	99.79	99.83	99.83	99.9	100	99.93	99.93	99.93	86.44	87.2
9/ZAF/1942_JN255963	68.69	95.26	95.19	99.9	99.86	99.86	99.76	99.76	99.83	99.93	100	100	100	86.37	87.13
9/ZAF/2014_KT885056	68.69	95.26	95.19	99.9	99.86	99.86	99.76	99.76	99.83	99.93	100	100	100	86.37	87.13
9/ZAF/2014_KT885076	68.69	95.26	95.19	99.9	99.86	99.86	99.76	99.76	99.83	99.93	100	100	100	86.37	87.13
9/USA/2008_KX164070	68.79	85.77	85.77	86.26	86.23	86.23	86.47	86.47	86.54	86.44	86.37	86.37	86.37	100	93.2
9/ECU/2015_KX442583	69.32	85.91	85.84	87.03	87.03	87.03	87.24	87.24	87.31	87.2	87.13	87.13	87.13	93.2	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-10 strains sequenced in this study and global reference western BTV-10 strains

	BTV-10	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	10/RSArrrr/1956_AJ585131	100	99	99.3	99.3	87.4	87.5	89.2	89.3	89.4	89.2	89.2	89.3	89.3	89.3	89.3	89.3	89.3	89.3	89.3	89.4	87.7	87.8	87.7
2	10_KX247939	99	100	99.7	99.6	87.6	87.5	88.9	89	89.1	88.7	88.7	88.9	88.9	89	89	89	89	89	89	89.1	87.4	87.5	87.5
3	10/ZAF_JX272520	99.3	99.7	100	99.9	87.6	87.5	88.9	89	89.1	88.8	88.8	88.9	88.9	89	89	89	89	89	89	89.1	87.5	87.6	87.6
4	10/ZAF/2011_KT317696	99.3	99.6	99.9	100	87.5	87.5	88.9	89	89	88.8	88.8	88.9	89	89	89	89	89	89	89	89	87.5	87.6	87.5
5	10/FRA/2006_HQ222821	87.4	87.6	87.6	87.5	100	96.3	89.5	89.4	89.5	89.2	89.2	89.5	89.5	89.5	89.5	89.5	89.5	89.5	89.5	89.6	88.3	88.6	88.4
6	10/GUF/2011_JQ436735	87.5	87.5	87.5	87.5	96.3	100	89.3	89.2	89.3	89.1	89.1	89.3	89.3	89.3	89.3	89.3	89.3	89.3	89.3	89.4	88.3	88.5	88.3
7	10/USA/1980_U06780	89.2	88.9	88.9	88.9	89.5	89.3	100	99.8	99.8	99.2	99.2	99.5	99.4	99.5	99.5	99.5	99.5	99.5	99.5	99.6	95.3	95.2	95.1
8	10/USA/1980_U06782	89.3	89	89	89	89.4	89.2	99.8	100	99.9	99.2	99.2	99.5	99.4	99.5	99.5	99.5	99.5	99.5	99.5	99.6	95.2	95.1	95.1
9	10/USA/1980_U06783	89.4	89.1	89.1	89	89.5	89.3	99.8	99.9	100	99.2	99.3	99.6	99.5	99.6	99.6	99.6	99.6	99.6	99.6	99.7	95.3	95.3	95.1
10	10/IND/2004_JN704634	89.2	88.7	88.8	88.8	89.2	89.1	99.2	99.2	99.2	100	99.5	99.4	99.7	99.7	99.7	99.7	99.5	99.5	99.5	99.6	95.2	95.2	95.1
11	10/IND/2003_JF727655	89.2	88.7	88.8	88.8	89.2	89.1	99.2	99.2	99.3	99.5	100	99.4	99.7	99.7	99.7	99.7	99.6	99.6	99.6	99.6	95.3	95.2	95.2
12	10/USA_U06786	89.3	88.9	88.9	88.9	89.5	89.3	99.5	99.5	99.6	99.4	99.4	100	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.8	95.3	95.3	95.2
13	10/IND/2004_JQ740772	89.3	88.9	88.9	89	89.5	89.3	99.4	99.4	99.5	99.7	99.7	99.7	100	100	100	100	99.8	99.8	99.8	99.9	95.3	95.3	95.3
14	10/IND/2003_KP339245	89.3	89	89	89	89.5	89.3	99.5	99.5	99.6	99.7	99.7	99.7	100	100	100	100	99.8	99.8	99.8	99.9	95.3	95.3	95.2
15	IND2014/BTV1_2014-12-06	89.3	89	89	89	89.5	89.3	99.5	99.5	99.6	99.7	99.7	99.7	100	100	100	100	99.8	99.8	99.8	99.9	95.3	95.3	95.2
16	IND2014/BTV2_2014-12-06	89.3	89	89	89	89.5	89.3	99.5	99.5	99.6	99.7	99.7	99.7	100	100	100	100	99.8	99.8	99.8	99.9	95.3	95.3	95.2
17	10/USA_L29026	89.3	89	89	89	89.5	89.3	99.5	99.5	99.6	99.5	99.6	99.7	99.8	99.8	99.8	99.8	100	99.9	99.9	99.9	95.4	95.3	95.3
18	10/USA_M11787	89.3	89	89	89	89.5	89.3	99.5	99.5	99.6	99.5	99.6	99.7	99.8	99.8	99.8	99.8	99.9	100	100	99.9	95.4	95.3	95.3
19	10/USA/1953_NC_006013	89.3	89	89	89	89.5	89.3	99.5	99.5	99.6	99.5	99.6	99.7	99.8	99.8	99.8	99.8	99.9	100	100	99.9	95.4	95.3	95.3
20	10/USA_L29027	89.4	89.1	89.1	89	89.6	89.4	99.6	99.6	99.7	99.6	99.6	99.8	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100	95.4	95.4	95.3
21	10/USA/1990_U06784	87.7	87.4	87.5	87.5	88.3	88.3	95.3	95.2	95.3	95.2	95.3	95.3	95.3	95.3	95.3	95.3	95.4	95.4	95.4	95.4	100	98.3	98.1
22	10/USA/1990_U06781	87.8	87.5	87.6	87.6	88.6	88.5	95.2	95.1	95.3	95.2	95.2	95.3	95.3	95.3	95.3	95.3	95.3	95.3	95.3	95.4	98.3	100	99.7
23	10/USA/1990_U06785	87.7	87.5	87.6	87.5	88.4	88.3	95.1	95.1	95.1	95.1	95.2	95.2	95.3	95.2	95.2	95.2	95.3	95.3	95.3	95.3	98.1	99.7	100

Appendix

Percentage of nucleotide identity in Seg-2 between Indian BTV-12 strains sequenced in this study and global reference western BTV-12 strains

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	12/IND/2011_KC662613	100	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	97.4	97.6	97.5	96.8	96.1	87.9	87.6	88.6
2	IND2015/22_2015-12-18	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
3	IND2015/494_2015-12-25	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
4	IND2015/406_2015-12-25	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
5	IND2015/470_2015-12-25	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
6	IND2015/472_2015-12-25	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
7	IND2015/473_2015-12-25	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
8	IND2015/474_2015-12-25	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
9	IND2015/480_2015-12-25	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
10	IND2015/483_2015-12-25	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
11	IND2015/484_2015-12-25	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
12	IND2015/CS4_2015-12-25	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
13	IND2015/CS6_2015-12-25	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
14	IND2015/34_tissue_2015-12-	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
15	IND2015/556_saliva_2015-12-	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
16	IND2015/V41_2015-11-26	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
17	IND2015/V44_lamb_2015-11-	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
18	IND2015/V44Severe_2015-11-	99.4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.4
19	12/IND/2016_MF615238	99.4	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100	99.9	97.1	97.2	97.1	96.4	95.7	87.9	87.5	88.3
20	12/IND/2015_KX905151	99.4	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100	97.1	97.2	97.2	96.5	95.8	87.9	87.6	88.4
21	12/RSArrrr/1941_AJ585133	97.4	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	100	99.8	99.8	99.0	98.1	88.9	88.5	89.6
22	12/ZAF_JX272500	97.6	97.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2	99.8	100	100	98.9	98.0	89.0	88.6	89.7
23	12/RSArrrr/1941_KP821100	97.5	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.1	97.2	99.8	100	100	98.9	98.0	89.0	88.5	89.6
24	12/JPN/1990_AB686216	96.8	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.4	96.5	99.0	98.9	98.9	100	99.0	88.6	88.0	88.9
25	12/TWN/2003_GU390659	96.1	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.8	98.1	98.0	98.0	99.0	100	88.1	87.7	88.5
26	12/USA/2008_KX164080	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.9	88.9	89.0	89.0	88.6	88.1	100	88.1	89.1
27	12/GUF/2011_KC633278	87.6	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.6	88.5	88.6	88.5	88.0	87.7	88.1	100	90.6
28	12/USA/2012_KX164090	88.6	88.4	88.4	88.4	88.4	88.4	88.4	88.4	88.4	88.4	88.4	88.4	88.4	88.4	88.4	88.4	88.4	88.4	88.3	88.4	89.6	89.7	89.6	88.9	88.5	89.1	90.6	100

ACHARYA, R. M. 1982. Sheep and goat breeds of India. *FAO Animal production and Health***,** 30.

AFSHAR, A. 1994. Bluetongue: laboratory diagnosis. *Comp Immunol Microbiol Infect Dis,* 17**,** 221-42.

AFSHAR, A., DUBUC, C., DULAC, G. C., THOMAS, F. C., NIELSEN, K. & HENNING, D. 1991. Dot immunoperoxidase assay using monoclonal antibody for detection of bluetongue virus antigens. *J Virol Methods,* 31**,** 105-12.

AFSHAR, A., EATON, B. T., WRIGHT, P. F., PEARSON, J. E., ANDERSON, J., JEGGO, M. & TROTTER, H. C. 1992a. Competitive ELISA for Serodiagnosis of Bluetongue: Evaluation of Group-Specific Monoclonal Antibodies and Expressed VP7 Antigen. *Journal of Veterinary Diagnostic Investigation,* 4**,** 231-237.

AFSHAR, A., SHAKARCHI, N. H., WRIGHT, P. F. & BOSSE, J. 1992b. Simultaneous screening of bovine sera for antibodies to bluetongue and epizootic hemorrhagic disease of deer viruses by enzyme-linked immunosorbent assay. *Zentralbl Veterinarmed B,* 39**,** 159-64.

AFSHAR, A., THOMAS, F. C., WRIGHT, P. F., SHAPIRO, J. L., SHETTIGARA, P. T. & ANDERSON, J. 1987. Comparison of competitive and indirect enzyme-linked immunosorbent assays for detection of bluetongue virus antibodies in serum and whole blood. *J Clin Microbiol,* 25**,** 1705-10.

AICRP 2014. All India coordinated research project on animal disease monitoring and surveillance. *In:* GAJENDRAGAD, M. R., REDDY, G. B. M. & SURESH, K. P. (eds.). NIVEDI, Yelahanka, Bengaluru: ICAR-National Institute of Veterinary Epidemiology & Disease Informatics.

AICRP 2016. All India coordinated research project on animal disease monitoring and surveillance, Annual report, 2015-16. *In:* HEMADRI, D., PATIL, S. S., SURESH, K. P. & PRAJAPATI, A. (eds.). NIVEDI, Yelahanka, Bengaluru:: ICAR-National Institute of Veterinary Epidemiology & Disease Informatics.

AINPBT 2010. Annual Report All India Network Project on Bluetongue. Indian Council of Agricultural Research, New Delhi, India.

AINPBT 2013. All India network programme on bluetongue. http://ainpbt.com/ (accessed 11April 2018).

AKITA, G. Y., CHINSANGARAM, J., OSBURN, B. I., IANCONESCU, M. & KAUFMAN, R. 1992. Detection of bluetongue virus serogroup by polymerase chain reaction. *J Vet Diagn Invest,* 4**,** 400-5.

AKITA, G. Y., IANCONESCU, M., MACLACHLAN, N. J. & OSBURN, B. I. 1994. Bluetongue disease in dogs associated with contaminated vaccine. *Vet Rec,* 134**,** 283-4.

References

ALEXANDER, K. A., MACLACHLAN, N. J., KAT, P. W., HOUSE, C., O'BRIEN, S. J., LERCHE, N. W., SAWYER, M., FRANK, L. G., HOLEKAMP, K., SMALE, L. & ET AL. 1994. Evidence of natural bluetongue virus infection among African carnivores. *Am J Trop Med Hyg,* 51**,** 568-76.

ALPAR, H. O., BRAMWELL, V. W., VERONESI, E., DARPEL, K. E., PASTORET, P. P. & MERTENS, P. P. C. 2009. Bluetongue virus vaccines past and present. *In:* MELLOR, P. S., BAYLIS, M. & MERTENS, P. P. C. (eds.) *Bluetongue.* 1st ed. London: Elsevier Academic Press.

ANDERSON, J. 1984. Use of monoclonal antibody in a blocking ELISA to detect group specific antibodies to bluetongue virus. *J Immunol Methods,* 74**,** 139-49.

ANDERSON, J., BREARD, E., LOVGREN BENGTSSON, K., GRONVIK, K. O., ZIENTARA, S., VALARCHER, J. F. & HAGGLUND, S. 2014. Purification, stability, and immunogenicity analyses of five bluetongue virus proteins for use in development of a subunit vaccine that allows differentiation of infected from vaccinated animals. *Clin Vaccine Immunol,* 21**,** 443-52.

ANDERSON, J., MERTENS, P. P. & HERNIMAN, K. A. 1993. A competitive ELISA for the detection of anti-tubule antibodies using a monoclonal antibody against bluetongue virus non-structural protein NS1. *J Virol Methods,* 43**,** 167-75.

ANDINO, R. & DOMINGO, E. 2015. Viral quasispecies. *Virology,* 479-480**,** 46-51.

ANTHONY, S., JONES, H., DARPEL, K. E., ELLIOTT, H., MAAN, S., SAMUEL, A., MELLOR, P. S. & 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.

ANTHONY, S., MAAN, S., SAMUEL, A. R., MELLOR, P. S. & MERTENS, P. P. 2004. Differential diagnosis of bluetongue virus using a reverse transcriptase-polymerase chain reaction for genome segment 7. *Vet Ital,* 40**,** 546-51.

ANTHONY, S. J., DARPEL, K. E., BELAGANAHALLI, M. N., MAAN, N., NOMIKOU, K., SUTTON, G., ATTOUI, H., MAAN, S. & MERTENS, P. P. C. 2011. RNA segment 9 exists as a duplex concatemer in an Australian strain of epizootic haemorrhagic disease virus (EHDV): Genetic analysis and evidence for the presence of concatemers as a normal feature of orbivirus replication. *Virology,* 420**,** 164-171.

ARADAIB, I. E., SCHORE, C. E., CULLOR, J. S. & OSBURN, B. I. 1998. A nested PCR for detection of North American isolates of bluetongue virus based on NS1 genome sequence analysis of BTV-17. *Vet Microbiol,* 59**,** 99-108.

ARADAIB, I. E., SMITH, W. L., OSBURN, B. I. & CULLOR, J. S. 2003. A multiplex PCR for simultaneous detection and differentiation of North American serotypes of bluetongue and epizootic hemorrhagic disease viruses. *Comp Immunol Microbiol Infect Dis,* 26**,** 77-87.

ARUN, S., JOHN, K., RAVISHANKAR, C., MINI, M., RAVINDRAN, R. & PREJIT, N. 2014. Seroprevalence of bluetongue among domestic ruminants in Northern Kerala, India. *Trop Biomed,* 31**,** 26-30.

ATTOUI, H., BILLOIR, F., BIAGINI, P., DE MICCO, P. & DE LAMBALLERIE, X. 2000a. Complete sequence determination and genetic analysis of Banna virus and Kadipiro virus: proposal

for assignment to a new genus (Seadornavirus) within the family Reoviridae. *J Gen Virol,* 81**,** 1507-15.

ATTOUI, H., BILLOIR, F., CANTALOUBE, J. F., BIAGINI, P., DE MICCO, P. & DE LAMBALLERIE, X. 2000b. Strategies for the sequence determination of viral dsRNA genomes. *J Virol Methods,* 89**,** 147-58.

ATTOUI, H., FANG, Q., MOHD JAAFAR, F., CANTALOUBE, J. F., BIAGINI, P., DE MICCO, P. & DE LAMBALLERIE, X. 2002. Common evolutionary origin of aquareoviruses and orthoreoviruses revealed by genome characterization of Golden shiner reovirus, Grass carp reovirus, Striped bass reovirus and golden ide reovirus (genus Aquareovirus, family Reoviridae). *J Gen Virol,* 83**,** 1941-51.

ATTOUI, H., MERTENS, P. P. C., BECNEL, J., BELAGANAHALLI, M., BERGOIN, M., BRUSSAARD, C. P., CHAPPELL, J. D., CIARLET, M., DEL VAS, M., DERMODY, T. S., DORMITZER, P. R., DUNCAN, R., FCANG, Q., GRAHAM, R., GUGLIELMI, K. M., HARDING, R. M., HILLMAN, B., MAKKAY, A., MARZACHÌ, C., MATTHIJNSSENS, J., MILNE, R. G., MOHD JAAFAR, F., MORI, H., NOORDELOOS, A. A., OMURA, T., PATTON, J. T., RAO, S., MAAN, S., STOLTZ, D., SUZUKI, N., UPADHYAYA, N. M., WEI, C. & ZHOU, H. 2012. Family Reoviridae. *In:* KING, A. M. Q., ADAMS, M. J., B., C. E. & J., L. E. (eds.) *Virus Taxonomy Ninth report of the international committee on taxonomy of viruses.* San Diego: Elsevier Academic Press.

ATTOUI, H., MOHD JAAFAR, F., BELHOUCHET, M., BIAGINI, P., CANTALOUBE, J.-F., DE MICCO, P. & DE LAMBALLERIE, X. 2005. Expansion of family Reoviridae to include nine-segmented dsRNA viruses: Isolation and characterization of a new virus designated aedes pseudoscutellaris reovirus assigned to a proposed genus (Dinovernavirus). *Virology,* 343**,** 212-223.

ATTOUI, H., STIRLING, J. M., MUNDERLOH, U. G., BILLOIR, F., BROOKES, S. M., BURROUGHS, J. N., DE MICCO, P., MERTENS, P. P. & DE LAMBALLERIE, X. 2001. Complete sequence characterization of the genome of the St Croix River virus, a new orbivirus isolated from cells of Ixodes scapularis. *J Gen Virol,* 82**,** 795-804.

ATTRI, S. D. & TYAGI, A. 2010. Climate profile. *Climate profile of India.* Indian Meteorological Department, New Delhi, India.

BACKX, A., HEUTINK, C. G., VAN ROOIJ, E. M. & VAN RIJN, P. A. 2007. Clinical signs of bluetongue virus serotype 8 infection in sheep and goats. *Vet Rec,* 161**,** 591-2.

BALARO, M. F., DOS SANTOS LIMA, M., DEL FAVA, C., DE OLIVEIRA, G. R., & PITUCO, E. M., & BRANDAO, F. Z. 2014 Outbreak of bluetongue virus serotype 4 in dairy sheep in Rio de Janeiro, Brazil. *Journal of Veterinary Diagnostic Investigation,* 26 567–570.

BALASURIYA, U. B., NADLER, S. A., WILSON, W. C., PRITCHARD, L. I., SMYTHE, A. B., SAVINI, G., MONACO, F., DE SANTIS, P., ZHANG, N., TABACHNICK, W. J. & MACLACHLAN, N. J. 2008. The NS3 proteins of global strains of bluetongue virus evolve into regional topotypes through negative (purifying) selection. *Vet Microbiol,* 126**,** 91-100.

BARBER, T. L. 1979. Temporal appearance, geographic distribution, and species of origin of bluetongue virus serotypes in the United States. *Am J Vet Res,* 40**,** 1654-6.

References

BARRATT-BOYES, S. M. & MACLACHLAN, N. J. 1994. Dynamics of viral spread in bluetongue virus infected calves. *Vet Microbiol,* 40**,** 361-71.

BARRATT-BOYES, S. M. & MACLACHLAN, N. J. 1995. Pathogenesis of bluetongue virus infection of cattle. *J Am Vet Med Assoc,* 206**,** 1322-9.

BARRATT-BOYES, S. M., ROSSITTO, P. V., TAYLOR, B. C., ELLIS, J. A. & MACLACHLAN, N. J. 1995. Response of the regional lymph node to bluetongue virus infection in calves. *Vet Immunol Immunopathol,* 45**,** 73-84.

BARROS, S. C., CRUZ, B., LUIS, T. M., RAMOS, F., FAGULHA, T., DUARTE, M., HENRIQUES, M. & FEVEREIRO, M. 2009. A DIVA system based on the detection of antibodies to non-structural protein 3 (NS3) of bluetongue virus. *Vet Microbiol,* 137**,** 252-9.

BARSOUM, G., WALTON, T. AND OSBURN, B. 1992. Bluetongue and African horse sickness situation in Egypt. Bluetongue, African horse sickness, and related orbiviruses. *Proceedings of the Second International Symposium, CRC***,** Press Inc, 42–43.

BASAK, A. K., GOUET, P., GRIMES, J., ROY, P. & STUART, D. 1996. Crystal structure of the top domain of African horse sickness virus VP7: comparisons with bluetongue virus VP7. *J Virol,* 70**,** 3797-806.

BASAK, A. K., STUART, D. I. & ROY, P. 1992. Preliminary crystallographic study of bluetongue virus capsid protein, VP7. *Journal of molecular biology,* 228**,** 687-689.

BATTEN, C., DARPEL, K., HENSTOCK, M., FAY, P., VERONESI, E., GUBBINS, S., GRAVES, S., FROST, L. & OURA, C. 2014. Evidence for transmission of bluetongue virus serotype 26 through direct contact. *PLoS One,* 9**,** e96049.

BATTEN, C. A., MAAN, S., SHAW, A. E., MAAN, N. S. & MERTENS, P. P. 2008. A European field strain of bluetongue virus derived from two parental vaccine strains by genome segment reassortment. *Virus Res,* 137**,** 56-63.

BATTEN, C. A., SANDERS, A. J., BACHANEK-BANKOWSKA, K., BIN-TARIF, A. & OURA, C. A. 2009. Bluetongue virus: European Community proficiency test (2007) to evaluate ELISA and RT-PCR detection methods with special reference to pooling of samples. *Vet Microbiol,* 135**,** 380-3.

BEATON, A. R., RODRIGUEZ, J., REDDY, Y. K. & ROY, P. 2002. The membrane trafficking protein calpactin forms a complex with bluetongue virus protein NS3 and mediates virus release. *Proc Natl Acad Sci U S A,* 99**,** 13154-9.

BEKKER, J., DE KOCK, G. & QUINLAN, J. 1934. The occurrence and identification of bluetongue in cattle – the so-called pseudo foot-and-mouth disease in South Africa. . *Onderstepoort Journal of Veterinary Science and Animal Industry* 2**,** 393-507.

BELAGANAHALLI, M. N., MAAN, S., MAAN, N. S., BROWNLIE, J., TESH, R., ATTOUI, H. & MERTENS, P. P. 2015. Genetic characterization of the tick-borne orbiviruses. *Viruses,* 7**,** 2185-209.

References

BELAGANAHALLI, M. N., MAAN, S., MAAN, N. S., NOMIKOU, K., PRITCHARD, I., LUNT, R., KIRKLAND, P. D., ATTOUI, H., BROWNLIE, J. & MERTENS, P. P. C. 2012. Full Genome Sequencing and Genetic Characterization of Eubenangee Viruses Identify Pata Virus as a Distinct Species within the Genus Orbivirus. *PLoS ONE,* 7**,** e31911.

BELAGANAHALLI, M. N., MAAN, S., MAAN, N. S., TESH, R., ATTOUI, H. & MERTENS, P. P. 2011. Umatilla virus genome sequencing and phylogenetic analysis: identification of stretch lagoon orbivirus as a new member of the Umatilla virus species. *PLoS One,* 6**,** e23605.

BELHOUCHET, M., MOHD JAAFAR, F., FIRTH, A. E., GRIMES, J. M., MERTENS, P. P. C. & ATTOUI, H. 2011. Detection of a Fourth Orbivirus Non-Structural Protein. *PLoS ONE,* 6**,** e25697.

BELHOUCHET, M., MOHD JAAFAR, F., TESH, R., GRIMES, J., MAAN, S., MERTENS, P. P. & ATTOUI, H. 2010. Complete sequence of Great Island virus and comparison with the T2 and outer-capsid proteins of Kemerovo, Lipovnik and Tribec viruses (genus Orbivirus, family Reoviridae). *J Gen Virol,* 91**,** 2985-93.

BEXIGA, R., GUYOT, H., SAEGERMAN, C., MAUROY, A., ROLLIN, F., THIRY, E., PHILBEY, A. W., LOGUE, D. N., MELLOR, D. J., BARRETT, D. C. & ELLIS, K. 2007. Clinical differentiation of malignant catarrhal fever, mucosal disease and bluetongue. *Vet Rec,* 161**,** 858-9.

BHAN, S. C., DURAI, V. R., DAS, A. K., KAUR, S., KUMAR, N., MITRA, A. K., MOHAPATRA, M., PATTANAIK, D. R. & RAMA RAO, Y. V. 2015. Monsoon 2014, A report. *In:* PAI, D. S. & BHAN, S. C. (eds.). National Climate Centre,India Meteorological Department,Pune,India.

BHANUPRAKASH, V., INDRANI, B. K., HOSAMANI, M., BALAMURUGAN, V. & SINGH, R. K. 2009. Bluetongue vaccines: the past, present and future. *Expert Rev Vaccines,* 8**,** 191-204.

BHATIA, S. & ARORA, R. 2005. Biodiversity and Conservation of Indian Sheep Genetic Resources-An Overview *Asian-Austrailan Journal of Animal Science* 18**,** 1387-1402.

BILLINIS, C., KOUMBATI, M., SPYROU, V., NOMIKOU, K., MANGANA, O., PANAGIOTIDIS, C. A. & PAPADOPOULOS, O. 2001. Bluetongue virus diagnosis of clinical cases by a duplex reverse transcription-PCR: a comparison with conventional methods. *J Virol Methods,* 98**,** 77-89.

BISWAS, S. K., CHAND, K., DE, A., PANDEY, L. K., MOHAPATRA, J. K., PRASAD, G. & 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. *Arch Virol,* 155**,** 2041-6.

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. *Vet Microbiol,* 176**,** 358-64.

BLACKSELL, S. D. & LUNT, R. A. 1996. A simplified fluorescence inhibition test for the serotype determination of Australian bluetongue viruses. *Aust Vet J,* 73**,** 33-4.

BOMMINENI, Y. R., REDDY, Y. N., RAO, P. P., SUDHEER, D., SUSMITHA, B., SARMA, B. J. & RAO, M. V. 2008. Isolation of bluetongue virus serotypes 2, 9 and 15 from sheep in India. *Vet Rec,* 163**,** 545-6.

BONNEAU, K. R., DEMAULA, C. D., MULLENS, B. A. & MACLACHLAN, N. J. 2002. Duration of viraemia infectious to Culicoides sonorensis in bluetongue virus-infected cattle and sheep. *Vet Microbiol,* 88**,** 115-25.

BONNEAU, K. R., MULLENS, B. A. & MACLACHLAN, N. J. 2001. Occurrence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/NS3A genes of bluetongue virus upon passage between sheep, cattle, and Culicoides sonorensis. *J Virol,* 75**,** 8298-305.

BONNEAU, K. R., ZHANG, N., ZHU, J., ZHANG, F., LI, Z., ZHANG, K., XIAO, L., XIANG, W. & MACLACHLAN, N. J. 1999. Sequence comparison of the L2 and S10 genes of bluetongue viruses from the United States and the People's Republic of China. *Virus Res,* 61**,** 153-60.

BOULANGER, P., RUCKERBAUER, G. M., BANNISTER, G. L., GRAY, D. P. & GIRARD, A. 1967. Studies on bluetongue. 3. Comparison of two complement-fixation methods. *Can J Comp Med Vet Sci,* 31**,** 166-70.

BOUWKNEGT, C., VAN RIJN, P. A., SCHIPPER, J. J. M., HÖLZEL, D., BOONSTRA, J., NIJHOF, A. M., VAN ROOIJ, E. M. A. & JONGEJAN, F. 2010. Potential role of ticks as vectors of bluetongue virus. *Experimental & Applied Acarology,* 52**,** 183-192.

BOWEN, R. A. & HOWARD, T. H. 1984. Transmission of bluetongue virus by intrauterine inoculation or insemination of virus-containing bovine semen. *American journal of veterinary research,* 45**,** 1386-1388.

BOYCE, M., CELMA, C. C. & ROY, P. 2012. Bluetongue virus non-structural protein 1 is a positive regulator of viral protein synthesis. *Virol J,* 9**,** 178.

BOYCE, M., MCCRAE, M. A., BOYCE, P. & KIM, J. T. 2016. Inter-segment complementarity in orbiviruses: a driver for co-ordinated genome packaging in the Reoviridae? *J Gen Virol,* 97**,** 1145-57.

BOYCE, M., WEHRFRITZ, J., NOAD, R. & ROY, P. 2004. Purified recombinant bluetongue virus VP1 exhibits RNA replicase activity. *J Virol,* 78**,** 3994-4002.

BOYLE, D. B., BULACH, D. M., AMOS-RITCHIE, R., ADAMS, M. M., WALKER, P. J. & WEIR, R. 2012. Genomic sequences of Australian bluetongue virus prototype serotypes reveal global relationships and possible routes of entry into Australia. *J Virol,* 86**,** 6724-31.

BREARD, 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. *Vet Res,* 34**,** 777-89.

BREARD, E., SAILLEAU, C., NOMIKOU, K., HAMBLIN, C., MERTENS, P. P., MELLOR, P. S., EL HARRAK, M. & ZIENTARA, S. 2007. Molecular epidemiology of bluetongue virus serotype 4 isolated in the Mediterranean Basin between 1979 and 2004. *Virus Res,* 125**,** 191-7.

BREARD, E., SAILLEAU, C., QUENAULT, H., LUCAS, P., VIAROUGE, C., TOUZAIN, F., FABLET, A., VITOUR, D., ATTOUI, H., ZIENTARA, S. & BLANCHARD, Y. 2016. Complete Genome

Sequence of Bluetongue Virus Serotype 8, Which Reemerged in France in August 2015. *Genome Announc,* 4.

BRENNER, J., OURA, C., ASIS, I., MAAN, S., ELAD, D., MAAN, N., FRIEDGUT, O., NOMIKOU, K., ROTENBERG, D., BUMBAROV, V., MERTENS, P., YADIN, H. & BATTEN, C. 2010. Multiple serotypes of bluetongue virus in sheep and cattle, Israel. *Emerg Infect Dis,* 16**,** 2003-4.

BREWER, A. W. & MACLACHLAN, N. J. 1992. Ultrastructural characterization of the interaction of bluetongue virus with bovine erythrocytes in vitro. *Vet Pathol,* 29**,** 356-9.

BREWER, A. W. & MACLACHLAN, N. J. 1994. The pathogenesis of bluetongue virus infection of bovine blood cells in vitro: ultrastructural characterization. *Arch Virol,* 136**,** 287-98.

BRODIE, S. J., WILSON, W. C., O'HEARN, P. M., MUTHUI, D., DIEM, K. & PEARSON, L. D. 1998. The effects of pharmacological and lentivirus-induced immune suppression on orbivirus pathogenesis: assessment of virus burden in blood monocytes and tissues by reverse transcription-in situ PCR. *J Virol,* 72**,** 5599-609.

BROOKES, S. M., HYATT, A. D. & EATON, B. T. 1993. Characterization of virus inclusion bodies in bluetongue virus-infected cells. *J Gen Virol,* 74 (Pt 3)**,** 525-30.

BROOKES, S. M., HYATT, A. D. & EATON, B. T. 1994. The use of immuno-gold silver staining in bluetongue virus adsorption and neutralisation studies. *J Virol Methods,* 46**,** 117-32.

BROWN-JOSEPH, T., BATTEN, C., HARRUP, L. E., FROST, L., FLANNERY, J., HICKS, H., RAMKISSOON, V., RAMDEEN, R., CARRINGTON, C. V. & OURA, C. A. L. 2017. Bluetongue virus infection in naïve cattle: Identification of circulating serotypes and associated Culicoides biting midge species in Trinidad. *Veterinary Microbiology,* 211**,** 1-5.

BROWN, C. C., RHYAN, J. C., GRUBMAN, M. J. & WILBUR, L. A. 1996. Distribution of bluetongue virus in tissues of experimentally infected pregnant dogs as determined by in situ hybridization. *Vet Pathol,* 33**,** 337-40.

BUMBAROV, V., GOLENDER, N., ERSTER, O. & KHINICH, Y. 2016. Detection and isolation of Bluetongue virus from commercial vaccine batches. *Vaccine,* 34**,** 3317-3323.

BUSTIN, S. A., BENES, V., NOLAN, T. & PFAFFL, M. W. 2005. Quantitative real-time RT-PCR--a perspective. *J Mol Endocrinol,* 34**,** 597-601.

BUTAN, C. & TUCKER, P. 2010. Insights into the role of the non-structural protein 2 (NS2) in Bluetongue virus morphogenesis. *Virus Res,* 151**,** 109-17.

CALISHER, C. H. & MERTENS, P. P. 1998. Taxonomy of African horse sickness viruses. *Arch Virol Suppl,* 14**,** 3-11.

CALVO-PINILLA, E., CASTILLO-OLIVARES, J., JABBAR, T., ORTEGO, J., DE LA POZA, F. & MARIN-LOPEZ, A. 2014. Recombinant vaccines against bluetongue virus. *Virus Res,* 182**,** 78-86.

References

CAPORALE, M., DI GIALLEONORADO, L., JANOWICZ, A., WILKIE, G., SHAW, A., SAVINI, G., VAN RIJN, P. A., MERTENS, P., DI VENTURA, M. & PALMARINI, M. 2014. Virus and host factors affecting the clinical outcome of bluetongue virus infection. *J Virol,* 88**,** 10399-411.

CARPI, G., HOLMES, E. C. & KITCHEN, A. 2010. The evolutionary dynamics of bluetongue virus. *J Mol Evol,* 70**,** 583-92.

CELMA, C. C. & ROY, P. 2009. A viral nonstructural protein regulates bluetongue virus trafficking and release. *J Virol,* 83**,** 6806-16.

CHAIGNAT, V., WORWA, G., SCHERRER, N., HILBE, M., EHRENSPERGER, F., BATTEN, C., CORTYEN, M., HOFMANN, M. & THUER, B. 2009. Toggenburg Orbivirus, a new bluetongue virus: initial detection, first observations in field and experimental infection of goats and sheep. *Vet Microbiol,* 138**,** 11-9.

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. *J Virol Methods,* 160**,** 189-92.

CHAND, K., BISWAS, S. K., SHARMA, G., SAXENA, A., TEWARI, N., MAHAJAN, S. & PANDEY, A. B. 2016. Full genome sequencing of the bluetongue virus-1 isolate MKD20/08/Ind from goat in India. *Braz J Microbiol,* 47**,** 527-8.

CHAUHAN, H. C., BISWAS, S. K., CHAND, K., REHMAN, W., DAS, B., DADAWALA, A. I., CHANDEL, B. S., KHER, H. N. & MONDAL, B. 2014. Isolation of bluetongue virus serotype 1 from aborted goat fetuses. *Rev Sci Tech,* 33**,** 803-12.

CHAUVEAU, E., DOCEUL, V., LARA, E., BREARD, E., SAILLEAU, C., VIDALAIN, P. O., MEURS, E. F., DABO, S., SCHWARTZ-CORNIL, I., ZIENTARA, S. & VITOUR, D. 2013. NS3 of bluetongue virus interferes with the induction of type I interferon. *J Virol,* 87**,** 8241-6.

CHIANG, E. T., PERSAUD-SAWIN, D. A., KULKARNI, S., GARCIA, J. G. & IMANI, F. 2006. Bluetongue virus and double-stranded RNA increase human vascular permeability: role of p38 MAPK. *J Clin Immunol,* 26**,** 406-16.

CHOUDHARY, P. G. Incidence of bluetongue in State of Maharashtra. *In:* M.L. & MEHROTRA, M. L., eds. Proceedings of National Workshop on Bluetongue. , 1982 Izatnagar, IVRI. 153-8.

CLAVIJO, A., HECKERT, R. A., DULAC, G. C. & AFSHAR, A. 2000. Isolation and identification of bluetongue virus. *J Virol Methods,* 87**,** 13-23.

CLAVIJO, A., SEPULVEDA, L., RIVA, J., PESSOA-SILVA, M., TAILOR-RUTHES, A. & LOPEZ, J. W. 2002. Isolation of bluetongue virus serotype 12 from an outbreak of the disease in South America. *Vet Rec,* 151**,** 301-2.

COEN, M. L., ELLIS, J. A., O'TOOLE, D. T. & WILSON, W. C. 1991. Cytokine modulation of the interaction between bluetongue virus and endothelial cells in vitro. *Vet Pathol,* 28**,** 524-32.

References

COETZEE, P., STOKSTAD, M., VENTER, E. H., MYRMEL, M. & VAN VUUREN, M. 2012a. Bluetongue: a historical and epidemiological perspective with the emphasis on South Africa. *Virol J,* 9**,** 198.

COETZEE, P., VAN VUUREN, M., STOKSTAD, M., MYRMEL, M. & VENTER, E. H. 2012b. Bluetongue virus genetic and phenotypic diversity: towards identifying the molecular determinants that influence virulence and transmission potential. *Vet Microbiol,* 161**,** 1-12.

CONRATHS, F. J., GETHMANN, J. M., STAUBACH, C., METTENLEITER, T. C., BEER, M. & HOFFMANN, B. 2009. Epidemiology of bluetongue virus serotype 8, Germany. *Emerg Infect Dis,* 15**,** 433-5.

COOKE, S. J., LAMBDEN, P. R., CAUL, E. O. & CLARKE, I. N. 1991. Molecular cloning, sequence analysis and coding assignment of the major inner capsid protein gene of human group C rotavirus. *Virology,* 184**,** 781-785.

COWLED, C., PALACIOS, G., MELVILLE, L., WEIR, R., WALSH, S., DAVIS, S., GUBALA, A., LIPKIN, W. I., BRIESE, T. & BOYLE, D. 2009. Genetic and epidemiological characterization of Stretch Lagoon orbivirus, a novel orbivirus isolated from Culex and Aedes mosquitoes in northern Australia. *The Journal of General Virology,* 90**,** 1433-1439.

COWLEY, J. A. & GORMAN, B. M. 1989. Cross-neutralization of genetic reassortants of bluetongue virus serotypes 20 and 21. *Vet Microbiol,* 19**,** 37-51.

COX, H. R. 1954. Bluetongue. *Bacteriol Rev,* 18**,** 239-53.

DACFW 2011. All India Report on Agriculture Census 2010-11. New Delhi: Agriculture census division, department of agriculture, cooperation & farmers welfare, ministry of agriculture & farmers welfare, government of India, 2015.

DADF, G. S. Strengthening Small Ruminant Based Livelihoods: A Compendium of Workshop Proceedings. South Asia Pro-Poor Livestock Policy Programme, 2016 New Delhi. 205.

DAHDF 2014. 19 livestock census-2012 All India report. New Delhi: Ministry of agriculture, department of animal husbandry, dairying and fisheries.

DAL POZZO, F., DE CLERCQ, K., GUYOT, H., VANDEMEULEBROUCKE, E., SARRADIN, P., VANDENBUSSCHE, F., THIRY, E. & SAEGERMAN, C. 2009a. Experimental reproduction of bluetongue virus serotype 8 clinical disease in calves. *Vet Microbiol,* 136**,** 352-8.

DAL POZZO, F., SAEGERMAN, C. & THIRY, E. 2009b. Bovine infection with bluetongue virus with special emphasis on European serotype 8. *Vet J,* 182**,** 142-51.

DARPEL, K. E., BATTEN, C. A., VERONESI, E., WILLIAMSON, S., ANDERSON, P., DENNISON, M., CLIFFORD, S., SMITH, C., PHILIPS, L., BIDEWELL, C., BACHANEK-BANKOWSKA, K., SANDERS, A., BIN-TARIF, A., WILSON, A. J., GUBBINS, S., MERTENS, P. P., OURA, C. A. & MELLOR, P. S. 2009. Transplacental transmission of bluetongue virus 8 in cattle, UK. *Emerg Infect Dis,* 15**,** 2025-8.

References

DARPEL, K. E., LANGNER, K. F., NIMTZ, M., ANTHONY, S. J., BROWNLIE, J., TAKAMATSU, H. H., MELLOR, P. S. & MERTENS, P. P. 2011. Saliva proteins of vector Culicoides modify structure and infectivity of bluetongue virus particles. *PLoS One,* 6**,** e17545.

DARRIBA, D., TABOADA, G. L., DOALLO, R. & POSADA, D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods,* 9**,** 772.

DAVIES, F. G., MUNGAI, J. N. & PINI, A. 1992. A new bluetongue virus serotype isolated in Kenya. *Vet Microbiol,* 31**,** 25-32.

DE CLERCQ, K., VANDENBUSSCHE, F., VANDEMEULEBROUCKE, E., VANBINST, T., DE LEEUW, I., VERHEYDEN, B., GORIS, N., MINTIENS, K., MEROC, E., HERR, C., HOOYBERGS, J., HOUDART, P., SUSTRONCK, B., DE DEKEN, R., MAQUET, G., BUGHIN, J., SAULMONT, M., LEBRUN, M., BERTELS, G. & MIRY, C. 2008. Transplacental bluetongue infection in cattle. *Vet Rec,* 162**,** 564.

DEDOLLI, K., KONI, A., KECI, R. & KOLECI, X. 2017. Clinical and serological study on bluetongue virus serotype 4 in cattle in Albania. *Anim Husb Dairy Vet Sci,* 1**,** 2-4.

DELLA-PORTA, A. J., PARSONS, I. M. & MCPHEE, D. A. 1985. Problems in the interpretation of diagnostic tests Due To cross-reactions between orbiviruses and broad serological

responses in animals. *In:* BARBER, T. L., JOCHIM, M. M. & OSBURN, B. I. (eds.) *Bluetongue and Related Orbiviruses.* New York Alan R. Liss, Inc, .

DEMAULA, C. D., BONNEAU, K. R. & MACLACHLAN, N. J. 2000. Changes in the outer capsid proteins of bluetongue virus serotype ten that abrogate neutralization by monoclonal antibodies. *Virus Res,* 67**,** 59-66.

DEMAULA, C. D., JUTILA, M. A., WILSON, D. W. & MACLACHLAN, N. J. 2001. Infection kinetics, prostacyclin release and cytokine-mediated modulation of the mechanism of cell death during bluetongue virus infection of cultured ovine and bovine pulmonary artery and lung microvascular endothelial cells. *J Gen Virol,* 82**,** 787-94.

DEMAULA, C. D., LEUTENEGGER, C. M., BONNEAU, K. R. & MACLACHLAN, N. J. 2002a. The role of endothelial cell-derived inflammatory and vasoactive mediators in the pathogenesis of bluetongue. *Virology,* 296**,** 330-7.

DEMAULA, C. D., LEUTENEGGER, C. M., JUTILA, M. A. & MACLACHLAN, N. J. 2002b. Bluetongue virus-induced activation of primary bovine lung microvascular endothelial cells. *Vet Immunol Immunopathol,* 86**,** 147-57.

DESAI, G. S., HOSAMANE, M., KATARIA, R. S., PATIL, S. S., PRABHUDAS, K., SINGH, R. K., BHANUPRAKASH, V. & MONDAL, B. 2009. Sequence analysis of the S10 gene of six Bluetongue Virus isolates from India. *Transbound Emerg Dis,* 56**,** 329-36.

DI EMIDIO, B., NICOLUSSI, P., PATTA, C., RONCHI, G. F., MONACO, F., SAVINI, G., CIARELLI, A. & CAPORALE, V. 2004. Efficacy and safety studies on an inactivated vaccine against bluetongue virus serotype 2. *Vet Ital,* 40**,** 640-4.

References

DIPROSE, J. M., BURROUGHS, J. N., SUTTON, G. C., GOLDSMITH, A., GOUET, P., MALBY, R., OVERTON, I., ZIENTARA, S., MERTENS, P. P., STUART, D. I. & GRIMES, J. M. 2001. Translocation portals for the substrates and products of a viral transcription complex: the bluetongue virus core. *EMBO J,* 20**,** 7229-39.

DOCEUL, V., CHAUVEAU, E., LARA, E., BREARD, E., SAILLEAU, C., ZIENTARA, S. & VITOUR, D. 2014. Dual modulation of type I interferon response by bluetongue virus. *J Virol,* 88**,** 10792-802.

DUNGU, B., GERDES, T. & SMIT, T. 2004a. The use of vaccination in the control of bluetongue in southern Africa. *Vet Ital,* 40**,** 616-22.

DUNGU, B., POTGIETER, C., VON TEICHMAN, B. & SMIT, T. 2004b. Vaccination in the control of bluetongue in endemic regions: the South African experience. *Dev Biol (Basel),* 119**,** 463-72.

EATON, B. T. & CRAMERI, G. S. 1989. The site of bluetongue virus attachment to glycophorins from a number of animal erythrocytes. *J Gen Virol,* 70 (Pt 12)**,** 3347-53.

EATON, B. T., HYATT, A. D. & WHITE, J. R. 1988. Localization of the nonstructural protein NS1 in bluetongue virus-infected cells and its presence in virus particles. *Virology,* 163**,** 527-37.

EKUE, F. N., NFI, A. N., TSANGUE, P., TAYLOR, W. P. & GUMM, I. D. 1985a. Bluetongue in exotic sheep in Cameroon. *Trop Anim Health Prod,* 17**,** 187-8.

EKUE, F. N., NFI, A. N., TSANGUE, P., TAYLOR, W. P. & GUMM, I. D. 1985b. Prevalence of bluetongue virus and antibodies in ruminants in Cameroon. *Trop Anim Health Prod,* 17**,** 189.

ELBERS, A. R., BACKX, A., EKKER, H. M., VAN DER SPEK, A. N. & VAN RIJN, P. A. 2008a. Performance of clinical signs to detect bluetongue virus serotype 8 outbreaks in cattle and sheep during the 2006-epidemic in The Netherlands. *Vet Microbiol,* 129**,** 156-62.

ELBERS, A. R., BACKX, A., MINTIENS, K., GERBIER, G., STAUBACH, C., HENDRICKX, G. & VAN DER SPEK, A. 2008b. Field observations during the Bluetongue serotype 8 epidemic in 2006. II. Morbidity and mortality rate, case fatality and clinical recovery in sheep and cattle in the Netherlands. *Prev Vet Med,* 87**,** 31-40.

EPIZONE 2017. 11th EPIZONE Annual Meeting Welcome in Paris. *ANSES, Paris, France*.

ERASMUS, B. J. 1975. Bluetongue in sheep and goats. *Aust Vet J,* 51**,** 165-70.

ESCHBAUMER, M., HOFFMANN, B., KONIG, P., TEIFKE, J. P., GETHMANN, J. M., CONRATHS, F. J., PROBST, C., METTENLEITER, T. C. & BEER, M. 2009. Efficacy of three inactivated vaccines against bluetongue virus serotype 8 in sheep. *Vaccine,* 27**,** 4169-75.

EVERMANN, J. F. 2013. Letter to the Editor, regarding Bluetongue virus and canine abortions. *J Vet Diagn Invest,* 25**,** 670.

FERNANDEZ-PACHECO, P., FERNANDEZ-PINERO, J., AGUERO, M. & JIMENEZ-CLAVERO, M. A. 2008. Bluetongue virus serotype 1 in wild mouflons in Spain. *Vet Rec,* 162**,** 659-60.

References

FERRARI, G., DE LIBERATO, C., SCAVIA, G., LORENZETTI, R., ZINI, M., FARINA, F., MAGLIANO, A., CARDETI, G., SCHOLL, F., GUIDONI, M., SCICLUNA, M. T., AMADDEO, D., SCARAMOZZINO, P. & AUTORINO, G. L. 2005. Active circulation of bluetongue vaccine virus serotype-2 among unvaccinated cattle in central Italy. *Prev Vet Med,* 68**,** 103-13.

FIRTH, A. E. 2008. Bioinformatic analysis suggests that the Orbivirus VP6 cistron encodes an overlapping gene. *Virology Journal,* 5**,** 48-48.

FORZAN, M., MARSH, M. & ROY, P. 2007. Bluetongue virus entry into cells. *J Virol,* 81**,** 4819-27.

FORZAN, M., WIRBLICH, C. & ROY, P. 2004. A capsid protein of nonenveloped Bluetongue virus exhibits membrane fusion activity. *Proc Natl Acad Sci U S A,* 101**,** 2100-5.

FOSTER, N. M., LUEDKE, A. J., PARSONSON, I. M. & WALTON, T. E. 1991. Temporal relationships of viremia, interferon activity, and antibody responses of sheep infected with several bluetongue virus strains. *Am J Vet Res,* 52**,** 192-6.

FRENCH, T. J., MARSHALL, J. J. & ROY, P. 1990. Assembly of double-shelled, viruslike particles of bluetongue virus by the simultaneous expression of four structural proteins. *J Virol,* 64**,** 5695-700.

FRENCH, T. J. & ROY, P. 1990. Synthesis of bluetongue virus (BTV) corelike particles by a recombinant baculovirus expressing the two major structural core proteins of BTV. *J Virol,* 64**,** 1530-6.

FU, H. 1995. *Mechanisms controlling the infection of Culicoides biting midges with bluetongue virus.*

GAITONDE, R. & GOPICHANDRAN, V. 2016. The Chennai floods of 2015 and the health system response. . *Indian Journal of Med Ethics.,* 1**,** 71-75.

GERDES, G. H. 2004. A South African overview of the virus, vectors, surveillance and unique features of bluetongue. *Vet Ital,* 40**,** 39-42.

GHIASI, H., PURDY, M. A. & ROY, P. 1985. The complete sequence of bluetongue virus serotype 10 segment 3 and its predicted VP3 polypeptide compared with those of BTV serotype 17. *Virus Res,* 3**,** 181-90.

GOLD, S., MONAGHAN, P., MERTENS, P. & JACKSON, T. 2010. A clathrin independent macropinocytosis-like entry mechanism used by bluetongue virus-1 during infection of BHK cells. *PLoS One,* 5**,** e11360.

GOLDSMIT, L. & BARZILAI, E. 1985. Isolation and propagation of bluetongue virus in embryonating chicken eggs. *Prog Clin Biol Res,* 178**,** 307-18.

GOLENDER, N., BRENNER, J., BUMBAROV, V., FRIEDGUT,O., ROTENBERG, D. & PANSHIN A. 2013. Genetic characterization of VP6 and NS4 genes of bluetongue viruses isolated in Israel during 2006 *Afr. J. Microbiol. Res,* 7**,** 661–668.

References

GOLLAPALLI, S. R., MALLAVARAPU, S., UMA, M., RAO, P. P., SUSMITHA, B., PRASAD, P. U., CHAITANYA, P., PRASAD, G., HEGDE, N. R. & REDDY, Y. N. 2012. Sequences of genes encoding type-specific and group-specific antigens of an Indian isolate of bluetongue virus serotype 10 (BTV-10) and implications for their origin. *Transbound Emerg Dis,* 59**,** 165-72.

GOMEZ-TEJEDOR, C. 2004. Brief overview of the bluetongue situation in Mediterranean Europe, 1998-2004. *Vet Ital,* 40**,** 57-60.

GOODWIN, S., MCPHERSON, J. D. & MCCOMBIE, W. R. 2016. Coming of age: ten years of next-generation sequencing technologies. *Nat Rev Genet,* 17**,** 333-51.

GOUET, P., DIPROSE, J. M., GRIMES, J. M., MALBY, R., BURROUGHS, J. N., ZIENTARA, S., STUART, D. I. & MERTENS, P. P. 1999. The highly ordered double-stranded RNA genome of bluetongue virus revealed by crystallography. *Cell,* 97**,** 481-90.

GOULD, A. R. 1987. The complete nucleotide sequence of bluetongue virus serotype 1 RNA3 and a comparison with other geographic serotypes from Australia, South Africa and the United States of America, and with other orbivirus isolates. *Virus Res,* 7**,** 169-83.

GOULD, A. R. & EATON, B. T. 1990. The amino acid sequence of the outer coat protein VP2 of neutralizing monoclonal antibody-resistant, virulent and attenuated bluetongue viruses. *Virus Res,* 17**,** 161-72.

GOULD, A. R., HYATT, A. D., EATON, B. T., WHITE, J. R., HOOPER, P. T., BLACKSELL, S. D. & LE BLANC SMITH, P. M. 1989. Current techniques in rapid bluetongue virus diagnosis. *Aust Vet J,* 66**,** 450-4.

GOULD, A. R. & PRITCHARD, L. I. 1991. Phylogenetic analyses of the complete nucleotide sequence of the capsid protein (VP3) of Australian epizootic haemorrhagic disease of deer virus (serotype 2) and cognate genes from other orbiviruses. *Virus Res,* 21**,** 1-18.

GOUY, M., GUINDON, S. & GASCUEL, O. 2010. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol,* 27**,** 221-4.

GRIMES, J. M., BURROUGHS, J. N., GOUET, P., DIPROSE, J. M., MALBY, R., ZIENTARA, S., MERTENS, P. P. & STUART, D. I. 1998. The atomic structure of the bluetongue virus core. *Nature,* 395**,** 470-8.

GROOCOCK, C. M. & CAMPBELL, C. H. 1982. Isolation of an exotic serotype of bluetongue virus from imported cattle in quarantine. *Can J Comp Med,* 46**,** 160-4.

GUIMARAES, L. L. B., ROSA, J. C. C., MATOS, A. C. D., CRUZ, R. A. S., GUEDES, M., DORELLA, F. A., FIGUEIREDO, H. C. P., PAVARINI, S. P., SONNE, L., LOBATO, Z. I. P. & DRIEMEIER, D. 2017. Identification of bluetongue virus serotypes 1, 4, and 17 co-infections in sheep flocks during outbreaks in Brazil. *Res Vet Sci,* 113**,** 87-93.

GUINDON, S. & GASCUEL, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol,* 52**,** 696-704.

References

GUIRAKHOO, F., CATALAN, J. A. & MONATH, T. P. 1995. Adaptation of bluetongue virus in mosquito cells results in overexpression of NS3 proteins and release of virus particles. *Arch Virol,* 140**,** 967-74.

GUMM, I. D. & NEWMAN, J. F. 1982. The preparation of purified bluetongue virus group antigen for use as a diagnostic reagent. *Arch Virol,* 72**,** 83-93.

HAMBLIN, C. 2004. Bluetongue virus antigen and antibody detection, and the application of laboratory diagnostic techniques. *Vet Ital,* 40**,** 538-45.

HAMERS, C., GALLEAU, S., CHERY, R., BLANCHET, M., BESANCON, L., CARIOU, C., WERLE-LAPOSTOLLE, B., HUDELET, P. & GOUTEBROZE, S. 2009. Use of inactivated bluetongue virus serotype 8 vaccine against virulent challenge in sheep and cattle. *Vet Rec,* 165**,** 369-73.

HAN, Z. & HARTY, R. N. 2004. The NS3 protein of bluetongue virus exhibits viroporin-like properties. *J Biol Chem,* 279**,** 43092-7.

HARBOLA, P. C., CHAUDHARY, P. G., LALKRISHNA, B. S., SRIGUPPI, B. S. & KOLE, R. S. 1982. Incidence of bluetongue in sheep in Maharashtra, India. . *Indian Journal of comparative Microbiology, Immunology and infectious Diseases* 3**,** 121-127.

HARDING, M. J., PRUD'HOMME, I. & ROLA, J. 1995. Identification of the major North American bluetongue viruses using nucleic acid amplification techniques. *Mol Cell Probes,* 9**,** 223-31.

HASSAN, S. H., WIRBLICH, C., FORZAN, M. & ROY, P. 2001. Expression and functional characterization of bluetongue virus VP5 protein: role in cellular permeabilization. *J Virol,* 75**,** 8356-67.

HASSAN, S. S. & ROY, P. 1999. Expression and functional characterization of bluetongue virus VP2 protein: role in cell entry. *J Virol,* 73**,** 9832-42.

HAWKES, R. A., KIRKLAND, P. D., SANDERS, D. A., ZHANG, F., LI, Z., DAVIS, R. J. & ZHANG, N. 2000. Laboratory and field studies of an antigen capture ELISA for bluetongue virus. *J Virol Methods,* 85**,** 137-49.

HE, C. Q., DING, N. Z., HE, M., LI, S. N., WANG, X. M., HE, H. B., LIU, X. F. & GUO, H. S. 2010. Intragenic recombination as a mechanism of genetic diversity in bluetongue virus. *J Virol,* 84**,** 11487-95.

HEMADRI, D., MAAN, S., CHANDA, M. M., RAO, P. P., PUTTY, K., KRISHNAJYOTHI, Y., REDDY, G. H., KUMAR, V., BATRA, K., REDDY, Y. V., MAAN, N. S., REDDY, Y. N., SINGH, K. P., SHIVACHANDRA, S. B., HEGDE, N. R., RAHMAN, H. & MERTENS, P. P. 2016. Dual Infection with Bluetongue Virus Serotypes and First-Time Isolation of Serotype 5 in India. *Transbound Emerg Dis*.

HERNIMAN, K. A., BOORMAN, J. P. & TAYLOR, W. P. 1983. Bluetongue virus in a Nigerian dairy cattle herd. 1. Serological studies and correlation of virus activity to vector population. *J Hyg (Lond),* 90**,** 177-93.

References

HEWAT, E. A., BOOTH, T. F., WADE, R. H. & ROY, P. 1992. 3-D reconstruction of bluetongue virus tubules using cryoelectron microscopy. *J Struct Biol,* 108**,** 35-48.

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. & 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. *Vet Microbiol,* 139**,** 1-23.

HOFMANN, M., GRIOT, C., CHAIGNAT, V., PERLER, L. & THUR, B. 2008a. [Bluetongue disease reaches Switzerland]. *Schweiz Arch Tierheilkd,* 150**,** 49-56.

HOFMANN, M. A., RENZULLO, S., MADER, M., CHAIGNAT, V., WORWA, G. & THUER, B. 2008b. Genetic Characterization of Toggenburg Orbivirus, a New Bluetongue Virus, from Goats, Switzerland. *Emerging Infectious Diseases,* 14**,** 1855-1861.

HOMAN, E. J., MO, C. L., THOMPSON, L. H., BARRETO, C. H., OVIEDO, M. T., GIBBS, E. P. & GREINER, E. C. 1990. Epidemiologic study of bluetongue viruses in Central America and the Caribbean: 1986-1988. Regional Bluetongue Team. *Am J Vet Res,* 51**,** 1089-94.

HORNYAK, A., MALIK, P., MARTON, S., DORO, R., CADAR, D. & BANYAI, K. 2015. Emergence of multireassortant bluetongue virus serotype 4 in Hungary. *Infect Genet Evol,* 33**,** 6-10.

HOWELL, P. G. & VERWOERD, D. W. 1971. Bluetongue virus. *Virol Monogr,* 9**,** 35-74.

HUANG, I. J., HWANG, G. Y., YANG, Y. Y., HAYAMA, E. & LI, J. K. 1995. Sequence analyses and antigenic epitope mapping of the putative RNA-directed RNA polymerase of five U.S. bluetongue viruses. *Virology,* 214**,** 280-8.

HUISMANS, H. 1979. Protein synthesis in bluetongue virus-infected cells. *Virology,* 92**,** 385-96.

HUISMANS, H. & ELS, H. J. 1979. Characterization of the tubules associated with the replication of three different orbiviruses. *Virology,* 92**,** 397-406.

HUISMANS, H. & ERASMUS, B. J. 1981. Identification of the serotype-specific and group-specific antigens of bluetongue virus. *Onderstepoort J Vet Res,* 48**,** 51-8.

HUISMANS, H., VAN DER WALT, N. T., CLOETE, M. & ERASMUS, B. J. 1987. Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. *Virology,* 157**,** 172-179.

HUISMANS, H. & VAN DIJK, A. A. 1990. Bluetongue virus structural components. *Curr Top Microbiol Immunol,* 162**,** 21-41.

HUISMANS, H., VAN STADEN, V., FICK, W. C., VAN NIEKERK, M. & MEIRING, T. L. 2004. A comparison of different orbivirus proteins that could affect virulence and pathogenesis. *Vet Ital,* 40**,** 417-25.

HUNTER, P. & MODUMO, J. 2001. A monovalent attenuated serotype 2 bluetongue virus vaccine confers homologous protection in sheep. *Onderstepoort J Vet Res,* 68**,** 331-3.

References

HYATT, A. D., EATON, B. T. & BROOKES, S. M. 1989. The release of bluetongue virus from infected cells and their superinfection by progeny virus. *Virology,* 173**,** 21-34.

HYATT, A. D., ZHAO, Y. & ROY, P. 1993. Release of bluetongue virus-like particles from insect cells is mediated by BTV nonstructural protein NS3/NS3A. *Virology,* 193**,** 592-603.

HYNES, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell,* 69**,** 11-25.

ICAR-NIVEDI 2010. Annual report 2010-11. *In:* REAHMAN, H., HEMADRI, D., SENGUPTA, P. P., BALAMURGAN, V., PATIL, S. S., HIREMATH, J. & NAGALINGAM, M. (eds.). Bangaluru, ICAR-NIVEDI: ICAR-NIVEDI.

ICAR-NIVEDI 2013. Annual report 2013-14. *In:* SENGUPTA, P. P., BALAMURGAN, V., SURESH, K. P., GOVINDRAJ, G. & KRISHNAMOORTHY, P. (eds.). Bangaluru: ICAR-NIVEDI.

ICAR-NIVEDI 2015. Annual report 2014-15. *In:* SENGUPTA, P. P., DESAI, G. S., BALAMURGAN, V., SHVACHANDRA, S. B. & KRISHNAMOORTHY (eds.). Bangaluru: ICAR-NIVEDI.

ICAR-NIVEDI 2016. Annual Report 2015-16. *In:* SENGUPTA, P. P., BALAMURUGAN, V., GOVINDRAJ, G., CHANDA, M. M., HIREMATH, J., REDDY, G. B. M. & PRAJAPATI, A. (eds.). Bangaluru, Karnataka: ICAR-NIVEDI.

ICAR 1945. Nomenclature of different breeds and types of domesticated livestock in India. *Indian Farming* 415-416.

ICTV 2011. Virus Taxonomy: The Classification and Nomenclature of Viruses The 9th Report of the ICTV (2011).

ILANGO, K. 2006. Bluetongue virus outbreak in Tamil Nadu, southern India: Need to study the Indian biting midge vectors, Culicoides Latreille (Diptera:Ceratopogonidae). *Current Science,* 90**,** 163-167.

INDIA 2017. *India 2017 A reference annual,* Ministry of information and broadcasting, Government of India, New Delhi.

INKSCAPE 2013. Inkscape Team,2013.Inkscape 0.48.4.

INUMARU, S. & ROY, P. 1987. Production and characterization of the neutralization antigen VP2 of bluetongue virus serotype 10 using a baculovirus expression vector. *Virology,* 157**,** 472-9.

JACQUOT, M., NOMIKOU, K., PALMARINI, M., MERTENS, P. & BIEK, R. 2017. Bluetongue virus spread in Europe is a consequence of climatic, landscape and vertebrate host factors as revealed by phylogeographic inference. *Proceedings of the Royal Society B: Biological Sciences,* 284**,** 20170919.

JAIN, N. C., SHARMA, R. & PRASAD, G. 1986. Isolation of bluetongue virus from sheep in India. *Vet Rec,* 119**,** 17-8.

References

JAUNIAUX, T. P., DE CLERCQ, K. E., CASSART, D. E., KENNEDY, S., VANDENBUSSCHE, F. E., VANDEMEULEBROUCKE, E. L., VANBINST, T. M., VERHEYDEN, B. I., GORIS, N. E. & COIGNOUL, F. L. 2008. Bluetongue in Eurasian lynx. *Emerg Infect Dis,* 14**,** 1496-8.

JENCKEL, M., BREARD, E., SCHULZ, C., SAILLEAU, C., VIAROUGE, C., HOFFMANN, B., HOPER, D., BEER, M. & ZIENTARA, S. 2015. Complete coding genome sequence of putative novel bluetongue virus serotype 27. *Genome Announc,* 3.

JIMENEZ-CLAVERO, M. A. 2012. Animal viral diseases and global change: bluetongue and West Nile fever as paradigms. *Front Genet,* 3**,** 105.

JIMENEZ-CLAVERO, M. A., AGUERO, M., SAN MIGUEL, E., MAYORAL, T., LOPEZ, M. C., RUANO, M. J., ROMERO, E., MONACO, F., POLCI, A., SAVINI, G. & GOMEZ-TEJEDOR, C. 2006. High throughput detection of bluetongue virus by a new real-time fluorogenic reverse transcription-polymerase chain reaction: application on clinical samples from current Mediterranean outbreaks. *J Vet Diagn Invest,* 18**,** 7-17.

JOARDAR, S. N., LODH, C., CHAKRABARTI, A., BAKSI, S. & AICH, R. 2009. Isolation of bluetongue virus serotypes 15 and 21 in West Bengal, India. *Vet Rec,* 165**,** 751-2.

JOCHIM, M. M. & CHOW, T. L. 1969. Immunodiffusion of bluetongue virus. *Am J Vet Res,* 30**,** 33-41.

JOHNSON, D. J., MERTENS, P. P. C., MAAN, S. & OSTLUND, E. N. Exotic bluetongue viruses identified from ruminants in the south-eastern U.S. from 1999-2006. . Annual AAVLD Conference, 2007 Reno, NV. . 118.

JOHNSON, P., DHANALAKSHMI, K., REDDY, Y., RAO, P., SUSMITHA, B. & SARMA, B. 2006. Epidemiology of bluetongue in domestic animals of Andhra Pradesh. *Indian J Comp Microbiol Immunol Infect Dis,* 27**,** 104-106.

KANTWA, S. C., MOHANTY, T. K., PATBANDHA, T. K., LAYEK, S. S. & KUMARESAN, A. 2017. Disease Incidence and Mortality Pattern of Migratory Sheep Flocks in Marwar Region of Rajasthan, India. *International Journal of Current Microbiology and Applied Sciences* 6**,** 684-690.

KAR, A. K., BHATTACHARYA, B. & ROY, P. 2007. Bluetongue virus RNA binding protein NS2 is a modulator of viral replication and assembly. *BMC Mol Biol,* 8**,** 4.

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. & HUNT, N. T. 2002. Studies on the epidemiology of bluetongue virus in China. *Epidemiol Infect,* 128**,** 257-63.

KOUMBATI, M., MANGANA, O., NOMIKOU, K., MELLOR, P. S. & PAPADOPOULOS, O. 1999. Duration of bluetongue viraemia and serological responses in experimentally infected European breeds of sheep and goats. *Vet Microbiol,* 64**,** 277-85.

KOWALIK, T. F. & LI, J. K. 1989. Sequence analyses and structural predictions of double-stranded RNA segment S1 and VP7 from United States prototype bluetongue virus serotypes 13 and 10. *Virology,* 172**,** 189-95.

References

KRISHNAJYOTHI, Y., MAAN, S., KANDIMALLA, K., MAAN, N. S., TUTIKA, R. B., REDDY, Y. V., KUMAR, A., MRUNALINI, N., REDDY, G. H., PUTTY, K., AHMED, S. M., REDDY, Y. N., HEMADRI, D., SINGH, K. P., MERTENS, P. P., HEGDE, N. R. & RAO, P. P. 2016. Isolation of Bluetongue Virus 24 from India - An Exotic Serotype to Australasia. *Transbound Emerg Dis,* 63**,** 360-4.

KULKARNI, D. D., BANNILIKAR, A. S., KARPE, A. S., GUJAR, M. B. & KULKARNI, M. N. 1992. Epidemiological observations on bluetongue in sheep in Marathwada region of Maharashtra state of India. *In:* WALTON, T. E. & OSBURN, B. I. (eds.) *Bluetongue, African Horse Sickness and Related Orbiviruses.* Boca Raton, Florida, USA: CRC

KUMAR, L., BATRA, K., CHAUDHARY, D., GUPTA, A. K., DALAL, A., KALYANARAMAN, B., IRULAPPAN, G. P., KUMAR, V. & MAAN, S. 2016. Full-Genome Sequence Analysis of a Reassortant Strain of Bluetongue virus Serotype 16 from Southern India. *Genome Announc,* 4.

LACETERA, N. & RONCHI, B. 2004. Evaluation of antibody response and nonspecific lymphocyte blastogenesis following inoculation of a live attenuated bluetongue virus vaccine in goats. *Am J Vet Res,* 65**,** 1331-4.

LAGER, I. A. 2004. Bluetongue virus in South America: overview of viruses, vectors, surveillance and unique features. *Vet Ital,* 40**,** 89-93.

LAMBDEN, P. R., COOKE, S. J., CAUL, E. O. & CLARKE, I. N. 1992. Cloning of noncultivatable human rotavirus by single primer amplification. *J Virol,* 66**,** 1817-22.

LE BLOIS, H., FRENCH, T., MERTENS, P. P., BURROUGHS, J. N. & ROY, P. 1992. The expressed VP4 protein of bluetongue virus binds GTP and is the candidate guanylyl transferase of the virus. *Virology,* 189**,** 757-61.

LEE, F., TING, L. J., LEE, M. S., CHANG, W. M. & WANG, F. I. 2011. Genetic analysis of two Taiwanese bluetongue viruses. *Vet Microbiol,* 148**,** 140-9.

LEGISA, D., GONZALEZ, F., DE STEFANO, G., PEREDA, A. & DUS SANTOS, M. J. 2013. Phylogenetic analysis of bluetongue virus serotype 4 field isolates from Argentina. *J Gen Virol,* 94**,** 652-62.

LEGISA, D. M., GONZALEZ, F. N. & DUS SANTOS, M. J. 2014. Bluetongue virus in South America, Central America and the Caribbean. *Virus Res,* 182**,** 87-94.

LEVINGS, R. L., WILBUR, L. A., EVERMANN, J. F., STOLL, I. R., STARLING, D. E., SPILLERS, C. A., GUSTAFSON, G. A., MCKEIMAN, A. J., RHYAN, J. C., HALVERSON, D. H. & ROSENBUSCH, R. F. 1996. Abortion and death in pregnant bitches associated with a canine vaccine contaminated with bluetongue virus. *Dev Biol Stand,* 88**,** 219-20.

LI, H., HANDSAKER, B., WYSOKER, A., FENNELL, T., RUAN, J., HOMER, N., MARTH, G., ABECASIS, G. & DURBIN, R. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics,* 25**,** 2078-9.

LIVESTOCK CENSUS 2014. 19 Livestock census-2012 All India report. Ministry of Agriculture department of animal husbandry,dairying and fisheries, krishi bhawan, New Delhi.

References

LONKAR, P. S., UPPAL, P. K., BELWAL, L. M. & MATHUR, P. B. 1983. Bluetongue in sheep in India. *Tropical Animal Health Production* 15**,** 83.

LORUSSO, A., MARCACCI, M., ANCORA, M., MANGONE, I., LEONE, A., MARINI, V., CAMMA, C. & SAVINI, G. 2014. Complete Genome Sequence of Bluetongue Virus Serotype 1 Circulating in Italy, Obtained through a Fast Next-Generation Sequencing Protocol. *Genome Announc,* 2.

LUEDKE, A. J. & ANAKWENZE, E. I. 1972. Bluetongue virus in goats. *Am J Vet Res,* 33**,** 1739-45.

LUEDKE, A. J., JOCHIM, M. M. & BOWNE, J. G. 1965. Preliminary bluetongue Transmission with the sheep ked Melophagus ovinus (L.). *Can J Comp Med Vet Sci,* 29**,** 229-31.

LUNT, R. A., WHITE, J. R. & BLACKSELL, S. D. 1988. Evaluation of a monoclonal antibody blocking ELISA for the detection of group-specific antibodies to bluetongue virus in experimental and field sera. *J Gen Virol,* 69 (Pt 11)**,** 2729-40.

LYMPEROPOULOS, K., NOAD, R., TOSI, S., NETHISINGHE, S., BRIERLEY, I. & ROY, P. 2006. Specific binding of Bluetongue virus NS2 to different viral plus-strand RNAs. *Virology,* 353**,** 17-26.

MAAN, N. S., MAAN, S., BELAGANAHALLI, M., PULLINGER, G., MONTES, A. J., GASPARINI, M. R., GUIMERA, M., NOMIKOU, K. & MERTENS, P. P. 2015a. A quantitative real-time reverse transcription PCR (qRT-PCR) assay to detect genome segment 9 of all 26 bluetongue virus serotypes. *J Virol Methods,* 213**,** 118-26.

MAAN, N. S., MAAN, S., GUIMERA, M., NOMIKOU, K., MORECROFT, E., PULLINGER, G., BELAGANAHALLI, M. N. & MERTENS, P. P. 2012a. The genome sequence of a reassortant bluetongue virus serotype 3 from India. *J Virol,* 86**,** 6375-6.

MAAN, N. S., MAAN, S., GUIMERA, M., PULLINGER, G., SINGH, K. P., NOMIKOU, K., BELAGANAHALLI, M. N. & MERTENS, P. P. 2012b. Complete genome sequence of an isolate of bluetongue virus serotype 2, demonstrating circulation of a Western topotype in southern India. *J Virol,* 86**,** 5404-5.

MAAN, N. S., MAAN, S., NOMIKOU, K., GUIMERA, M., PULLINGER, G., SINGH, K. P., BELAGANAHALLI, M. N. & MERTENS, P. P. 2012c. The genome sequence of bluetongue virus type 2 from India: evidence for reassortment between eastern and western topotype field strains. *J Virol,* 86**,** 5967-8.

MAAN, S., KUMAR, A., GUPTA, A. K., DALAL, A., CHAUDHARY, D., GUPTA, T. K., BANSAL, N., KUMAR, V., BATRA, K., SINDHU, N., KUMAR, A., MAHAJAN, N. K., MAAN, N. S. & MERTENS, P. P. 2017. Concurrent infection of Bluetongue and Peste-des-petits-ruminants virus in small ruminants in Haryana State of India. *Transbound Emerg Dis*.

MAAN, S., MAAN, N. S., BELAGANAHALLI, M. N., KUMAR, A., BATRA, K., RAO, P. P., HEMADRI, D., REDDY, Y. N., PUTTY, K., KRISHNAJYOTHI, Y., REDDY, G. H., SINGH, K. P., HEGDE, N. R., NOMIKOU, K., SREENIVASULU, D. & MERTENS, P. P. 2015b. Genome Sequence of Bluetongue Virus Type 2 from India: Evidence for Reassortment between Outer Capsid Protein Genes. *Genome Announc,* 3.

References

MAAN, S., MAAN, N. S., BELAGANAHALLI, M. N., POTGIETER, A. C., KUMAR, V., BATRA, K., WRIGHT, I. M., KIRKLAND, P. D. & MERTENS, P. P. 2016. Development and Evaluation of Real Time RT-PCR Assays for Detection and Typing of Bluetongue Virus. *PLoS One,* 11**,** e0163014.

MAAN, S., MAAN, N. S., BELAGANAHALLI, M. N., RAO, P. P., SINGH, K. P., HEMADRI, D., PUTTY, K., KUMAR, A., BATRA, K., KRISHNAJYOTHI, Y., CHANDEL, B. S., REDDY, G. H., NOMIKOU, K., REDDY, Y. N., ATTOUI, H., HEGDE, N. R. & MERTENS, P. P. 2015c. Full-Genome Sequencing as a Basis for Molecular Epidemiology Studies of Bluetongue Virus in India. *PLoS One,* 10**,** e0131257.

MAAN, S., MAAN, N. S., GUIMERA, M., NOMIKOU, K., SINGH, K. P., PULLINGER, G., BELAGANAHALLI, M. N. & MERTENS, P. P. 2012d. Genome sequence of a reassortant strain of bluetongue virus serotype 23 from western India. *J Virol,* 86**,** 7011-2.

MAAN, S., MAAN, N. S., NOMIKOU, K., ANTHONY, S. J., ROSS-SMITH, N., SAMUEL, A. R., SHAW, A. E. & MERTENS, P. P. 2009. Molecular epidemiology studies of bluetongue virus. *In:* MERTENS, P. P. C., BAYLIS, M. & MELLOR, P. S. (ed.) *Bluetongue.* London, UK: Elsevier Academic Press.

MAAN, S., MAAN, N. S., NOMIKOU, K., BATTEN, C., ANTONY, F., BELAGANAHALLI, M. N., SAMY, A. M., REDA, A. A., AL-RASHID, S. A., EL BATEL, M., OURA, C. A. & MERTENS, P. P. 2011a. Novel bluetongue virus serotype from Kuwait. *Emerg Infect Dis,* 17**,** 886-9.

MAAN, S., MAAN, N. S., NOMIKOU, K., VERONESI, E., BACHANEK-BANKOWSKA, K., BELAGANAHALLI, M. N., ATTOUI, H. & MERTENS, P. P. 2011b. Complete genome characterisation of a novel 26th bluetongue virus serotype from Kuwait. *PLoS One,* 6**,** e26147.

MAAN, S., MAAN, N. S., PULLINGER, G., NOMIKOU, K., MORECROFT, E., GUIMERA, M., BELAGANAHALLI, M. N. & MERTENS, P. P. 2012e. The genome sequence of bluetongue virus type 10 from India: evidence for circulation of a western topotype vaccine strain. *J Virol,* 86**,** 5971-2.

MAAN, S., MAAN, N. S., ROSS-SMITH, N., BATTEN, C. A., SHAW, A. E., ANTHONY, S. J., SAMUEL, A. R., DARPEL, K. E., VERONESI, E., OURA, C. A. L., SINGH, K. P., NOMIKOU, K., POTGIETER, A. C., ATTOUI, H., VAN ROOIJ, E., VAN RIJN, P., DE CLERCQ, K., VANDENBUSSCHE, F., ZIENTARA, S., BRÉARD, E., SAILLEAU, C., BEER, M., HOFFMAN, B., MELLOR, P. S. & MERTENS, P. P. C. 2008. Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. *Virology,* 377**,** 308-318.

MAAN, S., MAAN, N. S., SAMUEL, A. R., O'HARA, R., MEYER, A. J., RAO, S. & MERTENS, P. P. 2004. Completion of the sequence analysis and comparisons of genome segment 2 (encoding outer capsid protein VP2) from representative isolates of the 24 bluetongue virus serotypes. *Vet Ital,* 40**,** 484-8.

MAAN, S., MAAN, N. S., SAMUEL, A. R., RAO, S., ATTOUI, H. & MERTENS, P. P. 2007a. Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. *J Gen Virol,* 88**,** 621-30.

References

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., POTGIETER, A. C., NOMIKOU, K. & MERTENS, P. P. 2010. Full genome characterisation of bluetongue virus serotype 6 from the Netherlands 2008 and comparison to other field and vaccine strains. *PLoS One,* 5**,** e10323.

MAAN, S., RAO, S., MAAN, N. S., ANTHONY, S. J., ATTOUI, H., SAMUEL, A. R. & MERTENS, P. P. 2007b. Rapid cDNA synthesis and sequencing techniques for the genetic study of bluetongue and other dsRNA viruses. *J Virol Methods,* 143**,** 132-9.

MACLACHLAN, N. J. 1994. The pathogenesis and immunology of bluetongue virus infection of ruminants. *Comp Immunol Microbiol Infect Dis,* 17**,** 197-206.

MACLACHLAN, N. J. 2004. Bluetongue: pathogenesis and duration of viraemia. *Vet Ital,* 40**,** 462-7.

MACLACHLAN, N. J. 2010. Global implications of the recent emergence of bluetongue virus in Europe. *Vet Clin North Am Food Anim Pract,* 26**,** 163-71, table of contents.

MACLACHLAN, N. J. 2011. Bluetongue: history, global epidemiology, and pathogenesis. *Prev Vet Med,* 102**,** 107-11.

MACLACHLAN, N. J., CRAFFORD, J. E., VERNAU, W., GARDNER, I. A., GODDARD, A., GUTHRIE, A. J. & VENTER, E. H. 2008. Experimental reproduction of severe bluetongue in sheep. *Vet Pathol,* 45**,** 310-5.

MACLACHLAN, N. J., DREW, C. P., DARPEL, K. E. & WORWA, G. 2009. The Pathology and Pathogenesis of Bluetongue. *Journal of Comparative Pathology,* 141**,** 1-16.

MACLACHLAN, N. J., JAGELS, G., ROSSITTO, P. V., MOORE, P. F. & HEIDNER, H. W. 1990. The pathogenesis of experimental bluetongue virus infection of calves. *Vet Pathol,* 27**,** 223-9.

MACLACHLAN, N. J., NUNAMAKER, R. A., KATZ, J. B., SAWYER, M. M., AKITA, G. Y., OSBURN, B. I. & TABACHNICK, W. J. 1994. Detection of bluetongue virus in the blood of inoculated calves: comparison of virus isolation, PCR assay, and in vitro feeding of Culicoides variipennis. *Arch Virol,* 136**,** 1-8.

MAHAJAN, N. K., PRASAD, G., JAIN, N. C., DHANDA, J. S. & GUPTA, Y. 1991. Epizootiological studies on bluetongue at an organised sheep breeding farm near Hisar, Haryana. *Indian Journal of Animal Science* 61**,** 15.

MARCHI, P. R., RAWLINGS, P., BURROUGHS, J. N., WELLBY, M., MERTENS, P. P., MELLOR, P. S. & WADE-EVANS, A. M. 1995. Proteolytic cleavage of VP2, an outer capsid protein of African horse sickness virus, by species-specific serum proteases enhances infectivity in Culicoides. *J Gen Virol,* 76 (Pt 10)**,** 2607-11.

MARDIS, E. R. 2008. Next-generation DNA sequencing methods. *Annu Rev Genomics Hum Genet,* 9**,** 387-402.

MAREE, F. F. & HUISMANS, H. 1997. Characterization of tubular structures composed of nonstructural protein NS1 of African horsesickness virus expressed in insect cells. *J Gen Virol,* 78 (Pt 5)**,** 1077-82.

MARTIN, S. A. & ZWEERINK, H. J. 1972. Isolation and characterization of two types of bluetongue virus particles. *Virology,* 50**,** 495-506.

MARTINEZ-COSTAS, J., SUTTON, G., RAMADEVI, N. & ROY, P. 1998. Guanylyltransferase and RNA 5'-triphosphatase activities of the purified expressed VP4 protein of bluetongue virus. *J Mol Biol,* 280**,** 859-66.

MARTYN, J. C., GOULD, A. R. & EATON, B. T. 1991. High level expression of the major core protein VP7 and the non-structural protein NS3 of bluetongue virus in yeast: use of expressed VP7 as a diagnostic, group-reactive antigen in a blocking ELISA. *Virus Res,* 18**,** 165-78.

MASON, J. H., COLES, J. D. W. A. & ALEXANDER, R. A. 1940. Cultivation of bluetongue virus in fertile eggs produced on vitamin deficient diet. *Nature* 145**,** 1022–3.

MATSUO, E. & ROY, P. 2011. Bluetongue virus VP1 polymerase activity in vitro: template dependency, dinucleotide priming and cap dependency. *PLoS One,* 6**,** e27702.

MAXAM, A. M. & GILBERT, W. 1977. A new method for sequencing DNA. *Proc Natl Acad Sci U S A,* 74**,** 560-4.

MCCOLL, K. A. & GOULD, A. R. 1991. Detection and characterisation of bluetongue virus using the polymerase chain reaction. *Virus Res,* 21**,** 19-34.

MCCOLL, K. A. & GOULD, A. R. 1994. Bluetongue virus infection in sheep: haematological changes and detection by polymerase chain reaction. *Aust Vet J,* 71**,** 97-101.

MCLAUGHLIN, B. E., DEMAULA, C. D., WILSON, W. C., BOYCE, W. M. & MACLACHLAN, N. J. 2003. Replication of bluetongue virus and epizootic hemorrhagic disease virus in pulmonary artery endothelial cells obtained from cattle, sheep, and deer. *Am J Vet Res,* 64**,** 860-5.

MECHAM, J. O., STALLKNECHT, D. & WILSON, W. C. 2003. The S7 gene and VP7 protein are highly conserved among temporally and geographically distinct American isolates of epizootic hemorrhagic disease virus. *Virus Research,* 94**,** 129-133.

MEHROTRA, M. L., SINGH, R. & SHUKLA, D. C. 1991. Seroepidemiology and isolation of virus from and outbreak of bluetongue in Tamil Nadu. *Indian Journal of Animal Science* 1282-83.

MELLOR, P. S. 1990. The replication of bluetongue virus in Culicoides vectors. *Curr Top Microbiol Immunol,* 162**,** 143-61.

MELLOR, P. S. 2000. Replication of arboviruses in insect vectors. *J Comp Pathol,* 123**,** 231-47.

MENZIES, F. D., MCCULLOUGH, S. J., MCKEOWN, I. M., FORSTER, J. L., JESS, S., BATTEN, C., MURCHIE, A. K., GLOSTER, J., FALLOWS, J. G., PELGRIM, W., MELLOR, P. S. & OURA, C. A. 2008. Evidence for transplacental and contact transmission of bluetongue virus in cattle. *Vet Rec,* 163**,** 203-9.

MERTENS, P. 2004. The dsRNA viruses. *Virus Research,* 101**,** 3-13.

MERTENS, P. P., BROWN, F. & SANGAR, D. V. 1984. Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode. *Virology,* 135**,** 207-17.

MERTENS, P. P., BURROUGHS, J. N. & ANDERSON, J. 1987. Purification and properties of virus particles, infectious subviral particles, and cores of bluetongue virus serotypes 1 and 4. *Virology,* 157**,** 375-86.

MERTENS, P. P., BURROUGHS, J. N., WALTON, A., WELLBY, M. P., FU, H., O'HARA, R. S., BROOKES, S. M. & MELLOR, P. S. 1996. Enhanced infectivity of modified bluetongue virus particles for two insect cell lines and for two Culicoides vector species. *Virology,* 217**,** 582-93.

MERTENS, P. P., CROOK, N. E., RUBINSTEIN, R., PEDLEY, S. & PAYNE, C. C. 1989a. Cytoplasmic polyhedrosis virus classification by electropherotype; validation by serological analyses and agarose gel electrophoresis. *J Gen Virol,* 70 (Pt 1)**,** 173-85.

MERTENS, P. P. & DIPROSE, J. 2004. The bluetongue virus core: a nano-scale transcription machine. *Virus Res,* 101**,** 29-43.

MERTENS, P. P., DIPROSE, J., MAAN, S., SINGH, K. P., ATTOUI, H. & SAMUEL, A. R. 2004. Bluetongue virus replication, molecular and structural biology. *Vet Ital,* 40**,** 426-37.

MERTENS, P. P., MAAN, N. S., PRASAD, G., SAMUEL, A. R., SHAW, A. E., POTGIETER, A. C., ANTHONY, S. J. & MAAN, S. 2007a. Design of primers and use of RT-PCR assays for typing European bluetongue virus isolates: differentiation of field and vaccine strains. *J Gen Virol,* 88**,** 2811-23.

MERTENS, P. P., PEDLEY, S., COWLEY, J., BURROUGHS, J. N., CORTEYN, A. H., JEGGO, M. H., JENNINGS, D. M. & GORMAN, B. M. 1989b. Analysis of the roles of bluetongue virus outer capsid proteins VP2 and VP5 in determination of virus serotype. *Virology,* 170**,** 561-5.

MERTENS, P. P. & SANGAR, D. V. 1985. Analysis of the terminal sequences of the genome segments of four orbiviruses. *Virology,* 140**,** 55-67.

MERTENS, P. P. C. 1999. Orbiviruses and coltiviruses - general features. *In:* G, W. R. & GRANOFF, A. (eds.) *Encyclopaedia of Virology.* 2nd ed. London Academic Press.

MERTENS, P. P. C., ARELLA, M., ATTOUI, H., BELLONCIK, S., BERGOIN, M., BOCCARDO, G., BOOTH, T. F., CHIU, W., DIPROSE, J. M., DUNCAN, R., ESTES, M. K., GORZIGLIA, M., GOUET, P., GOULD, A. R., GRIMES, J. M., HEWAT, E., HILL, C.,HOLMES, I. H., HOSHINO, Y., JOKLIK, W. K., KNOWLES, N., LÓPEZ FERBER, M.L., MALBY, R., MARZACHI, C., MCCRAE, M. A., MILNE, R. G., NIBERT, M., NUNN, M., OMURA, T., PRASAD, B. V. V., PRITCHARD, I., SAMAL, S. K., SCHOEHN, G., SHIKATA, E., STOLTZ, D. B., STUART, D. I., SUZUKI, N., UPADHYAYA, N., UYEDA, I., WATERHOUSE, P., WILLIAMS, C.F., WINTON, J. R. AND ZHOU, H. Z. 2000. Reoviridae. *Virus Taxonomy. 7th Report of the International Committee for the Taxonomy of Viruses.* London Academic Press.

MERTENS, P. P. C., DIPROSE, J., MAAN, S., SINGH, K. P., ATTOUI, H. & SAMUEL, A. Bluetongue virus replication, molecular and structural biology. *In:* MACLACHLAN, N. J. & PEARSON, J. E.,

eds. Proceedings of the Third International Symposium Taormina, 2003 Teramo, Italy. Veterinaria Italiana, 462-437.

MERTENS, P. P. C., MAAN, N. S., JOHNSON, D. J., OSTLUND, E. N. & MAAN, S. 2007b. Sequencing and RT-PCR assays for genome segment 2 of the 24 bluetongue virus serotypes: identification of exotic serotypes in the south eastern USA (1999–2006). *World Association of Veterinary Laboratory Diagnosticians (WAVLD) 13th International Symposium Proceeding***,** 55.

MERTENS, P. P. C., MAAN, S., BATTEN, C., DARPEL, K. E., SHAW, A., MAAN, N. S., NOMIKOU, K., ANTHONY, S. J., VERONESI, E., OURA, C. A. I., CARPENTER, S., MELLOR, P. S. & 2009. Bluetongue virus diagnosis. *In:* MELLOR, P. S., BAYLIS, M. & MERTENS, P. P. C. (eds.) *Bluetongue.* 1st ed. London: Elsevier Academic Press.

MERTENS, P. P. C., MAAN, S., SAMUEL, A. & ATTOUI, H. 2005. Orbivirus, Reoviridae. *In:* FAUQUET, C. M., MAYO, M. A., MANILOFF, J., DESSELBERGER, U. & BALL, L. A. (eds.) *Virus Taxonomy, VIIIth Report of the ICTV.* London Academic Press.

METZKER, M. L. 2010. Sequencing technologies - the next generation. *Nat Rev Genet,* 11**,** 31-46.

MEYER, G., LACROUX, C., LEGER, S., TOP, S., GOYEAU, K., DEPLANCHE, M. & LEMAIRE, M. 2009. Lethal bluetongue virus serotype 1 infection in llamas. *Emerg Infect Dis,* 15**,** 608-10.

MINAKSHI, P., SINGH, R., RANJAN, K., KUMAR, P., JOSHI, C. G., REDDY, Y. K. M. & PRASAD, G. 2012. Complete Genome Sequence of Bluetongue Virus Serotype 16 of Goat Origin from India. *Journal of Virology,* 86**,** 8337-8338.

MIURA, Y., INABA, Y., TSUDA, T., TOKUHISA, S., SATO, K. & AKASHI, H. 1982. Seroepizootiological survey on bluetongue virus infection in cattle in Japan. *Natl Inst Anim Health Q (Tokyo),* 22**,** 154-8.

MO, C. L., THOMPSON, L. H., HOMAN, E. J., OVIEDO, M. T., GREINER, E. C., GONZALEZ, J. & SAENZ, M. R. 1994. Bluetongue virus isolations from vectors and ruminants in Central America and the Caribbean. Interamerican Bluetongue Team. *Am J Vet Res,* 55**,** 211-5.

MOHD JAAFAR, F., BELHOUCHET, M., BELAGANAHALLI, M., TESH, R. B., MERTENS, P. P. & ATTOUI, H. 2014. Full-genome characterisation of Orungo, Lebombo and Changuinola viruses provides evidence for co-evolution of orbiviruses with their arthropod vectors. *PLoS One,* 9**,** e86392.

MONACO, F., DE LUCA, N., MORELLI, D., PISCIELLA, M., PALMARINI, S., DI GIANDOMENICO, M. & SAVINI, G. 2004. Field vaccination of cattle using a bivalent modified-live vaccine against bluetongue virus serotypes 2 and 9: effect on milk production. *Vet Ital,* 40**,** 661-3.

MONDAL, B., BISHT, B., BISWAS, S. K. & CHAND, K. 2013. Bluetongue virus serotype-1 in goats in the Pithoragarh area of Uttarakahand, India. *Vet Ital,* 49**,** 375-80.

MONDAL, B., SEN, A., CHAND, K., BISWAS, S. K., DE, A., RAJAK, K. K. & CHAKRAVARTI, S. 2009. Evidence of mixed infection of peste des petits ruminants virus and bluetongue virus in a

flock of goats as confirmed by detection of antigen, antibody and nucleic acid of both the viruses. *Trop Anim Health Prod,* 41**,** 1661-7.

MOULTON, J. E. 1961. Pathology of bluetongue of sheep in California. *J Am Vet Med Assoc,* 138**,** 493-8.

NARAYANAN, B., SHARMA, S. V., KUMAR, S. & PANDEY, S. 2018. Land and the people. *In:* CHATTERJEE, R. (ed.) *India 2018 a reference annual.* Lodhi Road, New Delhi-110 003: Ministry of Information and Broadcasting, Government of India.

NASON, E. L., ROTHAGEL, R., MUKHERJEE, S. K., KAR, A. K., FORZAN, M., PRASAD, B. V. V. & ROY, P. 2004. Interactions between the Inner and Outer Capsids of Bluetongue Virus. *Journal of Virology,* 78**,** 8059-8067.

NCERT 2014. Climate. *In:* UPPAL, S. (ed.) *Social science contemporary India-I textbook in geography for class IX.* New Delhi: Publication division, NCERT.

NIEDBALSKI, W. 2011. Bluetongue vaccines in Europe. *Pol J Vet Sci,* 14**,** 299-304.

NIEDBALSKI, W. 2013. The evolution of bluetongue virus: genetic and phenotypic diversity of field strains. *Pol J Vet Sci,* 16**,** 611-6.

NIEDRINGHAUS, T. P., MILANOVA, D., KERBY, M. B., SNYDER, M. P. & BARRON, A. E. 2011. Landscape of next-generation sequencing technologies. *Anal Chem,* 83**,** 4327-41.

NIKOLAKAKI, S. V., NOMIKOU, K., KOUMBATI, M., MANGANA, O., PAPANASTASSOPOULOU, M., MERTENS, P. P. & PAPADOPOULOS, O. 2005. Molecular analysis of the NS3/NS3A gene of Bluetongue virus isolates from the 1979 and 1998-2001 epizootics in Greece and their segregation into two distinct groups. *Virus Res,* 114**,** 6-14.

NIKOLAKAKI, S. V., NOMIKOU, K., MANGANA-VOUGIOUKA, O., PAPANASTASSOPOULOU, M., KOUMBATI, M. & PAPADOPOULOS, O. 2004. S10 segment sequence analysis of some Greek bluetongue virus strains. *Vet Ital,* 40**,** 468-72.

NOAD, R. & ROY, P. 2009. Bluetongue vaccines. *Vaccine,* 27 Suppl 4**,** D86-9.

NOMIKOU, K., DOVAS, C. I., MAAN, S., ANTHONY, S. J., SAMUEL, A. R., PAPANASTASSOPOULOU, M., MAAN, N. S., MANGANA, O. & MERTENS, P. P. 2009. Evolution and phylogenetic analysis of full-length VP3 genes of Eastern Mediterranean bluetongue virus isolates. *PLoS One,* 4**,** e6437.

NOMIKOU, K., HUGHES, J., WASH, R., KELLAM, P., BREARD, E., ZIENTARA, S., PALMARINI, M., BIEK, R. & MERTENS, P. 2015. Widespread Reassortment Shapes the Evolution and Epidemiology of Bluetongue Virus following European Invasion. *PLoS Pathog,* 11**,** e1005056.

OBERST, R. D., STOTT, J. L., BLANCHARD-CHANNELL, M. & OSBURN, B. I. 1987. Genetic reassortment of bluetongue virus serotype 11 strains in the bovine. *Vet Microbiol,* 15**,** 11-8.

OIE 2017. Bluetongue (infection with bluetongue virus). *Manual of diagnostic tests and vaccines for terrestrial animals* Paris, France

ORGI. 2011. *Census of India 2011 Office of the Registrar General & Census Commissioner, India,Ministry of Home Affairs, Government of India* [Online]. Available: http://censusindia.gov.in/.

ORRU, G., FERRANDO, M. L., MELONI, M., LICIARDI, M., SAVINI, G. & DE SANTIS, P. 2006. Rapid detection and quantitation of Bluetongue virus (BTV) using a Molecular Beacon fluorescent probe assay. *J Virol Methods,* 137**,** 34-42.

OSBURN, B. I., DE MATTOS, C. A., DE MATTOS, C. C. & MACLACHLAN, N. J. 1996. Bluetongue disease and the molecular epidemiology of viruses from the western United States. *Comp Immunol Microbiol Infect Dis,* 19**,** 181-90.

OSBURN, B. I., JOHNSON, R. T., SILVERSTEIN, A. M., PRENDERGAST, R. A., JOCHIM, M. M. & LEVY, S. E. 1971. Experimental viral-induced congenital encephalopathies. II. The pathogenesis of bluetongue vaccine virus infection in fetal lambs. *Lab Invest,* 25**,** 206-10.

OWENS, R. J., LIMN, C. & ROY, P. 2004. Role of an arbovirus nonstructural protein in cellular pathogenesis and virus release. *J Virol,* 78**,** 6649-56.

PAGES, N., BREARD, E., URIEN, C., TALAVERA, S., VIAROUGE, C., LORCA-ORO, C., JOUNEAU, L., CHARLEY, B., ZIENTARA, S., BENSAID, A., SOLANES, D., PUJOLS, J. & SCHWARTZ-CORNIL, I. 2014. Culicoides midge bites modulate the host response and impact on bluetongue virus infection in sheep. *PLoS One,* 9**,** e83683.

PARSONSON, I. M. 1990. Pathology and pathogenesis of bluetongue infections. *Curr Top Microbiol Immunol,* 162**,** 119-41.

PARSONSON, I. M. & MCCOLL, K. A. 1995. Retrospective diagnosis of bluetongue virus in stored frozen and fixed tissue samples using PCR. *Vet Microbiol,* 46**,** 143-9.

PARSONSON, I. M. & SNOWDON, W. A. 1985. Bluetongue, epizootic haemorrhagic disease of deer and related viruses: current situation in Australia. *Prog Clin Biol Res,* 178**,** 27-35.

PATTA, C., GIOVANNINI, A., ROLESU, S., NANNINI, D., SAVINI, G., CALISTRI, P., SANTUCCI, U. & CAPORALE, V. 2004. Bluetongue vaccination in Europe: the Italian experience. *Vet Ital,* 40**,** 601-10.

PEARSON, J. E., CARBREY, E. A. & GUSTAFSON, G. A. 1985. Bluetongue and related orbivirus diagnosis in the United States. *Prog Clin Biol Res,* 178**,** 469-75.

PEDLEY, S., MOHAMED, M. E. & MERTENS, P. P. 1988. Analysis of genome segments from six different isolates of bluetongue virus using RNA-RNA hybridisation: a generalised coding assignment for bluetongue viruses. *Virus Res,* 10**,** 381-90.

PEREZ DE DIEGO, A. C., ATHMARAM, T. N., STEWART, M., RODRIGUEZ-SANCHEZ, B., SANCHEZ-VIZCAINO, J. M., NOAD, R. & ROY, P. 2011. Characterization of protection afforded by a

bivalent virus-like particle vaccine against bluetongue virus serotypes 1 and 4 in sheep. *PLoS One,* 6**,** e26666.

PINI, A. 1976. Study on the pathogenesis of bluetongue: replication of the virus in the organs of infected sheep. *Onderstepoort J Vet Res,* 43**,** 159-64.

POLCI, A., CAMMA, C., SERINI, S., DI GIALLEONARDO, L., MONACO, F. & SAVINI, G. 2007. Real-time polymerase chain reaction to detect bluetongue virus in blood samples. *Vet Ital,* 43**,** 77-88.

POSADA, D. 2008. jModelTest: phylogenetic model averaging. *Mol Biol Evol,* 25**,** 1253-6.

POTGIETER, A. C., PAGE, N. A., LIEBENBERG, J., WRIGHT, I. M., LANDT, O. & VAN DIJK, A. A. 2009. Improved strategies for sequence-independent amplification and sequencing of viral double-stranded RNA genomes. *J Gen Virol,* 90**,** 1423-32.

POTGIETER, A. C., STEELE, A. D. & VAN DIJK, A. A. 2002. Cloning of complete genome sets of six dsRNA viruses using an improved cloning method for large dsRNA genes. *J Gen Virol,* 83**,** 2215-23.

PRASAD, G., GARG, A. K., MINAKSHI, KAKKER, N. K. & SRIVASTAVA, R. N. 1994. Isolation of bluetongue virus from sheep in Rajasthan State, India. *Rev Sci Tech,* 13**,** 935-8.

PRASAD, G., JAIN, N. C. & GUPTA, Y. 1992. Bluetongue virus infection in India: a review. *Rev Sci Tech,* 11**,** 699-711.

PRASAD, G., SREENIVASULU, D., SINGH, K. P., MERTENS, P. P. C. & MAAN, S. 2009. Bluetongue in the Indian subcontinent. *In:* MELLOR, P. S., BAYLIS, M. & MERTENS, P. P. C. (eds.) *Bluetongue.* London: Elsevier Academic Press.

PRICE, D. A. & HARDY, W. T. 1954. Isolation of the bluetongue virus from Texas sheep-Culicoides shown to be a vector. *J Am Vet Med Assoc,* 124**,** 255-8.

PRITCHARD, L. I., DANIELS, P. W., MELVILLE, L. F., KIRKLAND, P. D., JOHNSON, S. J., LUNT, R. & EATON, B. T. 2004. Genetic diversity of bluetongue viruses in Australasia. *Vet Ital,* 40**,** 438-45.

PRITCHARD, L. I., GOULD, A. R., WILSON, W. C., THOMPSON, L., MERTENS, P. P. & WADE-EVANS, A. M. 1995. Complete nucleotide sequence of RNA segment 3 of bluetongue virus serotype 2 (Ona-A). Phylogenetic analyses reveal the probable origin and relationship with other orbiviruses. *Virus Res,* 35**,** 247-61.

PUDUPAKAM, R. S., RAGHUNATH, S., PUDUPAKAM, M. & DAGGUPATI, S. 2017. Genetic characterization of the non-structural protein-3 gene of bluetongue virus serotype-2 isolate from India. *Vet World,* 10**,** 348-352.

PURDY, M. A., GHIASI, H., RAO, C. D. & ROY, P. 1985. Complete sequence of bluetongue virus L2 RNA that codes for the antigen recognized by neutralizing antibodies. *J Virol,* 55**,** 826-30.

References

RAMADEVI, N., BURROUGHS, N. J., MERTENS, P. P., JONES, I. M. & ROY, P. 1998. Capping and methylation of mRNA by purified recombinant VP4 protein of bluetongue virus. *Proc Natl Acad Sci U S A,* 95**,** 13537-42.

RAMADEVI, N. & ROY, P. 1998. Bluetongue virus core protein VP4 has nucleoside triphosphate phosphohydrolase activity. *J Gen Virol,* 79 (Pt 10)**,** 2475-80.

RAMBAUT, A., DRUMMOND, A. 2009. FigTree version 1.3.1 .computer program and documentation distribution by the author. .

RANJAN, K., MINAKSHI, P. & PRASAD, G. 2015. Bluetongue: Indian perspective. *Acta Virol,* 59**,** 317-37.

RAO, P., KRISHNAJYOTH, Y., REDDY, V., SUSMITHA, B., REDDY, H., SREENIVASULU, D., HEGDE, N. & REDDY, N. 2016a. A decade of research on Bluetongue virus in Andhra Pradesh, a Southern state of India. *Vet Ital,* 52**,** 299-304.

RAO, P. P., HEGDE, N. R. & REDDY, Y. N. 2012a. Intercontinental movement of bluetongue virus and potential consequences to trade. *J Virol,* 86**,** 8341; author reply 8342-3.

RAO, P. P., HEGDE, N. R., REDDY, Y. N., KRISHNAJYOTHI, Y., REDDY, Y. V., SUSMITHA, B., GOLLAPALLI, S. R., PUTTY, K. & REDDY, G. H. 2016b. Epidemiology of Bluetongue in India. *Transbound Emerg Dis,* 63**,** e151-64.

RAO, P. P., REDDY, Y. N. & HEGDE, N. R. 2012b. Complete genome sequence of bluetongue virus serotype 9: implications for serotyping. *J Virol,* 86**,** 8333.

RAO, P. P., REDDY, Y. V. & HEGDE, N. R. 2015. Isolation and Complete Genome Sequencing of Bluetongue Virus Serotype 12 from India. *Transbound Emerg Dis,* 62**,** e52-9.

RAO, P. P., REDDY, Y. V., MEENA, K., KARUNASREE, N., SUSMITHA, B., UMA, M., PRASAD, P. U., CHAITANYA, P., REDDY, Y. N. & HEGDE, N. R. 2012c. Genetic characterization of bluetongue virus serotype 9 isolates from India. *Virus Genes,* 44**,** 286-94.

RATINIER, M., CAPORALE, M., GOLDER, M., FRANZONI, G., ALLAN, K., NUNES, S. F., ARMEZZANI, A., BAYOUMY, A., RIXON, F., SHAW, A. & PALMARINI, M. 2011. Identification and characterization of a novel non-structural protein of bluetongue virus. *PLoS Pathog,* 7**,** e1002477.

RAVISHANKAR, C., KRISHNAN NAIR, G., MINI, M. & JAYAPRAKASAN, V. 2005. Seroprevalence of bluetongue virus antibodies in sheep and goats in Kerala State, India. *Rev Sci Tech,* 24**,** 953-8.

REDDINGTON, J. J., REDDINGTON, G. M. & MACLACHLAN, N. J. 1991. A competitive ELISA for detection of antibodies to the group antigen of bluetongue virus. *J Vet Diagn Invest,* 3**,** 144-7.

REDDY, B. Y., REDDY, Y. N., SUSMITHA, B., RAO, P. P., SUDHEER, D., SREENIVASULU, D. & SUBBA RAO, M. V. 2010. Involvement of two bluetongue virus serotypes in single outbreak. *Indian Veterinary Journal***,** 1071–1074.

References

REDDY, Y. K., BRINDHA, K., GANESAN, P. I., SRINIVAS, K., REDDY, G. S. & MINAKSHI, P. 2016a. Occurrence of Bluetongue in ruminants in Tamil Nadu, South India. *Vet Ital,* 52**,** 293-297.

REDDY, Y. K. M., MANOHAR, B. M., PANDEY, A. B., REDDY, Y. N., PRASAD, G. & R.S, C. 2010 Development and evaluation of inactivated pentavalent adjuvanted vaccine for Bluetongue. *Indian Veterinary Journal***,** 434-436.

REDDY, Y. V., KRISHNAJYOTHI, Y., SUSMITHA, B., DEVI, B. V., BRUNDAVANAM, Y., GOLLAPALLI, S. R., KARUNASRI, N., SONALI, B., KAVITHA, K., PATIL, S. R., SUNITHA, G., PUTTY, K., REDDY, G. H., REDDY, Y. N., HEGDE, N. R. & RAO, P. P. 2016b. Molecular Typing of Bluetongue Viruses Isolated Over a Decade in South India. *Transbound Emerg Dis,* 63**,** e412-8.

REDDY, Y. V., SUSMITHA, B., PATIL, S., KRISHNAJYOTHI, Y., PUTTY, K., RAMAKRISHNA, K. V., SUNITHA, G., DEVI, B. V., KAVITHA, K., DEEPTHI, B., KROVVIDI, S., REDDY, Y. N., REDDY, G. H., SINGH, K. P., MAAN, N. S., HEMADRI, D., MAAN, S., MERTENS, P. P., HEGDE, N. R. & RAO, P. P. 2018. Isolation and evolutionary analysis of Australasian topotype of bluetongue virus serotype 4 from India. 65**,** 547-556.

REOID. 2017. *Isolates of Bluetongue virus in the dsRNA virus collection at IAH Pirbright* [Online]. Available: http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/btv-12.htm [Accessed December 12 2017].

ROY, P. 1989. Bluetongue virus genetics and genome structure. *Virus Research,* 13**,** 179-206.

ROY, P. 1992. Bluetongue virus proteins. *J Gen Virol,* 73 (Pt 12)**,** 3051-64.

ROY, P. 2008a. Bluetongue virus: dissection of the polymerase complex. *J Gen Virol,* 89**,** 1789-804.

ROY, P. 2008b. Functional mapping of bluetongue virus proteins and their interactions with host proteins during virus replication. *Cell Biochem Biophys,* 50**,** 143-57.

ROY, P., ADACHI, A., URAKAWA, T., BOOTH, T. F. & THOMAS, C. P. 1990a. Identification of bluetongue virus VP6 protein as a nucleic acid-binding protein and the localization of VP6 in virus-infected vertebrate cells. *J Virol,* 64**,** 1-8.

ROY, P., BISHOP, D. H., LEBLOIS, H. & ERASMUS, B. J. 1994. Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: evidence for homologous and partial heterologous protection. *Vaccine,* 12**,** 805-11.

ROY, P., FUKUSHO, A., RITTER, G. D. & LYON, D. 1988. Evidence for genetic relationship between RNA and DNA viruses from the sequence homology of a putative polymerase gene of bluetongue virus with that of vaccinia virus: conservation of RNA polymerase genes from diverse species. *Nucleic Acids Res,* 16**,** 11759-67.

ROY, P., MARSHALL, J. J. & FRENCH, T. J. 1990b. Structure of the bluetongue virus genome and its encoded proteins. *Curr Top Microbiol Immunol,* 162**,** 43-87.

ROY, P., PURDY, M. A., PETRE, J. & RAO, C. D. 1985. Cloning and nucleotide sequencing of bluetongue virus genomes. *Prog Clin Biol Res,* 178**,** 363-70.

RUSSELL, H., O'TOOLE, D. T., BARDSLEY, K., DAVIS, W. C. & ELLIS, J. A. 1996. Comparative effects of bluetongue virus infection of ovine and bovine endothelial cells. *Vet Pathol,* 33**,** 319-31.

SAEGERMAN, C., BERKVENS, D. & MELLOR, P. S. 2008. Bluetongue epidemiology in the European Union. *Emerg Infect Dis,* 14**,** 539-44.

SAILLEAU, C., BREARD, E., VIAROUGE, C., VITOUR, D., ROMEY, A., GARNIER, A., FABLET, A., LOWENSKI, S., GORNA, K., CAIGNARD, G., PAGNEUX, C. & ZIENTARA, S. 2017. Re-Emergence of Bluetongue Virus Serotype 8 in France, 2015. *Transbound Emerg Dis,* 64**,** 998-1000.

SAMAL, S. K., LIVINGSTON, C. W., JR., MCCONNELL, S. & RAMIG, R. F. 1987. Analysis of mixed infection of sheep with bluetongue virus serotypes 10 and 17: evidence for genetic reassortment in the vertebrate host. *J Virol,* 61**,** 1086-91.

SANGER, F., NICKLEN, S. & COULSON, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A,* 74**,** 5463-7.

SAPRE, S. N. 1964. An outbreak of bluetongue in goats and sheep. *Vet. Rev.,* 15**,** 78-80.

SARAVANABAVA, K. 1992. *Studies on bluetongue virus in sheep.* Tamil Nadu Veterinary and Animal Sciences University, Madras, India.

SAVINI, G., CANNAS, A., CASACCIA, C., DI GIALLEONARDO, L., LEONE, A., PATTA, C. & NICOLUSSI, P. 2010. Risk factors associated with the occurrence of undesired effects in sheep and goats after field vaccination with modified-live vaccine against bluetongue virus serotypes 2, 4 and 16. *Vet Microbiol,* 146**,** 44-50.

SAVINI, G., LORUSSO, A., PALADINI, C., MIGLIACCIO, P., DI GENNARO, A., DI PROVVIDO, A., SCACCHIA, M. & MONACO, F. 2014. Bluetongue serotype 2 and 9 modified live vaccine viruses as causative agents of abortion in livestock: a retrospective analysis in Italy. *Transbound Emerg Dis,* 61**,** 69-74.

SAVINI, G., MACLACHLAN, N. J., SANCHEZ-VIZCAINO, J. M. & ZIENTARA, S. 2008. Vaccines against bluetongue in Europe. *Comp Immunol Microbiol Infect Dis,* 31**,** 101-20.

SAVINI, G., PUGGIONI, G., MELONI, G., MARCACCI, M., DI DOMENICO, M., MARIA ROCCHIGIANI, A., SPEDICATO, M., OGGIANO, A., MANUNTA, D., TEODORI, L., LEONE, A., PORTANTI, O., CITO, F., CONTE, A., ORSINI, M., CAMMA, C., CALISTRI, P., GIOVANNINI, A. & LORUSSO, A. 2017a. *Novel putative Bluetongue virus in healthy goats from Sardinia, Italy*.

SAVINI, G., PUGGIONI, G., MELONI, G., MARCACCI, M., DI DOMENICO, M., ROCCHIGIANI, A. M., SPEDICATO, M., OGGIANO, A., MANUNTA, D., TEODORI, L., LEONE, A., PORTANTI, O., CITO, F., CONTE, A., ORSINI, M., CAMMÀ, C., CALISTRI, P., GIOVANNINI, A. & LORUSSO, A. 2017b. Novel putative Bluetongue virus in healthy goats from Sardinia, Italy. *Infection, Genetics and Evolution,* 51**,** 108-117.

SAVINI, G., RONCHI, G. F., LEONE, A., CIARELLI, A., MIGLIACCIO, P., FRANCHI, P., MERCANTE, M. T. & PINI, A. 2007. An inactivated vaccine for the control of bluetongue virus serotype 16 infection in sheep in Italy. *Vet Microbiol,* 124**,** 140-6.

SCHULZ, C., BREARD, E., SAILLEAU, C., JENCKEL, M., VIAROUGE, C., VITOUR, D., PALMARINI, M., GALLOIS, M., HOPER, D., HOFFMANN, B., BEER, M. & ZIENTARA, S. 2016. Bluetongue virus serotype 27: detection and characterization of two novel variants in Corsica, France. *J Gen Virol,* 97**,** 2073-83.

SCHWARTZ-CORNIL, I., MERTENS, P. P., CONTRERAS, V., HEMATI, B., PASCALE, F., BREARD, E., MELLOR, P. S., MACLACHLAN, N. J. & ZIENTARA, S. 2008. Bluetongue virus: virology, pathogenesis and immunity. *Vet Res,* 39**,** 46.

SCOTT, M. D. & JAMES, M. N. 2015. Vaccines for Prevention of Bluetongue and Epizootic Hemorrhagic Disease in Livestock: A North American Perspective. *Vector-Borne and Zoonotic Diseases,* 15**,** 385-396.

SELLERS, R. & TAYLOR, W. 1980. Epidemiology of bluetongue and the import and export of livestock, semen and embryos. *Bulletin of Office international des Epizooties* 92**,** 587–592.

SENDOW, I., DANIELS, P. W., CYBINSKI, D. H., YOUNG, P. L. & RONOHARDJO, P. 1991. Antibodies against certain bluetongue and epizootic haemorrhagic disease viral serotypes in Indonesian ruminants. *Vet Microbiol,* 28**,** 111-8.

SHAD, G., WILSON, W. C., MECHAM, J. O. & EVERMANN, J. F. 1997. Bluetongue virus detection: a safer reverse-transcriptase polymerase chain reaction for prediction of viremia in sheep. *J Vet Diagn Invest,* 9**,** 118-24.

SHAFIQ, M., MINAKSHI, P., BHATEJA, A., RANJAN, K. & PRASAD, G. 2013. Evidence of genetic reassortment between Indian isolate of bluetongue virus serotype 21 (BTV-21) and bluetongue virus serotype 16 (BTV-16). *Virus Res,* 173**,** 336-43.

SHARMA, M. M., LONKAR, P. S., SRIVASTAVA, C. P., DUBEY, S. C., MARU, A. & KALRA, D. B. 1985. Epidemiology of bluetongue in sheep at an organised farm in semi arid part of Rajasthan. *Indian Journal of comparative Microbiology, Immunology and infectious diseases* 6 188-192.

SHAW, A. E., MONAGHAN, P., ALPAR, H. O., ANTHONY, S., DARPEL, K. E., BATTEN, C. A., GUERCIO, A., ALIMENA, G., VITALE, M., BANKOWSKA, K., CARPENTER, S., JONES, H., OURA, C. A., KING, D. P., ELLIOTT, H., MELLOR, P. S. & MERTENS, P. P. 2007. Development and initial evaluation of a real-time RT-PCR assay to detect bluetongue virus genome segment 1. *J Virol Methods,* 145**,** 115-26.

SHAW, A. E., RATINIER, M., NUNES, S. F., NOMIKOU, K., CAPORALE, M., GOLDER, M., ALLAN, K., HAMERS, C., HUDELET, P., ZIENTARA, S., BREARD, E., MERTENS, P. & PALMARINI, M. 2013. Reassortment between two serologically unrelated bluetongue virus strains is flexible and can involve any genome segment. *J Virol,* 87**,** 543-57.

SHENDURE, J. & JI, H. 2008. Next-generation DNA sequencing. *Nature Biotechnology,* 26**,** 1135.

SHENDURE, J. & LIEBERMAN AIDEN, E. 2012. The expanding scope of DNA sequencing. *Nat Biotechnol,* 30**,** 1084-94.

SIEVERS, F., WILM, A., DINEEN, D., GIBSON, T. J., KARPLUS, K., LI, W., LOPEZ, R., MCWILLIAM, H., REMMERT, M., SODING, J., THOMPSON, J. D. & HIGGINS, D. G. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol,* 7**,** 539.

SIMON-LORIERE, E. & HOLMES, E. C. 2011. Why do RNA viruses recombine? *Nat Rev Microbiol,* 9**,** 617-26.

SINGER, R. S., MACLACHLAN, N. J. & CARPENTER, T. E. 2001. Maximal predicted duration of viremia in bluetongue virus-infected cattle. *J Vet Diagn Invest,* 13**,** 43-9.

SINGH, B., ANANTWAR, L. G. & SAMOD, A. 1982. A severe outbreak of bluetongue in sheep in Maharastra. *National Seminar on Sheep and Goat Diseases* CSWRI, Avikanagar, Rajasthan.

SINGH, K. P., MAAN, S., SAMUEL, A. R., RAO, S., MEYER, A. J. & MERTENS, P. P. 2004. Phylogenetic analysis of bluetongue virus genome segment 6 (encoding VP5) from different serotypes. *Vet Ital,* 40**,** 479-83.

SPERLOVA, A. & ZENDULKOVA, D. 2011 Bluetongue: a review. *Veterinarni Medicina, 56,* 9**,** 430–452.

SPREULL, J. 1905. Malarial catarrhal fever (bluetongue) of sheep in South Africa. *Journal of Comparative Pathology and Therapeutics,* 18**,** 321-337.

SREENIVASULU, D., RAO, M. V. & GARD, G. P. 1999. Isolation of bluetongue virus serotype 2 from native sheep in India. *Vet Rec,* 144**,** 452-3.

SREENIVASULU, D., SUBBA RAO, M. V., REDDY, Y. N. & GARD, G. P. 2004. Overview of bluetongue disease, viruses, vectors, surveillance and unique features: the Indian sub-continent and adjacent regions. *Vet Ital,* 40**,** 73-7.

SRIGUPPI, B. S. A note on seroepidemiology of bluetongue. *In:* MEHROTRA, M. L., ed. Proceedings of National Workshop on Bluetongue, 1982 Izatnagar, IVRI. 114-22.

SRINIVAS, R. P., MOOGI, V. M., ANANTH, M., GOPAL, T. & KESHAVMURTHY, B. S. Bluetongue disease outbreaks among sheep and goats of Karnataka. National Seminar on Sheep and Goat Diseases, 1982 CSWRI, Avikanagar, Rajasthan. 15.

STAUBER, N., MARTINEZ-COSTAS, J., SUTTON, G., MONASTYRSKAYA, K. & ROY, P. 1997. Bluetongue virus VP6 protein binds ATP and exhibits an RNA-dependent ATPase function and a helicase activity that catalyze the unwinding of double-stranded RNA substrates. *J Virol,* 71**,** 7220-6.

STEWART, M., DOVAS, C. I., CHATZINASIOU, E., ATHMARAM, T. N., PAPANASTASSOPOULOU, M., PAPADOPOULOS, O. & ROY, P. 2012. Protective efficacy of Bluetongue virus-like and subvirus-like particles in sheep: presence of the serotype-specific VP2, independent of its geographic lineage, is essential for protection. *Vaccine,* 30**,** 2131-9.

STEWART, M., DUBOIS, E., SAILLEAU, C., BREARD, E., VIAROUGE, C., DESPRAT, A., THIERY, R., ZIENTARA, S. & ROY, P. 2013. Bluetongue virus serotype 8 virus-like particles protect

sheep against virulent virus infection as a single or multi-serotype cocktail immunogen. *Vaccine,* 31**,** 553-8.

STEWART, M., HARDY, A., BARRY, G., PINTO, R. M., CAPORALE, M., MELZI, E., HUGHES, J., TAGGART, A., JANOWICZ, A., VARELA, M., RATINIER, M. & PALMARINI, M. 2015. Characterization of a second open reading frame in genome segment 10 of bluetongue virus. *J Gen Virol,* 96**,** 3280-93.

STOTT, J. L., OBERST, R. D., CHANNELL, M. B. & OSBURN, B. I. 1987. Genome segment reassortment between two serotypes of bluetongue virus in a natural host. *J Virol,* 61**,** 2670-4.

STUART, D. I., GOUET, P., GRIMES, J., MALBY, R., DIPROSE, J., ZIENTARA, S., BURROUGHS, J. N. & MERTENS, P. P. 1998. Structural studies of orbivirus particles. *Arch Virol Suppl,* 14**,** 235-50.

SUSMITHA, B., SUDHEER, D., RAO, P. P., UMA, M., PRASAD, G., MINAKSHI, P., HEGDE, N. R. & REDDY, Y. N. 2012. Evidence of bluetongue virus serotype 21 (BTV-21) divergence. *Virus Genes,* 44**,** 466-9.

SUTTON, G., GRIMES, J. M., STUART, D. I. & ROY, P. 2007. Bluetongue virus VP4 is an RNA-capping assembly line. *Nat Struct Mol Biol,* 14**,** 449-51.

TABACHNICK, W. J. 1996. Culicoides variipennis and bluetongue-virus epidemiology in the United States. *Annu Rev Entomol,* 41**,** 23-43.

TABACHNICK, W. J. 2004. Culicoides and the global epidemiology of bluetongue virus infection. *Vet Ital,* 40**,** 144-50.

TABACHNICK, W. J., ROBERTSON, M. A. & MURPHY, K. E. 1996. Culicoides variipennis and Bluetongue Disease: Research on Arthropod-Borne Animal Diseases for Control and Prevention in the Year 2000a. *Annals of the New York Academy of Sciences,* 791**,** 219-226.

TAKAMATSU, H., MELLOR, P. S., MERTENS, P. P., KIRKHAM, P. A., BURROUGHS, J. N. & PARKHOUSE, R. M. 2003. A possible overwintering mechanism for bluetongue virus in the absence of the insect vector. *J Gen Virol,* 84**,** 227-35.

TAN, B. H., NASON, E., STAEUBER, N., JIANG, W., MONASTRYRSKAYA, K. & ROY, P. 2001. RGD tripeptide of bluetongue virus VP7 protein is responsible for core attachment to Culicoides cells. *J Virol,* 75**,** 3937-47.

TAYLOR, W. P. 1986. The epidemiology of bluetongue. *Rev. Sci. Tech. OIE,* 5**,** 351–356.

TEMBHURNE, P. A., MONDAL, B., PATHAK, K. B., BISWAS, S. K., SANYAL, A., YADAV, M. P., BANDYOPADHYAY, S. K. & SINGH, R. K. 2010. Segment-2 sequence analysis and cross-neutralization studies on some Indian bluetongue viruses suggest isolates are VP2-variants of serotype 23. *Arch Virol,* 155**,** 89-95.

THEVASAGAYAM, J. A., WELLBY, M. P., MERTENS, P. P., BURROUGHS, J. N. & ANDERSON, J. 1996. Detection and differentiation of epizootic haemorrhagic disease of deer and bluetongue viruses by serogroup-specific sandwich ELISA. *J Virol Methods,* 56**,** 49-57.

References

THIRY, E., SAEGERMAN, C., GUYOT, H., KIRTEN, P., LOSSON, B., ROLLIN, F., BODMER, M., CZAPLICKI, G., TOUSSAINT, J. F., DE CLERCQ, K., DOCHY, J. M., DUFEY, J., GILLEMAN, J. L. & MESSEMAN, K. 2006. Bluetongue in northern Europe. *Vet Rec,* 159**,** 327.

THOMAS, C. P., BOOTH, T. F. & ROY, P. 1990. Synthesis of bluetongue virus-encoded phosphoprotein and formation of inclusion bodies by recombinant baculovirus in insect cells: it binds the single-stranded RNA species. *J Gen Virol,* 71 (Pt 9)**,** 2073-83.

THUENEMANN, E. C., MEYERS, A. E., VERWEY, J., RYBICKI, E. P. & LOMONOSSOFF, G. P. 2013. A method for rapid production of heteromultimeric protein complexes in plants: assembly of protective bluetongue virus-like particles. *Plant Biotechnol J,* 11**,** 839-46.

TOUSSAINT, J. F., SAILLEAU, C., BREARD, E., ZIENTARA, S. & DE CLERCQ, K. 2007. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. *J Virol Methods,* 140**,** 115-23.

TOYE, P. G., BATTEN, C. A., KIARA, H., HENSTOCK, M. R., EDWARDS, L., THUMBI, S., POOLE, E. J., HANDEL, I. G., BRONSVOORT, B. M., HANOTTE, O., COETZER, J. A., WOOLHOUSE, M. E. & OURA, C. A. 2013. Bluetongue and epizootic haemorrhagic disease virus in local breeds of cattle in Kenya. *Res Vet Sci,* 94**,** 769-73.

UPPAL, P. K. & VASUDEVAN, B. 1980. Occurrence of bluetongue in India. *Journal of comparative Microbiology,Immunology and infectious Diseases,* 1**,** 18-20.

URAKAWA, T., RITTER, D. G. & ROY, P. 1989. Expression of largest RNA segment and synthesis of VP1 protein of bluetongue virus in insect cells by recombinant baculovirus: association of VP1 protein with RNA polymerase activity. *Nucleic Acids Res,* 17**,** 7395-401.

URAKAWA, T. & ROY, P. 1988. Bluetongue virus tubules made in insect cells by recombinant baculoviruses: expression of the NS1 gene of bluetongue virus serotype 10. *J Virol,* 62**,** 3919-27.

VAN DEN BERGH, C., COETZEE, P., GUTHRIE, A. J., LE GRANGE, M. & VENTER, E. H. 2016. Complete Genome Sequences of Five Bluetongue Virus (BTV) Vaccine Strains from a Commercial Live Attenuated Vaccine, a BTV-4 Field Strain from South Africa, and a Reassortant Strain Isolated from Experimentally Vaccinated Cattle. *Genome Announc,* 4.

VAN REGENMORTEL, M. H. V. & MAHY, B. W. J. 2004. Emerging Issues in Virus Taxonomy. *Emerging Infectious Diseases,* 10**,** 8-13.

VASUDEVAN, B. A note on bluetongue infection among sheep in India. National Workshop on Bluetongue, 1982 IVRI, Izatnagar. 129-144.

VENKATARAMANAN, R., Y.K., R., KRISHNAKUMAR, S. & B.M., M. 2010. Occurrence of bluetongue in the Nilgiri Hills of Tamil Nadu. *Indian Vet J,* 87**,** 724-725.

VERONESI, E., DARPEL, K. E., HAMBLIN, C., CARPENTER, S., TAKAMATSU, H. H., ANTHONY, S. J., ELLIOTT, H., MERTENS, P. P. & MELLOR, P. S. 2010. Viraemia and clinical disease in Dorset Poll sheep following vaccination with live attenuated bluetongue virus vaccines serotypes 16 and 4. *Vaccine,* 28**,** 1397-403.

VERONESI, E., HAMBLIN, C. & MELLOR, P. S. 2005. Live attenuated bluetongue vaccine viruses in Dorset Poll sheep, before and after passage in vector midges (Diptera: Ceratopogonidae). *Vaccine,* 23**,** 5509-16.

VERWOERD, D. W., ELS, H. J., DE VILLIERS, E.-M. & HUISMANS, H. 1972. Structure of the Bluetongue Virus Capsid. *Journal of Virology,* 10**,** 783-794.

VERWOERD, D. W. & ERASMUS, B. J. 2004. Bluetongue *In:* 2ND (ed.) *Infectious Diseases of Livestock.* Cape Town, South Africa Oxford University Press.

VERWOERD, D. W. & HUISMANS, H. 1972. Studies on the in vitro and the in vivo transcription of the bluetongue virus genome. *Onderstepoort J Vet Res,* 39**,** 185-91.

VITOUR, D., DOCEUL, V., RUSCANU, S., CHAUVEAU, E., SCHWARTZ-CORNIL, I. & ZIENTARA, S. 2014. Induction and control of the type I interferon pathway by Bluetongue virus. *Virus Res,* 182**,** 59-70.

VOSDINGH, R. A., TRAINER, D. O. & EASTERDAY, B. C. 1968. Experimental Bluetongue Disease in White-Tailed Deer. *Canadian Journal of Comparative Medicine and Veterinary Science,* 32**,** 382-387.

VREEDE, F. T., CLOETE, M., NAPIER, G. B., VAN DIJK, A. A. & VILJOEN, G. J. 1998. Sequence-independent amplification and cloning of large dsRNA virus genome segments by poly(dA)-oligonucleotide ligation. *Journal of Virological Methods,* 72**,** 243-247.

WACKERLIN, R., ESCHBAUMER, M., KONIG, P., HOFFMANN, B. & BEER, M. 2010. Evaluation of humoral response and protective efficacy of three inactivated vaccines against bluetongue virus serotype 8 one year after vaccination of sheep and cattle. *Vaccine,* 28**,** 4348-55.

WADE-EVANS, A. M., MERTENS, P. P. & BELSHAM, G. J. 1992. Sequence of genome segment 9 of bluetongue virus (serotype 1, South Africa) and expression analysis demonstrating that different forms of VP6 are derived from initiation of protein synthesis at two distinct sites. *J Gen Virol,* 73 (Pt 11)**,** 3023-6.

WADE-EVANS, A. M., MERTENS, P. P. & BOSTOCK, C. J. 1990. Development of the polymerase chain reaction for the detection of bluetongue virus in tissue samples. *J Virol Methods,* 30**,** 15-24.

WALDVOGEL, A. S., STOTT, J. L., SQUIRE, K. R. & OSBURN, B. I. 1986. Strain-dependent virulence characteristics of bluetongue virus serotype 11. *J Gen Virol,* 67 (Pt 4)**,** 765-9.

WECHSLER, S. J. & LUEDKE, A. J. 1991. Detection of bluetongue virus by using bovine endothelial cells and embryonated chicken eggs. *J Clin Microbiol,* 29**,** 212-4.

WECHSLER, S. J. & MCHOLLAND, L. E. 1988. Susceptibilities of 14 cell lines to bluetongue virus infection. *J Clin Microbiol,* 26**,** 2324-7.

WILBUR, L. A., EVERMANN, J. F., LEVINGS, R. L., STOLL, I. R., STARLING, D. E., SPILLERS, C. A., GUSTAFSON, G. A. & MCKEIRNAN, A. J. 1994. Abortion and death in pregnant bitches

associated with a canine vaccine contaminated with bluetongue virus. *J Am Vet Med Assoc,* 204**,** 1762-5.

WILLIAMSON, S., WOODGER, N. & DARPEL, K. 2008. Differential diagnosis of bluetongue in cattle and sheep. *Practise* 30**,** 242–251.

WILSON, A. & MELLOR, P. 2008. Bluetongue in Europe: vectors, epidemiology and climate change. *Parasitol Res,* 103 Suppl 1**,** S69-77.

WILSON, W. C. & CHASE, C. C. 1993. Nested and multiplex polymerase chain reactions for the identification of bluetongue virus infection in the biting midge, Culicoides variipennis. *J Virol Methods,* 45**,** 39-47.

WILSON, W. C., MA, H. C., VENTER, E. H., VAN DJIK, A. A., SEAL, B. S. & MECHAM, J. O. 2000. Phylogenetic relationships of bluetongue viruses based on gene S7. *Virus Res,* 67**,** 141-51.

WILSON, W. C., STALLKNECHT, D. E. & MECHAM, J. O. 2004. Field-deployable real-time polymerase chain reaction detection of bluetongue and epizootic haemorrhagic disease viral ribonucleic acid. *Vet Ital,* 40**,** 587-93.

WITTMANN, E. J., MELLO, P. S. & BAYLIS, M. 2002. Effect of temperature on the transmission of orbiviruses by the biting midge, Culicoides sonorensis. *Med Vet Entomol,* 16**,** 147-56.

WRIGHT, I. 2014. *Serological and genetic characterisation of putative new serotypes of bluetongue virus and epizootic haemorrhagic disease virus isolated from an Alpaca.* North-West University,Potchefstroom Campus,South Africa.

WU, X., CHEN, S. Y., IWATA, H., COMPANS, R. W. & ROY, P. 1992. Multiple glycoproteins synthesized by the smallest RNA segment (S10) of bluetongue virus. *J Virol,* 66**,** 7104-12.

XU, G., WILSON, W., MECHAM, J., MURPHY, K., ZHOU, E. M. & TABACHNICK, W. 1997. VP7: an attachment protein of bluetongue virus for cellular receptors in Culicoides variipennis. *J Gen Virol,* 78 (Pt 7)**,** 1617-23.

YANG, H., XIAO, L., WANG, J., MENG, J., LV, M., LIAO, D., SONG, J., GAO, L., XIONG, H., HE, Y., NIU, B., CHUANG, X. & LI, H. 2017. Phylogenetic Characterization Genome Segment 2 of Bluetongue Virus Strains Belonging to Serotypes 5, 7 and 24 Isolated for the First Time in China During 2012 to 2014. *Transbound Emerg Dis,* 64**,** 1317-1321.

YANG, H., ZHU, J., LI, H., XIAO, L., WANG, J., LI, N., ZHANG, N. & KIRKLAND, P. D. 2012. Full genome sequence of bluetongue virus serotype 4 from China. *J Virol,* 86**,** 13122-3.

YOUNG, S. & CORDY, D. R. 1964. An Ovine Fetal Encephalopathy Caused by Bluetongue Vaccine Virus. *J Neuropathol Exp Neurol,* 23**,** 635-59.

ZHANG, J., CHIODINI, R., BADR, A. & ZHANG, G. 2011. The impact of next-generation sequencing on genomics. *J Genet Genomics,* 38**,** 95-109.

References

ZHANG, X., BOYCE, M., BHATTACHARYA, B., ZHANG, X., SCHEIN, S., ROY, P. & ZHOU, Z. H. 2010. Bluetongue virus coat protein VP2 contains sialic acid-binding domains, and VP5 resembles enveloped virus fusion proteins. *Proc Natl Acad Sci U S A,* 107**,** 6292-7.

ZIENTARA, S., MACLACHLAN, N. J., CALISTRI, P., SANCHEZ-VIZCAINO, J. M. & SAVINI, G. 2010. Bluetongue vaccination in Europe. *Expert Rev Vaccines,* 9**,** 989-91.

ZIENTARA, S., SAILLEAU, C., VIAROUGE, C., HOPER, D., BEER, M., JENCKEL, M., HOFFMANN, B., ROMEY, A., BAKKALI-KASSIMI, L., FABLET, A., VITOUR, D. & BREARD, E. 2014. Novel bluetongue virus in goats, Corsica, France, 2014. *Emerg Infect Dis,* 20**,** 2123-5.