HUMORAL IMMUNE RESPONSES TO BLUETONGUE VIRAL PROTEINS VP2, VP5 and NS1

By

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Thesis submitted to

**P.V.NARSIMHA RAO TELANGANA
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SEPTEMBER, 2019.

CERTIFICATE

This is to certify that Dr.J.Shiva Jyothi has satisfactorily prosecuted the course of research and that the thesis entitled entitled **"HUMORAL IMMUNE RESPONSES TO BLUETONGUE VIRAL PROTEINS VP2, VP5 and NS1"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by her for a degree of any University.

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Hyderabad

DECLARATION

I, J. Shiva Jyothi, RVD/15-05 hereby declare that the thesis entitled **"HUMORAL IMMUNE RESPONSES TO BLUETONGUE VIRAL PROTEINS VP2, VP5 and NS1"** submitted to **P.V.NARSIMHA RAO TELANGANA VETERINARY UNIVERSITY** for the degree of *DOCTOR of PHILOSOPHY* is the result of original research work done by me. It is further declared that no part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged.

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Place: Hyderabad

CERTIFICATE

This is to certify that the thesis entitled **"HUMORAL IMMUNE RESPONSES TO BLUETONGUE VIRAL PROTEINS VP2, VP5 and NS1."** submitted in partial fulfillment of the requirements for the degree of **"DOCTOR OF PHILOSOPHY"** of the **P.V.Narsimha Rao Telangana Veterinary University**, is a record of the bonafide research work carried out by **J. SHIVA JYOTHI, RVD/15-05** under our guidance and supervision. The subject of the thesis has been approved by the student's Advisory Committee.

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

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Shiva Jyothi

ABREVIATIONS AND SYMBOLS USED

aa	:	Accession
AHSV	:	African Horse Sickness Virus
Amp	:	Ampicillin
APS	:	Ammonium per sulphate
ATP	:	Adenosine triphosphate
BLAST	:	Basic Alignment Search Tool
BHK-21	:	Baby hamster Kidney cells
BSA	:	Bovine serum albumin
Bp	:	Base pair
BT	:	Bluetongue
BTV	:	Bluetongue virus
I-ELISA	:	Indirect Enzyme Linked Immunosorbent Assay
CMI	:	Cell mediated immunity
cDNA	:	Complementary dioxynuclic acid
CO_2	:	Carbon dioxide
CPE	:	Cytopathic effect
C-terminus	:	Carboxy terminus
DISC	:	Disabled infectious single cycled vaccines
DIVA	:	Differentiation of infected from vaccinated Animals
DMEM	:	Dulbecco’s minimum essential medium
DNA	:	Deoxyribonucleic acid
dN/dS	:	Non-synonymous to Synonymous substitutions
dNTP	:	Deoxy nucleotide triphosphates
ds RNA	:	Double stranded Ribonucleic acid

ECE : Embryonated chicken egg

EDTA : Ethylenediamine tetra acetic acid

EHDV : Epizootic hemorrhagic disease virus

et al : Et alia (and others)

Fig : Figure

FBS : Fetal Bovine Serum

FP : Forward primer

h : Hour

His-Tag : Histidine Tagged

H_2O : Water

H_2SO_4 : Sulphuric acid

HCl : Hydrochloride

HRPO : Horse raddish peroxidase

IEDB : Immune epitope data base

IFN : Interferon

IFNAR : Interferon-alpha/beta receptor

IgG : Immunoglobulin G

IgM : Immunoglobulin M

IL : Interleukin

IPTG : Isopropyl-β –D- thiogalactoside

Kan : Kanamycin

Kb : kilobase pairs

KDa : Kilodaltons

KH_2PO_4 : Potassium dihydrogenphosphate

LB : Luria-Bertani

Log_{10} : logarithm to the base 10

Mab	:	Monoclonal antibody
MCS	:	Multiple cloning site
MEGA	:	Molecular evolutionary genetic analysis
MEM	:	Minimum essential medium
MHC I	:	Major histocompatability complex I
MHC II	:	Major histocompatability complex II
MLV	:	Modified Live Vaccine
min	:	Minute
ml	:	Milli liter
MLV	:	Modified live Vaccine
mM	:	Milli molar
mm	:	Millimeter
Mr	:	Molecular range
mRNA	:	messenger RNA
MUSCLE	:	Multiple sequence comparison by log expectation
Nab	:	Neutralizing antibodies
NaCl	:	Sodium chloride
Na_2HPO_4	:	Disodium phosphate
NCBI	:	National Center for Biotechnology Information
NFW	:	Nuclease free water
$(NH_4)_2SO_4$	:	Ammonium sulfate
ng	:	Nano gram
nm	:	Nanometre
NS	:	Non-structural
nt	:	Nucleotides
N-terminus	:	Amino terminus

Ni-NTA	:	Nickel-nitrilotriacetic acid
OD	:	optical density
OIE	:	*Office International des Epizooties*
ORF	:	Open reading frame
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PBST	:	Phosphate buffered saline with Tween-20
PCR	:	Polymerase chain reaction
PEG	:	Polyethylene glycol
pH	:	Negative logarithm of hydrogen ions concentration in moles per litre
PPR	:	Peste- des- petits ruminants
PVDF	:	Polyvinylidene difluoride
RBC	:	Red blood cell
RNA	:	Ribonucleic acid
RP	:	Reverse primer
rpm	:	Revolutions per minute
RPMI	:	Roswell Park Memorial Institute
RT	:	Reverse transcription
RT-PCR	:	Reverse transcription polymerase chain reaction
SDS	:	Sodium dodecyl sulphate
SLAC	:	Sinle-Likelihood Ancestor Counting
SMP	:	Skim milk powder
SNT	:	Serum neutralization test
ssRNA	:	Single stranded ribo nucleic acid
TAE	:	Tris Acetate ethylenediaminetetraacidic acid

Taq	:	*Thermus aquaticus*
TBE	:	Tris Boric acid EDTA
TBS	:	Tris base sodium chloride
TBST	:	TBS-tween
TE buffer	:	Tris EDTA buffer
TEMED	:	N,N,N´, N´-Tetramethylethylenediamine
USA		United States of America
UK	:	United Kingdom
UV	:	Ultra violet
VLP	:	Virus like particle
VP	:	Viral protein

Unit of Measurement and other symbol

%	:	Percent
®	:	Registered trade
α	:	Alpha
bp	:	Base pair
IU	:	International Unit
Kbp	:	Kilo base pair
Lbs	:	Pounds
mg	:	Milligram
min	:	Minute
ml	:	Millilitre
mM	:	Millimole
Ng	:	Nanogram
Nm	:	Nanometer
^{o}C	:	Degree Celsius
$TCID_{50}$	:	50% Tissue culture infective dose
V	:	Volts
w/v	:	Weight/volume
µl	:	Microlitre

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ABSTRACT

Bluetongue (BT) disease, caused by bluetongue virus (BTV) is an acute hemorrhagic fever of domesticated and wild ruminants. India is endemic to BTV, and twenty-nine distinct serotypes have been identified worldwide so far. Although live attenuated and inactivated vaccines are being developed, and used in response to endemic serotypes or a particular outbreak, they are not without their share of drawbacks. Insufficient inactivation or attenuation of the virus in the development of the vaccine can cause possible teratological effects, severe clinical disease with outbreaks, and emergence of novel virus strains due to gene reassortment with wild type viruses. Shortcomings as mentioned above warrant rigorous quality control, which is tricky in developing countries like India. Moreover, the threat posed by the range of different BTV serotypes has highlighted the need for more widely cross-protective BTV vaccines. Hence the current study is proposed with the central objectives to develop a recombinant subunit protein based vaccine that is cross-protective against all the known serotypes of BTV.

Data sets of complete sequences for segments 2 (encoding VP2), segment 6 (encoding VP5) and segment 5 (encoding NS1) of the BTV genome were compiled from GenBank. In the Seg-2 alignment, a number of gaps due to insertions and deletions among the different serotypes were observed where as VP5, NS1 nucleotide and protein sequences were rather conserved. The amino acid sequence alignment showed that, although VP2 is very variable, several regions are relatively more conserved between serotypes. According to the present study analysis, most conserved region of VP2 is evident from 338-383 aa. Interestingly, 3D homology modeling of VP2 protein of different serotypes of BTV revealed different structures whereas conserved VP2 (cVP2) region of those serotypes revealed similar protein structure. Being rather conserved, VP5 of different BTV serotypes revealed similar protein structure on homology modelling. To better understand the evolutionary dynamics, selection pressures acting on individual codons of VP2 and VP5 was next determined. The global estimate of *dN*/*dS* was 0.16 for VP2 and 0.058 for VP5 indicating strong purifying selection. Moreover, high number of negatively selected codons was identified in both (850/988 for VP2 and 506/528 for VP5). Even more interesting was the observation that all the codons in the putative conserved region of VP2 (cVP2) are under strong negative selection. Two major histocompatability complex (MHC) class I, two MHC class II binding, and three B cell epitopes were predicted with significant percentile ranks suggesting a strong immunogenicity potential of cVP2.

Primers were designed for the amplification of sequences of BTV-16 (that was isolated from 2016 field outbreaks of BT in Andhra Pradesh) cVP2, and codon optimized sequence of VP5 and NS1. Following amplification of the cVP2 by RT-PCR the amplified 265 bp cVP2 and codon optimized 1610 bp VP5, 1671 bp NS1 were subjected to restriction enzyme digestion before cloning into pRSET-B (cVP2) and pET-28b (+) for VP5 and NS1 vectors, respectively. Recombinant plasmids (cVP2, VP5 and NS1) were then transformed into the *E. coli* BL21 (DE3) expression hosts. Protein expression was isopropyl β-D-1-thiogalactopyranoside (IPTG) dependent for cVP2 and autoinduction was used for VP5 and NS1 recombinant proteins. The expressed proteins were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. All the three recombinant proteins were found to be in inclusion bodies. Hence, optimized conditions were used for the purification of the recombinant proteins cVP2, VP5 and NS1 by Ni-NTA column. The purity of the eluted His-Tag fusion protein was checked by SDS-PAGE that revealed a band with molecular weights of expected size of 9.35 KDa for cVP2, 59.8 KDa for VP5 and 61.2 KDa for NS1. Purified proteins were then desalted by dialysis against desired buffer and were quantified by Bradford's method of protein estimation with BSA as standard.

Immunization studies were carried with 36 BALB/c female mice of 4 weeks age. Mice were divided into 6 groups (G1 to G6) of 6 mice in each group. Immunization was done subcutaneously in the back of neck on day 0, day14 and day 28 with recombinant proteins cVP2, VP5 and NS1. Group 1 received 25 µg of cVP2, Group 2 received 25 µg of VP5 and Group 3 received 25 µg of NS1, Group 4 received a mixture of all the three recombinant proteins at a concentration of 25 µg each. Group 5 received the commercial pentavalent vaccine (Raksha Blu, Indian Immunologicals). All the above groups also received equal volume of Montanide as adjuvant. The control group (Group 6) received only Montanide. Blood samples were obtained on day 41 by cardiac puncture, serum was separated and stored at -80°C till use.

Detection of protein-specific antibodies was done by standardizing the indirect-enzyme linked immuno sorbent assay (I-ELISA) by checker-board titration method. Results showed that the animals developed antibodies pertaining to the given recombinant proteins (protein-specific antibodies). Furthermore, the ability of these antibodies to neutralize the BTV serotypes was checked by serum neutralization test. Neutralization assay was carried out with eleven available BTV serotypes (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23, and 24). These serotypes represent 8 of the 10 BTV nucleotype groups. Varying neutralizing titres were observed for different BTV serotypes against different groups. Recombinant proteins elicited higher neutralizing antibody (Nab) titres as compared to inactivated vaccine group, except for neutralization of BTV-1 where the vaccine group elicited higher Nab titres. Interestingly, the cocktail of recombinant proteins did not elicit higher Nab titres than individual protein alone as was expected; Nab titre in the mixed group was either similar to or less than the titre of individual protein groups. BTV-12 could only be neutralized by serum raised against inactivated vaccine. Moreover, BTV-5 and 24 could not be neutralized by any of the groups tested. Consistent absence of neutralization with all the serotypes was seen with the control group serum.

NS1 recombinant protein developed for use in subunit vaccine in the current study was also found to be a tool in the detection of BTV antibodies from field samples by I-ELISA. Since, the developed subunit vaccine was shown to induce specific immune response to the administered proteins and that additionally could neutralize several BTV serotypes, it is tempting to consider that the conserved recombinant proteins of cVP2, VP5 and NS1 are of significance as recombinant vaccine. Additional investigations in the determinants of cellular immune response of these proteins and immunogenicity studies in the natural host will shed more light into the immune response generated by these vaccines to be used as successful recombinant broad-spectrum vaccines.

CHAPTER 1

INTRODUCTION

Bluetongue virus (BTV) is the causative agent of bluetongue (BT) disease, a hemorrhagic disease of sheep and some species of wild ruminants (Spruell 1905 & MacLachlan 1994). Although BTV has many vertebrate hosts including goats, cattle, buffaloes, and deer, it is a major disease of sheep which show the apparent clinical manifestation of the disease with signs *viz.*, fever, edema of the lips, tongue, and head, conjunctivitis, coronitis, excessive salivation, and nasal discharges. In cattle and goats, the disease is subclinical and these animals act as reservoirs of the disease. BTV is a member of the family *Reoviridae* and is the prototype virus of the genus *Orbivirus* (Murphy *et al.,* 1995). So far, 29 serotypes (Maan *et al.,* 2016 & Kartika Lakshmi *et al.,* 2018) of BTV have been identified worldwide and a total of 24 serotypes have been reported from India (Krishnajyothi *et al.,* 2016 & Hemadri *et al.,* 2017). Curtailing the initial introduction into regions which harbour susceptible host and vector species and vaccination of the susceptible animal species may aid in effective prevention and control of BT (Maclacchlan and Mayo 2013). An inactivated pentavalent vaccine consisting of BTV serotypes 1, 2, 10, 16, 23 is currently being used in some parts of India. However, circulation of multiple serotypes, often within the same animal (Hemadri *et al.,* 2017) and little or no cross-protection between serotypes makes BTV control and prevention problematic (MacLachlan and Mayo 2013; Baetza 2014; Pioz *et al.,* 2014; and Breard *et al.,* 2015).

The BTV particle consists of an icosahedral core formed by five proteins (VP1, VP3, VP4, VP6 and VP7) and a diffuse outer coat composed of VP2 and VP5 (Verwoerd *et al.,* 1972; Roy *et al.,* 1992 and Mertens *et al.,* 1984). Protein VP2, coded by L2 segment, is the major cell adhesion protein (Huismans *et al.,* 1987 & Mertens *et* *al.,* 1995) and the most variable protein in BTV. It can induce neutralizing antibody in the infected host. Although the VP2 protein encoded by seg-2 being highly variable is the determinant of serotype, the other viral protein VP5 is now found to codetermine serotype along with VP2 (Schulz *et al.,* 2016). In contrast to VP2, VP5 is reasonably well conserved and has been shown to possess membrane penetration and membrane fusion-like activities (Forzan *et al.,* 2004 and Hassan *et al.,* 2001). Hybridization analyses have suggested that the most highly conserved RNA segment for BTV serotypes from all geographical regions was that coding for NS1 (Huismans *et al.,* 1987).

Protein-based vaccines for BT are based on the initial observation that the serotype determining outer capsid protein of the virus, VP2, elicits protective immunity in vaccinated sheep, following which several studies reported vaccine potential for other immunogenic proteins of BTV (Huismans *et al.,* 1987; Roy *et al.,* 1990; Jones *et al.,* 1996; Anderson *et al.,* 2013*;* Anderson *et al.,* 2014; Mohd Jaffar *et al.,* 2014 and Marin-Lopez *et al.,* 2014). It was also shown that combining both virus outer capsid proteins (VP2 + VP5) results in better protection than VP2 alone (Roy *et al.,* 1990). The nonstructural proteins NS1 and NS2 have both been shown to produce specific humoral and cell-mediated immunity (Jones *et al.,* 1996).

Conventional live and attenuated BTV vaccines have certain inherent deficiencies (Roy *et al.,* 1990). Genetic engineering techniques offer the possibility of preparing subunit vaccines without the need to grow pathogenic organisms. In accordance, vaccination with recombinant pox viruses expressing VP2 and VP5 together induced greater protection in sheep than vaccination with pox viruses expressing either protein alone (Lobato *et al.,* 1997; Boone *et al.,* 2007). Likewise, virus-like particles (VLPs) expressing VP2, VP5, VP7 and VP3 of BTV were highly immunogenic and conferred protective immunity in sheep (Noad & Roy, 2009). However, as a number of different BTV serotypes are in circulation, a drawback to the use of recombinant proteins and VLPs is that they require the expression of subunit immunogenic proteins from each serotype. This could explain why any of the recombinant vaccines mentioned above did not reach the market yet.

Advent and latest advances of genetics and bioinformatics have made a plethora of sequence information of BTV and its different serotypes available. Utilizing such information to identify conserved protein regions of highly immunogenic VP2, VP5 should help us in developing a broad-spectrum vaccine against all the available serotypes of BTV. Drawbacks of currently available BT vaccines, BT emergence with outbreaks and the development of a BTV reverse genetic system has boosted the research field of BT vaccine development.

For different field situations, vaccine candidates with suitable vaccine profiles are available, but there is a need for further development and licensing of these vaccine candidates for many serotypes in order to be prepared for future outbreaks (Femke Feenstra and Piet A van Rijn, 2017). The development of veterinary vaccine from discovery to regulatory approval and their use in the market is an extremely costly and lengthy process. Hence, vaccines for diseases, particularly those caused by multiple distinct serotypes are an especially big challenge for the industry. It can take approximately 1 to 6 months for a vaccine manufacturer to acquire a new BTV strain from the field, and further 14 to 20 months to develop test, produce a new vaccine and take it through provisional market authorization. These are conservative figures assuming that every step runs smoothly (Sandro Filipe Nunes *et al.,* 2014).

Keeping this in mind, the current study was aimed to identify conserved regions in the VP2 (cVP2) of various BTV serotypes, and testing the broad spectrum immunogenicity potential of cVP2, VP5 and NS1 recombinant proteins. The objectives of the study are as follows.

Objectives of the investigation

- Identification of conserved epitopes in VP2.
- To clone, express and purify cVP2, VP5, and NS1 as recombinant proteins.
- To determine the humoral antibody response of cVP2, VP5, and NS1 in mice.

CHAPTER II

REVIEW OF LITERATURE

2.1 HISTORY

Bluetongue (BT) disease is thought to have long existed, though it has only been described in scientific literature since the late eighteenth century (Spruell, 1905). The first confirmed outbreak of BT outside Africa occurred in Cyprus in 1943 (Gambles, 1949). Subsequently, the disease rapidly spread all over the world, and was reported in Israel in 1949 (Komarov and Goldsmit, 1951), in California in 1952 (Alexander, 1959), in Pakistan in 1958 (Sarwar, 1962), in India in 1963 (Sapre, 1964), in China in 1979 (Zhang *et al.,* 1996), and in Australia in 1977 (St George *et al.,* 1978). Since then, outbreaks of BT have been reported from several other regions in India such as in 1973 in Himachal Pradesh (Uppal and Vasudevan, 1980), in 1975 in Haryana (Vasudevan, 1982), in Karanataka (Srinivas *et al.,* 1982), in Maharashtra (Singh *et al.,* 1982), in Rajasthan (Lonkar *et al.,* 1983), in Andhra Pradesh (Babu *et al.*,1983; Kahlon *et al.,* 1983) and in 1985 in Haryana (Jain *et al.,* 1986).

The virus attracted attention following the introduction of foreign sheep breeds to southern Africa during the years of British and Dutch colonization. Since then, BTV has made numerous incursions onto every continent except Antarctica, including North America in the 1950s (Hardy & Price, 1952) and Australia in 1975 (Ward, 1994). In the early 1970s researchers from the Onderstepoort Veterinary Institute, South Africa, showed that the BTV genome consisted of double-stranded (ds) ribonucleic acid (RNA) (Verwoerd *et al.,* 1970); a controversial observation at a time when viruses were thought to possess only dsDNA (deoxyribonucleic acid) or single-stranded (ss) DNA or RNA genomes (Palmarini, 2014).

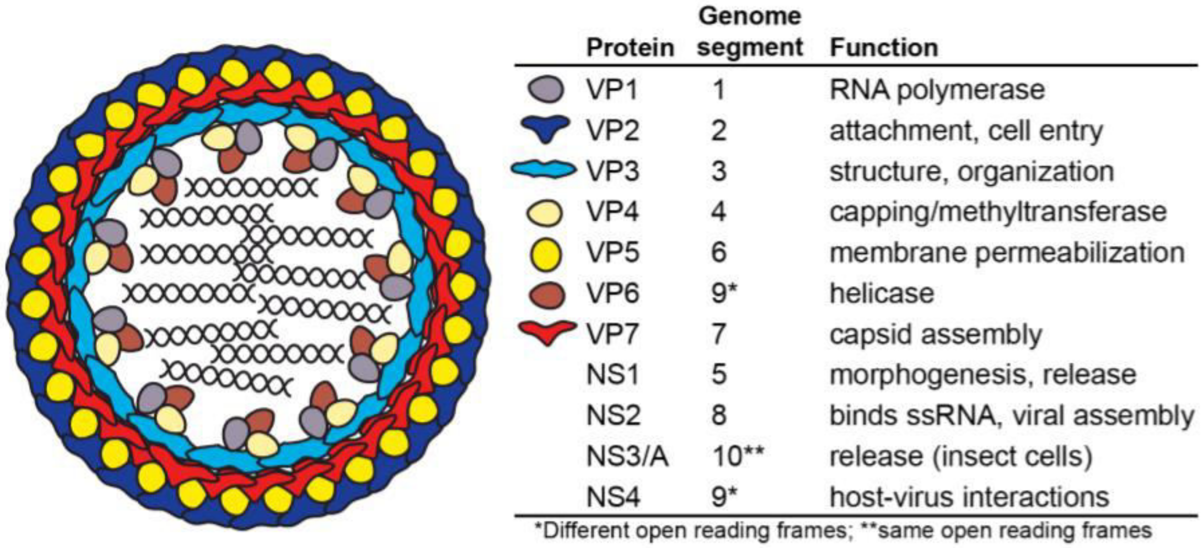
Furthermore, while trying to determine if *Culicoides* insects transmitted BTV or epizootic hemorrhagic disease virus, researchers at Onderstepoort improved the design of a light trap to better collect midges (Du Toit, 1944), thereby contributing to the field of entomology. Additionally, advances in egg-based virus attenuation, application of lyophilization for vaccine production, and understanding the concept of virus serotypes kept BTV on the cutting edge of vaccinology throughout the twentieth century (Verwoerd, 2009).

2.2 Bluetongue Virus (BTV)

2.2.1BTV classification and viral characteristics

Bluetongue virus is a non-enveloped RNA virus classified as a Group III virus of the family *Reoviridae* (International Committee on Taxonomy of Viruses, 2012). It is the type species of the genus *Orbivirus*, which includes other economically important viruses such as African horse sickness virus and epizootic hemorrhagic disease virus. Like other orbiviruses, BTV has a dsRNA genome composed of ten linear segments of different lengths, surrounded by a double-capsid icosahedral shell that is approximately 85 nm in diameter (Gouet *et al.,* 1999).

The BTV genome consists of 10 linear double-stranded RNA segments, which encode a total of seven structural proteins (VP1 to VP7) and six distinct nonstructural proteins (NS1, NS2 and NS3/ NS3a) (Table 1). The two inner layers of the BTV capsid (identified as the ‘sub-core’ and ‘core’) are composed of major structural proteins VP3 and VP7 (encoded by genome segments 3 and 7 respectively). The innermost subcore-shell surrounds the ten segments of the virus genome (one copy of each segment per particle) as well as three minor enzyme proteins VP1, VP4 and VP6 (encoded by Seg-1, 4 and 9, respectively). These core proteins and two of the non-structural proteins that are also synthesised in infected cells (NS1 and NS2 - encoded by Seg-5 and 8 respectively) are highly conserved and are antigenically cross-reactive between different strains of BTV (Verwoerd 1970; Mertens *et al.,* 1984; Mertens *et al.,* 1987a; Mertens *et al.,* 1987b; Roy 1992; Verwoerd *et al.,* 1970) (Fig. 1).



	Protein	Genome segment	Function
●	VP1	1	RNA polymerase
▼	VP2	2	attachment, cell entry
▬	VP3	3	structure, organization
●	VP4	4	capping/methyltransferase
●	VP5	6	membrane permeabilization
●	VP6	9*	helicase
▼	VP7	7	capsid assembly
	NS1	5	morphogenesis, release
	NS2	8	binds ssRNA, viral assembly
	NS3/A	10**	release (insect cells)
	NS4	9*	host-virus interactions

*Different open reading frames; **same open reading frames

Fig. 1: BTV structure (adapted from Gouet *et al.,* 1999)

Schematic illustration of the BTV virion, including VP1-7 surrounding 10 dsRNA genome segments. The genome segment that encodes for each protein is indicated in the chart, as are the primary functions of the individual proteins.

The outer capsid is composed of major proteins VP2 and VP5 which is laid onto the foundation provided by the core. The three minor proteins (VP1, VP4 and VP6) are enclosed by the sub-core that is made up of VP3. The core-surface layer consists of VP7, both VP3 and VP7 are major proteins (Mertens and Diprose, 2004; Mertens *et al.,* 2004; Roy, 1992; Roy, 2004). The minor proteins together with genomic RNA form the virus replication complex, whereas the four major proteins make up the capsid of the virus. In addition to the structural proteins BTV has six non-structural (NS) proteins which are involved in virus replication and assembly in BTV-infected cells (Roy, 2004)

2.2.2 Three-dimensional (3-D) structure of BTV

The 3-D structure of BTV cores and virions has been determined by cryoelectron microscopy (Cryo-EM) and reconstructed using computational methods. Cryo-EM of the BTV core showed that it has a diameter of 69 nm, with the surface exhibiting icosahedral symmetry (Grimes *et al.,* 1997). Figure 2B and 2C show that the core is divided into two layers (VP3 and VP7) that enclose the ten dsRNA segments and the proteins that are responsible for replication. A total of 120 molecules of VP3 (Figure 2B) provide a smooth scaffold for the VP7 trimers to associate with (Figure 2C). The surface of the core consists of clusters of VP7 trimers that have knob-like protrusions organised into pentameric and hexameric units with channels between them. In total there are 780 VP7 molecules per particle. The layer formed by VP7 is essential for the deposition of VP2 and VP5, which constitute the outer capsid (Roy, 2004).

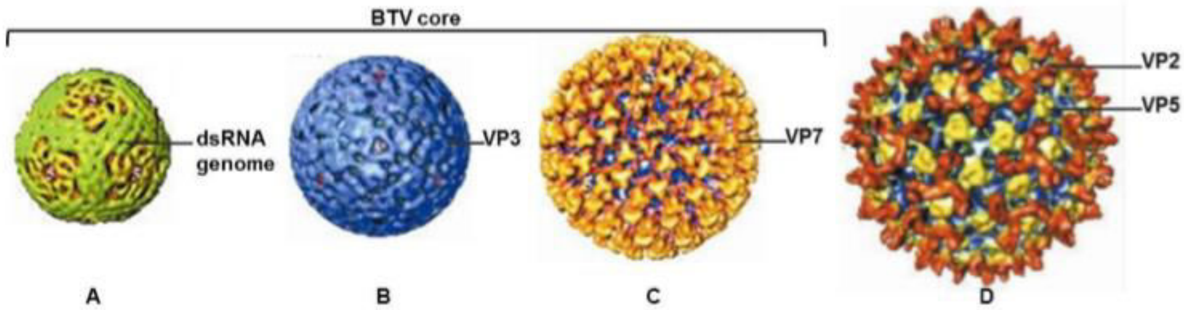


Fig. 2: A surface representation of a cryoelectron micrograph of BTV (adapted from Roy 2004)

(A) The RNA genome and the replication complex that is formed by VP1, VP4 and VP6, (B) the smooth inner layer formed by 120 molecules of VP3 and (C) protrusions of the 260 VP7 trimers in the outer layer. Together A, B and C form the BTV core. (D) The topography of the globular-shaped VP5 and the sail-shaped VP2 displayed on a whole virus particle.

Analysis of cryoelectron micrographs of BTV virions show that the two outer capsid proteins, VP2 and VP5 (Fig. 2), attach in an orderly fashion to the foundation provided by the surface (VP7) of the core (Roy, 2004). The capsid is icosahedral and the two outer capsid proteins have very distinctive morphologies. VP5 is globular and 120 of VP5 molecules sit in the channels that are formed by the VP7 trimers. The sail-shaped VP2 spikes are located above 180 of the VP7 trimers and form triskelion-type motifs that cover most of the VP7 molecules. The VP2 spikes protrude 4 nm above the globular proteins and contain the virus neutralising epitopes. VP2 and VP5 form a continuous layer around the core, except for holes on the five-fold axis (Hewat *et al.,* 1992a; Hewat *et al.,* 1992b;Roy,92004).

Table 1. Details of BTV genome segments, the encoded proteins and their characteristics and functions

Genome segment	Segment Size (bp)	Proteins (encoded)	Protein length (aa)	Protein molecular weight (kDa)	Estimated subunits per virion	Location in the virion	Characteristics/functions
Seg-1	3954	VP1(Pol)	1302	150.6	10-12	Transcriptase complex (TC) within the sub-core	RNA-dependent RNA polymerase (Pol; transcriptase and replicase)
Seg-2	2926	VP2(OC1)	956	111.1	180 (60 trimers)	Outer shell	Cell attachment, entry, haemagglutination, virus neutralization antigen, determines serotype
Seg-3	2770	VP3(T2)	901	103.3	120 (12 decamers)	Sub-core capsid shell	T=2 icosahedral symmetry, scaffold for addition of core surface layer, outer coat proteins, localizes TCs on internal surface of the capsid, at 5-fold axes
Seg-4	1981	VP4(CaP)	644	76.4	5–24	TC within the sub-core	Capping enzyme (CaP) – nucleoside phosphohydrolase, guanylyltransferase, transmethylase, covalently binds GMP from GTP
Seg-5	1769	NS1(TuP)	552	64.4	NA	Nonstructural	Abundant in infected cell cytoplasm, Forms tubules (TuP), with unknown function, aids virus release from insect cells, a viral protein translation enhancer, co-localizes with the centrosome – may play a role in disruption and blocking of cell division in mammalian cells
Seg-6	1638	VP5(OC2)	526	59.1	360	Outer capsids	Has coiled coil domain – induces membrane permeabilization during initiation of infection and can cause syncytia formation, affects specificity of virus neutralization
Seg-	1156	VP7(T13)	349	38.5	780 (260 trimers)	Core surface	Core structural protein, T=13 icosahedral symmetry, receptor binding for *Culicoides* cells,group-/virus-species-specific antigen.
Seg-8	1124	NS2(ViP)	357	41.0	NA	Nonstructural	Forms inclusion bodies (ViP), aids in early morphogenesis, binds mRNA, phosphorylated by protein kinase 1, blocks spindle formation and cell division.

Genome segment	**Segment Size (bp)**	**Proteins (encoded)**	**Protein length (aa)**	**Protein molecular weight (kDa)**	**Estimated subunits per virion**	**Location in the virion**	**Characteristics/functions**
Seg-9	1046	VP6(Hel)	329	35.7	37/72	Inner core	Helicase (Hel) binds ss/dsRNA, ATPase
		NS4	78	17	NA	Non structural	Imparts viral fitness to interferon (IFN) response
Seg-10	822	NS3	229	25.6	NA	Non structural	Glycoprotein, binds cellular exocytotic components, Aids virus release, Contains coiled coil domain, Localizes to cell membrane late in infection – interacts with lipid droplets in infected cell
		NS3A	216	24.0	NA	Non structural	Glycoprotein binds cellular exocytotic components Viroporin – aids virus release from insect cells
		NS5	50-59	7.6	NA	Non structural	Localizes to nucleus IFN antagonist?

Table adapted from (Rao *et al.,* 2017).

2.3 Epidemiology and transmission

2.3.1 BTV Serotypes, Topotypes and Nucleotypes

BTV epidemiology is described by the geographical distribution of different BTV serotypes as well as the presence of potential hosts and vectors. The virus neutralizing ability of antibodies that are produced against them determines the BTV serotype. This serotype-specificity is attributed to the outer capsid protein VP2 (Huismans & Erasmus, 1981; Kahlon *et al.,* 1983; Roy *et al.,* 1990; Mertens *et al.,* 2007; Maan *et al.,* 2011) and to some extent VP5 (Roy *et al.,* 1990).

So far, 29 serotypes (Maan *et al.,* 2016 & Kartika Lakshmi *et al.,* 2018) of BTV have been identified worldwide and a total of 24 serotypes have been reported from India (Krishnajyothi *et al.,* 2016 & Hemadri *et al.,* 2017). Variations in the sequence of Seg-2 and of its translated protein VP2 (outer capsid protein-1) [VP2 (OC1) correlate with BTV serotype. Although the sequence of Seg-6 and its product VP5 (outer capsid protein-2) [VP5 (OC2)] can also show a partial correlation with serotype, large variations are sometimes detected in different isolates of the same serotype, and isolates of different serotypes may contain Seg-6/VP5 (OC2) with almost identical sequences (Maan *et al.,* 2009). Identifying BTV serotypes, and analysing their appearance and reappearance, provides important epidemiological data and is essential for the design and implementation of effective control and prevention strategies for BT, particularly vaccine matching (Rao *et al.,* 2017).

The BTV serotypes can be further divided into topotypes by genetic analysis of certain RNA genome segments, such as segment 3 (encoding VP3) or segment 10 (encoding NS3). These topotypes indicate regional differences among serotypes and include "western" (the Americas, Africa, Europe) or "eastern" (Asia, Australia) genetic variations (Gould & Pritchard, 1990; Bonneau *et al.,* 1999; Balasuriya *et al.,* 2008), though it has been suggested that there are likely other topotypes corresponding to additional geographical lineages as well (Maan *et al.,* 2012). The major eastern group includes isolates from Australasia, the Middle East and the Mediterranean, while the western group includes viruses from Africa, the Mediterranean and the Americas. In addition, there is evidence for several further diverse groups including the recently discovered BTV-25 (SWI2008/01), BTV-26 (KUW2010/02) and BTV-27. The nucleotide/amino acid identity levels are shown in Fig. 3.

The eastern topotypes for Seg-2 and Seg-6 (the serotype-determining segments) of BTV-1, BTV-2, BTV-3, BTV-4, BTV-9 and BTV-16 have 25–30 % nucleotide sequence variation with their western topotype counterparts. Similar levels of sequence variation have also been observed for all the non-serotype-determining segments (Tan *et al.,* 2001; Celma and Roy 2009). The amino acid sequence alignment showed that although VP2 was very variable among serotypes, several regions were relatively more conserved between serotypes; most conserved region (cVP2) of which was evident from 338-383aa (Shiva jyothi *et al.,* 2018).

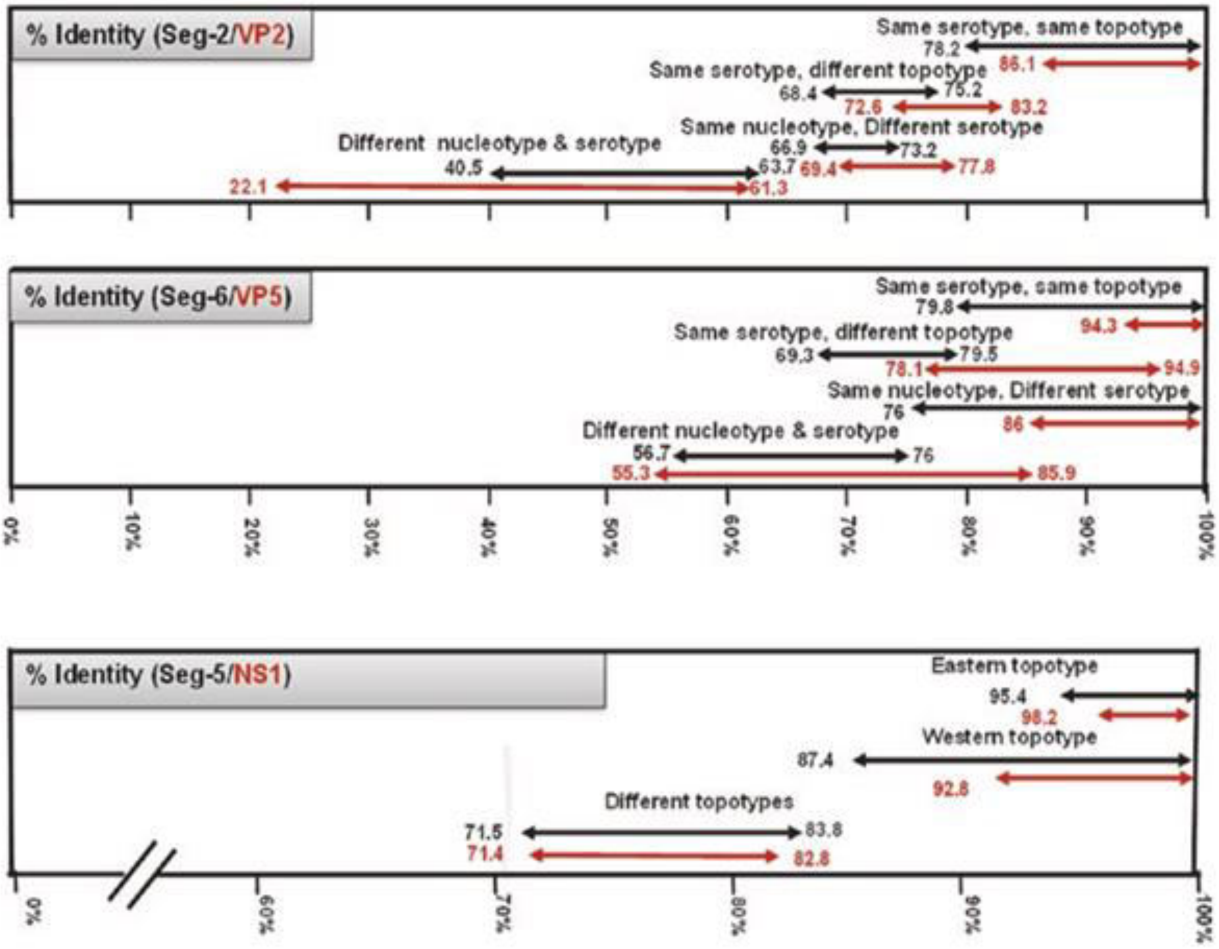


Fig. 3: Sequence identities in Seg-2/VP2 (OC1), Seg-6/VP5 (OC2) and Seg-5/NS1(TuP) within and between BTV serotypes and topotypes (adapted from Rao *et al.,* 2017)

The levels of sequence identity detected within the major eastern and western topotypes are given for Seg-2, Seg-5 and Seg-6 (in black) and proteins VP2 (OC1), VP5 (OC2) and NS1(ViP) (in red).

The presence of the same BTV serotypes within different regions (topotypes) suggests that these serotypes initially emerged from a common ancestor before spreading out to different regions, gradually acquiring point mutations through successive rounds of virus replication in the vertebrate and invertebrate hosts, as well as adaptation to local ecosystems (and vector populations) over long periods of separation. The processes of random mutation, genome segment reassortment and selection may have contributed to the divergence of BTV into topotypes in most of the BTV genome segments. However, new strains of BTV, representing exotic topotypes or serotypes, have occasionally entered naïve or endemic areas via natural or anthropogenic routes, potentially changing the genome segment 'mix' and local genotype, and are frequently associated with severe clinical disease outbreaks (Nomikou *et al.,* 2015).

By considering the major topotypes separately, it is possible to set levels of sequence variation that may be used as a guide to identify different BTV serotypes. Overall, BTV strains within the same serotype can show up to 31.6 % nucleotide (nt) and 27.4 % amino acid (aa) variation in Seg-2 and VP2(OC1) (Maan *et al.,* 2009). Viruses belonging to different serotypes can show up to 26.8 % nt and 22.2 % aa identity, making the delineation of clear limits for identification of distinct serotypes difficult. However, the maximum level of variation in Seg-2/VP2(OC1) for isolates of the same serotype and the same major topotype drops to a 21.8 % nt and 13.9 % aa sequences, respectively, 5.0 % and 8.3 % lower than the minimum levels of nucleotide and amino acid variation detected between different BTV serotypes. These levels, based on Seg-2 sequences, can therefore be used as a guide for the identification of existing serotypes, regardless of their geographic origin. If a novel isolate falls into the 'gap' [between 26.8 % and 21.8 % nt and

22.2 % and13.9 % aa variation in Seg-2/VP2(OC1) compared to known BTV isolates], it could represent either an entirely novel serotype that is related to but distinct from other established serotypes or an isolate of an existing serotype but belonging to a different and distinct 'major' topotype (Rao *et al.,* 2017). In either case, additional serological analyses would be required. Such 'new viruses' would then become 'reference strains' for the new serotype or the topotype. It is therefore important to accurately identify and curate reference strains.

2.3.2 Phylogenetic relationships of the orbivirus proteins

In BTV, AHSV or EHDV, it is the larger of the two outer capsid proteins (VP2 – encoded by genome segment 2) that mediates cell attachment and is the major target for neutralizing antibodies (Mertens *et al.,* 2005). VP2 shows up to 27% aa sequence variation within a single serotype, while different BTV types can show as much as 73% variation in the sequence of this protein (Maan *et al.,* 2007). Variations in the aa sequence of VP2 can also reflect the serological relationships (low-level cross-reactions) that exist between different serotypes (Erasmus, 1990; Anthony, 2007) (Figures 3.2 and 3.3). The smaller orbivirus outer-coat protein (VP5) can also influence virus serotype, and variations in VP5 of BTV show a partial correlation with virus type (Maan *et al.,* 2008).

The variations that have been detected in VP2/Seg-2 and VP5/Seg-6 within individual serotypes indicate that the different BTV types emerged as an early step in the evolution of the virus species. Strains of each serotype subsequently became geographically dispersed and then acquired further point mutations that distinguish the different topotypes of each serotype. Reverse transcription polymerase chain reaction assays and sequence analyses of BTV Seg2/VP2 provide rapid and reliable diagnostic methods to determine both the serotype and the origin of individual isolates (molecular epidemiology) (Mertens *et al.,* 2007; Maan *et al.,* 2008). These molecular assays are currently replacing serological methods for BTV-type identification within reference laboratories although viral neutralization test (VNT) and serum neutralization test (SNT) remain the gold standard for identification of virus serotype (Mertens *et al.,* 2008).

The Seg-2/VP2 sequences of the reference strains of different BTV serotypes cluster as 10 distinct evolutionary lineages, identified as nucleotypes A–J (Fig. 4). The grouping of the different BTV types within the 10 nucleotypes also reflects the serological relationships (low-level cross-reactions) between different BTV serotypes (Fig. 5).

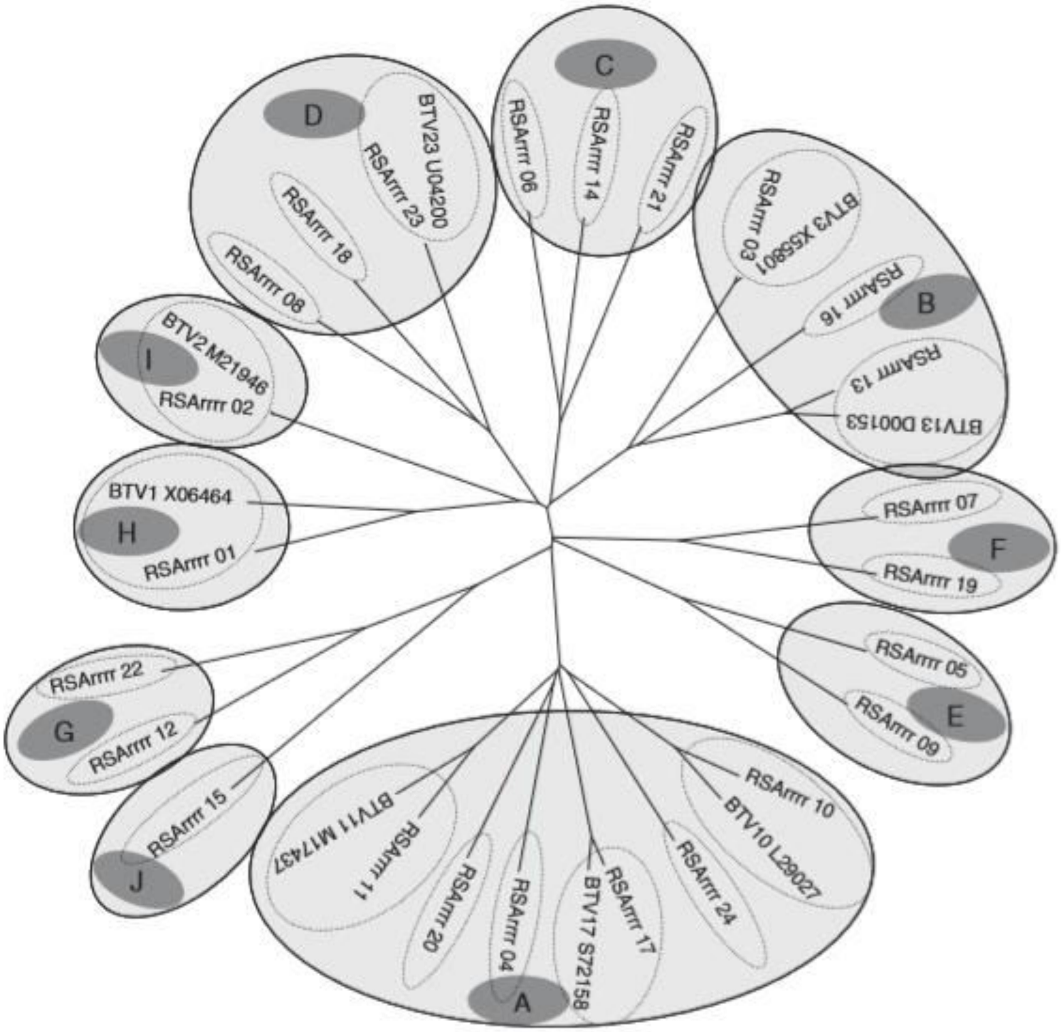


Fig. 4: Phylogenetic relationships of bluetongue virus (BTV) outer capsid protein VP2 (adapted from Rao *et al.,* 2017)

Unrooted neighbour-joining tree showing the relationships between deduced amino acid (aa) sequences of VP2 (encoded by genome segment 2) from the 24 BTV serotypes. This neighbour-joining tree was constructed using MEGA program, version 3.1 and using the p-distance algorithm and the full-length VP2 sequences of the 24 BTV types. The different serotypes (indicated by dotted circles) are distinct but show some relationships (grey bubbles) that mirror the serological relatedness (cross-reactions) that are known to exist between different serotypes. These groupings are reflected in the nucleotide sequences of genome segment 2 and 10. Distinct groups have previously been identified as nucleotypes A–J.

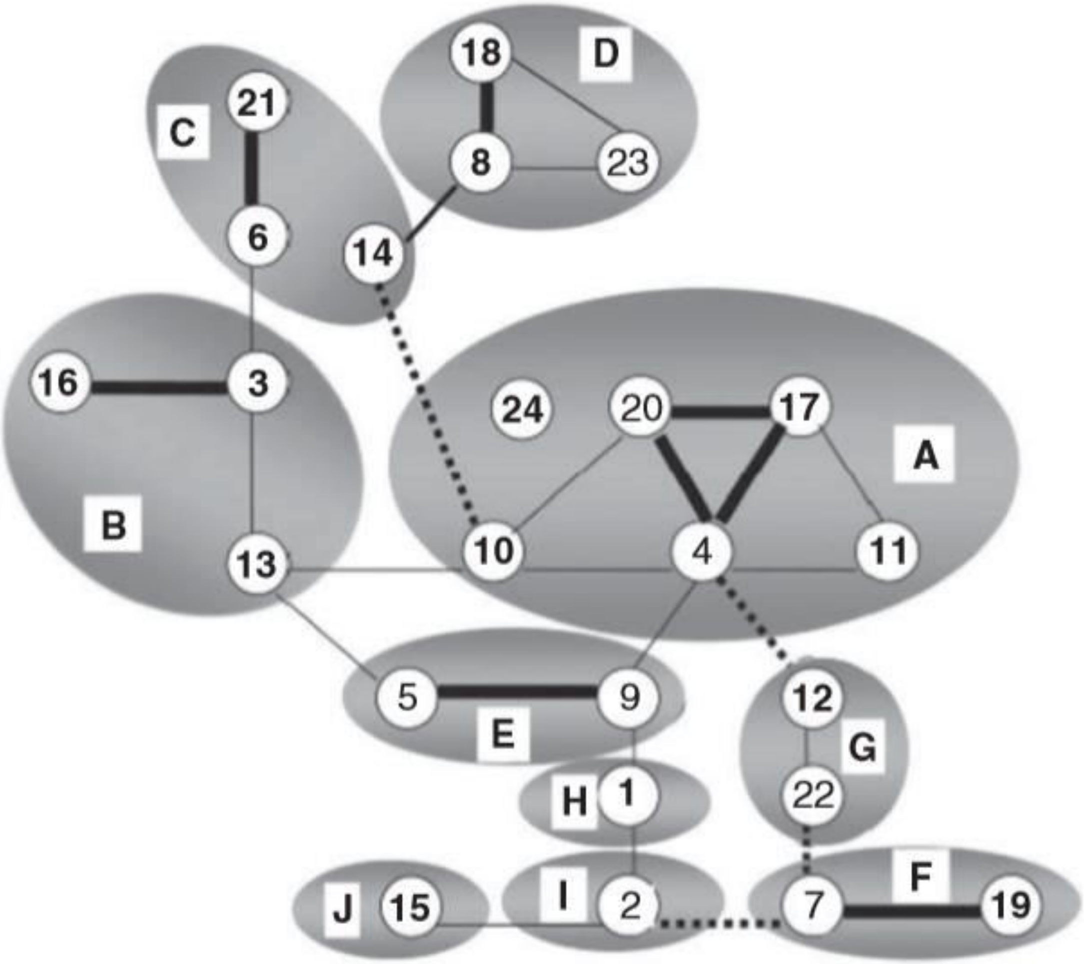


Fig.5: Schematic illustrating the serological relationships between bluetongue virus (BTV) serotypes [adapted from Erasmus (1990)]

The thicker lines shown between serotypes (represented by appropriate numbers) indicate stronger serological relationships, as detected in plaque reduction assays. The thinner lines represent relationships that are evident only as cross- or heterotypic antibody responses in cross-protection assays. The black dotted lines represent inter-relationships that are very weak. The different BTV nucleotypes (A–J) that were identified by phylogenetic analyses are represented by grey ovals. Serotype 24 is phylogenetically related to serotypes 4, 10, 11, 17 and 20, but the serological data concerning relationships between this and other types are limited. Therefore it is included it within the oval shown for nucleotype ‘A’ but without a line connecting it to other serotypes.

The open reading frame (ORF) and non-coding region (NCRs) of Seg-2, from the different serotypes within each nucleotype of BTV, are uniform in length. However, Seg-2 can vary in length between different BTV serotypes (Maan *et al.,* 2007). These variations appear to be the result of insertion and deletion events, some of which are unique to individual types, suggesting that at least in some cases they may have played a role in the emergence of the different nucleotypes and serotypes. For example, BTV-8 shows an insertion of 12 nt at position 122–134 when aligned to Seg-2 of the reference strain of BTV-1 (RSArrrr/01 – position 112–113) and an insertion of 6 nt at position 830–835 when aligned with Seg-2 sequence of BTV-19 (position 773–774 with reference to RSArrrr/01). Seg-2 of types 12, 15 and 22 contains several longer insertions (e.g. at nt 360–369) as well as deletions (between positions 1888 and 1926) in their aligned sequences compared to Seg-2 of other serotypes. This reflects the relatively close genetic relationship detected between types 12 and 22 (nucleotype G) and type 15 (nucleotype J) even though they are classified within separate nucleotypes. There are also several areas of Seg-2 from the 24 serotypes where no insertions or deletions are evident (e.g. nt 370–424, 900–1250, 1580–1850 in the aligned sequences).

Seg-2 encodes the VP2 protein, which ranges in size from 950 aa (2852 bp ORF) in BTV-12 to 962 aa (2888 bp ORF) in BTV-19. VP2 shows between 22.7% (BTV-4 and BTV-20) and 72.9% (BTV-6 and BTV-22) aa variation between BTV types. Only the carboxy-terminus (aa 946–961) and residues at positions 338–379 appeared to be relatively conserved. The amino-terminus of VP2 is one of the least conserved regions (Maan *et al.,* 2007). Comparisons of VP2s from the members of different *Orbivirus* species [(AHSV, EHDV, Broadhaven virus (BRDV), SCRV and Chuzan virus)] showed only low levels of aa sequence identity. Phylogenetic analyses confirmed that Seg-2s/VP2s of these orbiviruses exist as distinct monophyletic groups, despite their overlapping global distributions. This provides further evidence that the members of distinct *Orbivirus* species do not exchange genetic information by reassortment. It also confirms previous serological and sequencing studies, which indicate that BTVs are more closely related to EHDVs than to AHSVs or Palyam viruses (PALVs) (Maan, 2004; Anthony, 2007).

2.3.3 BTV Episystems, Climatic Zones, 'Source' and 'Sink' Populations

It has been suggested that the distribution of different BTV serotypes in different geographical areas may be linked to the *Culicoides* species inhabiting those areas (episystems) (Tabachnick 2004). Rather, the presence and spread of BTV into different climatic regions fits well into 'source' and 'sink' population dynamics. Tropical regions, with large vertebrate and invertebrate populations and conducive climate, support the circulation of multiple BTV serotypes throughout the year, acting as virus 'source' areas. In contrast, in temperate areas, BTV is often not sustained over long periods due to increasing herd immunity over time, and the massive reduction in adult vector populations during winters, favouring seasonal incursions caused by one or a few serotypes (Sellers 1980; Rao *et al.,* 2016).

Phylogenetic and evolutionary analyses show that the seasonal outbreaks in these virus 'sink areas', which may be interspersed with disease-free periods, reflect novel virus introductions from 'source areas' (Carpi *et al.,* 2010; Nomikou *et al.,* 2015). The extent of source and sink areas depends on the availability of susceptible vertebrate host populations and competent vectors. South and Central America, North Africa and the Mediterranean and southeast Asia represent source areas, while most parts of North America, northern

Europe and parts of Australia represent the associated sink areas, respectively (Sellers and Maarouf 1989; Mellor and Wittmann 2002; Johnson *et al.,* 2007; Purse *et al.,* 2008; Daniels *et al.,* 2009; Boyce *et al.,* 2012).

Combining source and sink systems with climatic zones, Rao *et al.,* 2017 proposed the division of tropical areas into four episystems: sub-Saharan Africa, the Mediterranean, Australasia and the Americas. Each episystem contained its own source and sink areas overlapping with climatic zones (Fig. 6). Evidence exists for sharing and circulation of virus populations within these proposed episystems, and more specifically, each source and sink area within each episystem has closely related populations of circulating viruses (Wilson *et al.,* 2000; Pritchard *et al.,* 2004; Potgieter *et al.,* 2005; Breard *et al.,* 2007; Johnson *et al.,* 2007; MacLachlan *et al.,* 2007; Balasuriya *et al.,* 2008; Daniels *et al.,* 2009; Boyce *et al.,* 2012; Legisa *et al.,* 2013; Viarouge *et al.,* 2014; Maan *et al.,* 2015; Nomikou *et al.,* 2015; Rao & Hegde 2015).

The *Culicoides* species and populations in different regions may have different vector competencies and capacity for different BTV strains, serotypes and topotypes (MacLachlan *et al.,* 2007; Purse *et al.,* 2008; Mellor *et al.,* 2009), and this may redefine source and sink areas within an episystem.

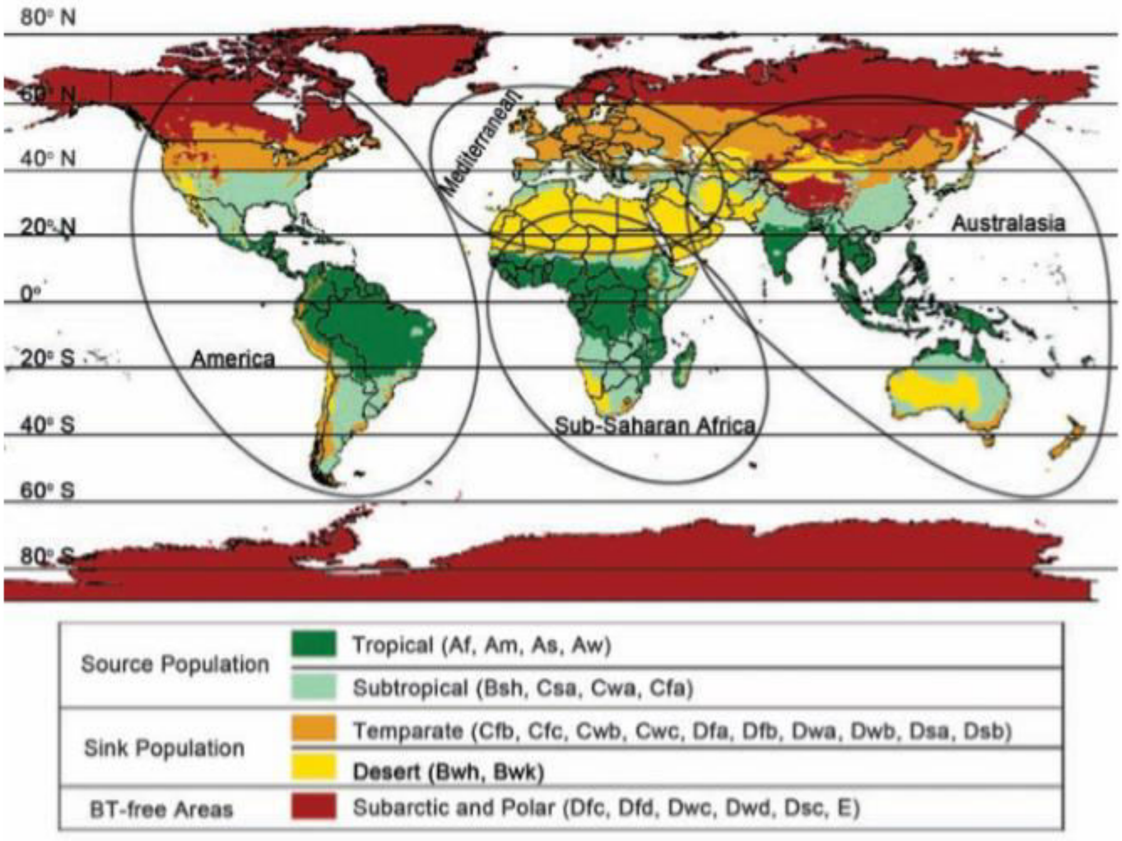


Fig. 6: Climatic zones and proposed BT episystems. (adapted from Rao *et al.,* 2017)

Source areas include tropical (dark green) and subtropical areas including the Mediterranean (light green), which are most congenial for *Culicoides* propagation. Sink areas include hot and cold deserts (yellow) and temperate areas (orange), where only part of the year is suitable for the sustenance of *Culicoides* populations. Subarctic and polar regions (red) are considered as BTV-free zones, where harsh weather hampers the survival of *Culicoides*. Four distinct episystems (sub-Saharan Africa, the Mediterranean, the Americas and Australasia) are shown with each system having sink and source areas. The boundaries of both climatic zones and episystems are indicative and not definitive and may overlap.

2.3.4 Host and vector species

All ruminants are potential hosts of BTV, though species and breed, among other factors, can play a role in whether BTV infection manifests as clinical disease. BTV is typically transmitted among susceptible hosts through the bite of a competent *Culicoides* midge. Although there are over 1400 recognized species of *Culicoides*, only about 30 species are known to be competent for transmitting BTV (Aiello & Moses, 2012). Traditionally, *C. imicola* is considered to be the most important species for transmitting the virus in Europe, Africa, and the Middle East (Mellor, 2004), *C. sonorensis* (formerly referred to as *C. variipennis*) in North America (Tabachnick, 1996), and *C. insignis* in Central America (Mo *et al.,* 1994).

2.3.5 Routes of transmission

Since BTV is primarily considered a vector-borne virus, its epidemiology is strongly linked to the presence of competent vectors. However, in some cases, vertical (transplacental) transmission has been implicated for certain strains or serotypes (Luedke *et al.,* 1977; Wouda *et al.,* 2008; Saegerman *et al.,* 2011), and there are even recent reports of direct contact transmission of some BTV serotypes in goats and cattle (Rasmussen *et al.,* 2013; Batten *et al.,* 2014).

Only female *Culicoides* midges are hematophagous (Mellor *et al.,* 2000) and a single bite of an infected midge is sufficient to infect a susceptible sheep (Foster *et al.,* 1968). Conversely, the quantity of virus in the host blood considered necessary to infect a competent *Culicoides* midge is relatively low, at approximately 2.5-3 log10 $TCID_{50}$ (50% tissue culture infective dose) per milliliter (Fu *et al.,* 1999; Savini *et al.,* 2008). Based on studies primarily performed in the United Kingdom and United States using *C. sonorensis*, the mechanisms of BTV infection and replication within its vector have been well elucidated (Fig. 7.). Briefly, the transmission of BTV to a susceptible ruminant begins with the bite of a competent female midge. The presence of trypsin-like proteins in the saliva of competent midges may aid the infectivity of BTV in insects, likely by cleaving VP2 from the virion to generate virus subparticles that have been shown to be up to ten times more infectious to *Culicoides*-derived, but not mammalian, cells (Darpel *et al.,* 2011).

The importance of these infectious virus subparticles in BTV transmission is unknown but they appear to facilitate virus entry into insect cells and may be linked to variations in the competencies of different *Culicoides* species (Mertens *et al.,* 1996). The virus replicates first in the midgut cells (day 0), then in the fat body cells (day 1) and neural tissues (day 3) (Fu *et al.,* 1999). During these first three days following ingestion of the virus, an eclipse or partial eclipse phase occurs, such that either no BTV in the former case, or some BTV in the latter case, are detectable within the insect vector (Mellor *et al.,* 2009). It is thought that this phase occurs because the virus is being inactivated in the insect's gut lumen, or perhaps due to virus excretion from the gut cells before proliferation is evident (Mellor *et al.,* 2009). By day 5, BTV can be detected in the salivary glands of the infected insect, where the virus replicates to approximately 1000 to 10000 times its day 0 titers and plateaus at these levels for the remainder of the insect's life (Foster & Jones, 1979).

Researchers have speculated that these final BTV titers may be limited by either the number of susceptible cells within a midge, or by a virus-vector balance that modulates the negative impact of virus replication on the vector (Mellor *et al.,* 2009). The lifespan of a

Culicoides midge is generally only 20 days but under consistently mild ambient conditions, it can approach 90 days in the field (Mellor *et al.,* 2000) or laboratory (Goffredo *et al.,* 2004).

Following the first infectious bite from a female *Culicoides* midge, BTV transmission from ruminant host to vector is possible beginning between one and two weeks (Fig.7.). Since females feed multiple times over their lifespan at three-to-four-day intervals, virus transmission may occur already at the third feeding (Mehlhorn *et al.,* 2007). It has been shown under both field and laboratory conditions that different midge populations demonstrate varying susceptibilities to different BTV serotypes, as well as to the same BTV serotype (Jones & Foster, 1978; Jennings & Mellor, 1987), at least partly due to species-specific saliva proteins.

Furthermore, ambient temperature has also been shown to play a role in transmission, with transmission likelihood based on the balance between high temperatures that decrease vector lifespan but encourage an increase in vector bites, and conversely, low temperatures that increase vector lifespan yet result in a decrease of virus replication (Mellor *et al.,* 2009). For example, a study of experimental infection of competent South African *Culicoides* species demonstrated that in *C. bolitinos* held at 25°C and 15°C, titers of BTV-1 reached transmission potential (defined as ≥3 log10 TCID50 per midge) two and eight days following infection, respectively, while in *C. imicola* held at 30°C and 23.5°C, BTV-1 titers reached this transmission potential four and ten days following experimental infection (Paweska *et al.,* 2002). At temperatures under 10-15°C, BTV replication is considered to cease entirely (Mullens *et al.,* 1995; Paweska *et al.,* 2002; Carpenter *et al.,* 2011), but like African horse sickness virus, BTV appears to persist for at least three weeks in surviving infected insects and can resume replication with warmer temperatures (Mullens *et al.,* 1995; Wellby *et al.,* 1996).

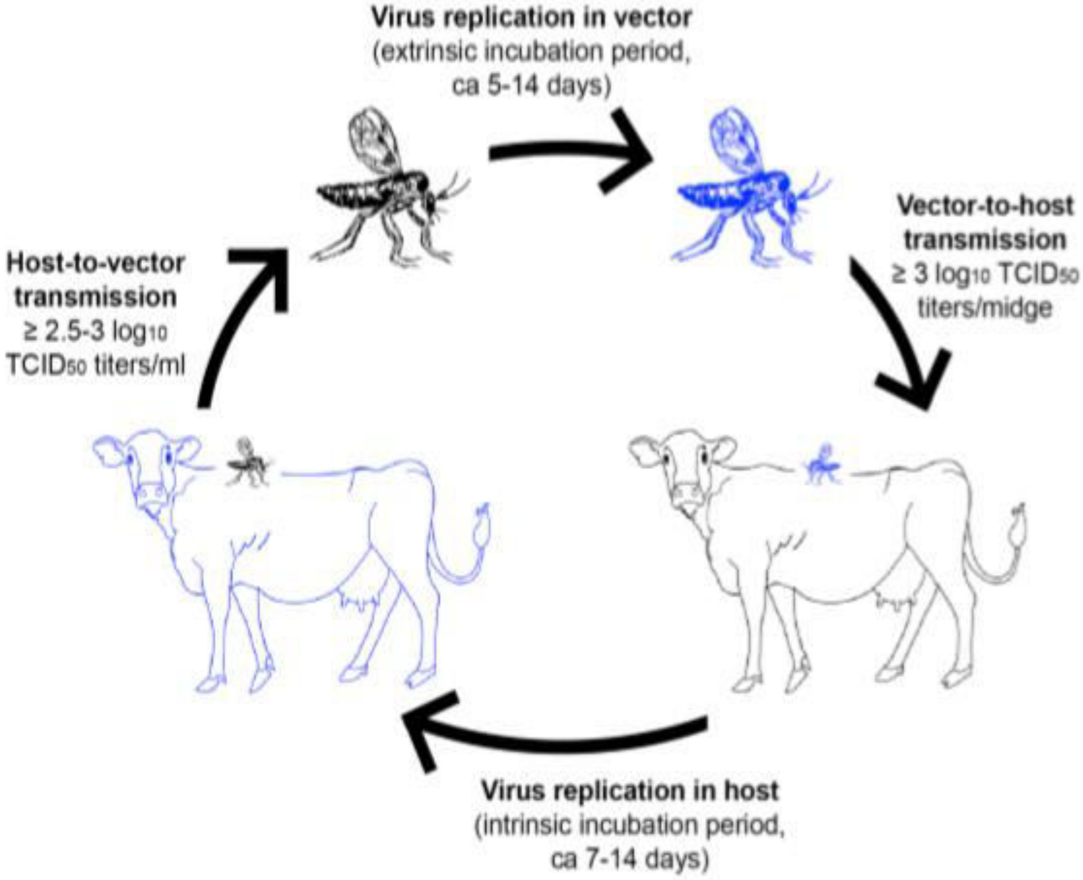


Fig.7: BTV vector-borne transmission cycle (adapted from (Purse *et al.,* 2005; Wilson *et al.,* 2009))

On rare occasions some strains of BTV can be transmitted by secondary mechanisms, such as oral or transplacental transmission within ruminant populations (Fig. 8.), however these mechanisms are not important in the long-term survival and spread of the virus (Mellor *et al.,* 2009; Mellor and Wittmann, 2002; Wilson *et al.*, 2009).

In regions of the world where enzootic BTV infection occurs throughout the year, BT is uncommon. This can be attributed to population immunity or it can be a result of co-evolution of the animal host and the virus (Huang *et al.,* 2009).

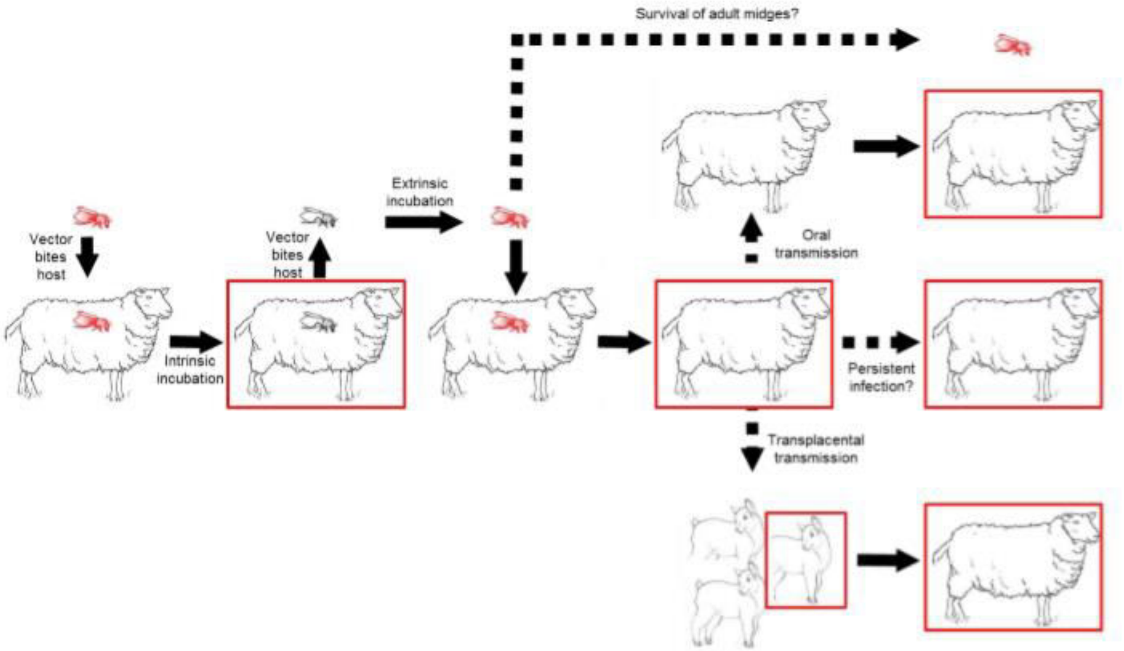


Fig. 8: Transmission routes of BTV (adapted from Wilson *et al.,* 2009)

Possible secondary transmission routes (oral and transplacental) are also indicated.

2.4 Replication

BTV can replicate in both wild and domestic ruminants. Replication takes place in both the host and the *Culicoides* insect vector (Roy *et al.,* 2009). Positive sense single stranded (ss) RNA copies that act as messenger RNAs are transcribed by VP1 in the BTV core from each of the ten genome segments (Boyce *et al.,* 2004). The RNA molecules are capped by the guanylyl-transferase and transmethylase activities of VP4 and exit the particles *via* channels that are situated at the five-fold axes of the core particle (Mertens and Diprose, 2004). Viral RNA is directed to the VIB, where encapsidation of different segments within the VP3 shell involves interactions with VP6 (helicase), NS2 (ssRNA binding protein), VP1 and VP4. DsRNA is synthesized from the positive strand RNA by VP1 (Boyce *et al.,* 2004); each dsRNA segment associates with a different transcriptase complex (Nason *et al.,* 2004).

BTV interacts with the cell surface via the VP2 trimers and enters the cell *via* a clathrin-dependent endocytosis pathway (Forzan *et al.,* 2004; Hyatt *et al.,* 1993). The reduction of pH in the endosome induces the association of VP5 with the endosomal membrane and the delivery of the transcriptionally active core into the cytoplasm (Forzan *et al.,* 2004; Mertens *et al.,* 2004). The transcriptase function of the core particle is activated by the removal of VP2 and VP5 during cell entry (Mertens and Diprose, 2004).

The fragile VP3 sub-cores act as a scaffold for the addition of VP7 trimers, thereby forming stable cores (Grimes *et al.,* 1997; Tanaka and Roy, 1994). VP2 and VP5 are added to the core surface at the periphery of the VIB upon entry into the host cytoplasm. The mature particles are transported within the cytoplasm on microtubules that involve the interaction of VP2 with vimentin (Bhattacharya *et al.,* 2007). The virions are released from infected cells when NS3 mediates the destabilisation of the cell membrane; budding and cell death/lysis also play a role in release of the virions (Mertens *et al.,* 2004) (Fig. 9).

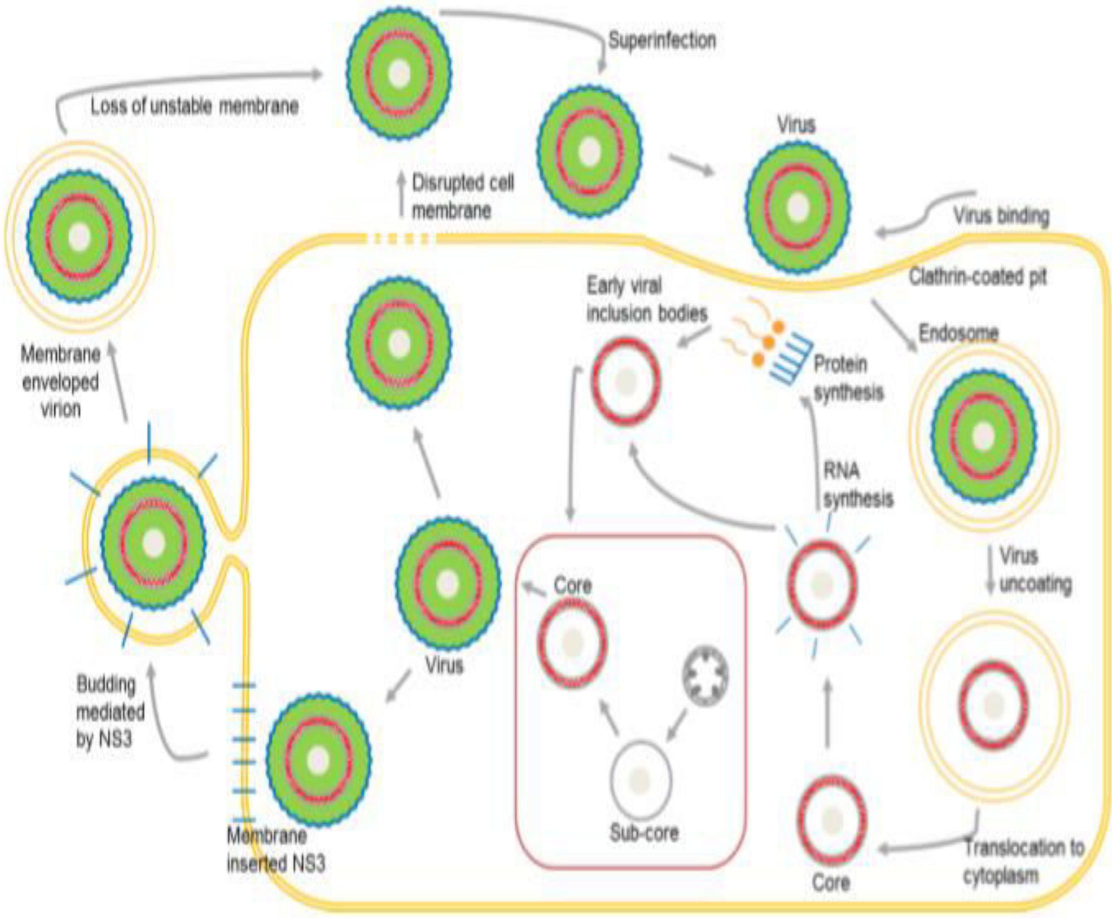


Fig. 9: A schematic presentation of the lytic replication cycle of BTV (adapted from Mertens *et al.,* 2004)

2.5 Clinical signs

BTV can infect all known species of domestic and wild ruminants. Severe disease usually occurs in the fine-wool and mutton breeds of sheep as well as some species of deer (Mellor and Boorman, 1995). BTV infection of cattle, goats and wild ruminant species is mostly asymptomatic or subclinical. In BTV endemic areas, BTV-infected sheep develop only mild or no obvious disease (MacLachlan, 2004). The blue tongue after which the disease is named is seen only in serious clinical cases (Wilson *et al.,* 2009).

Disease in sheep is typically characterised by high fever lasting 5 - 7 days (Roy, 2004). Clinical signs of disease can include fever, depression, excessive salivation, nasal discharge, facial oedema, hyperaemia and ulceration of the oral mucosa, coronitis, lameness and death (Darpel *et al.,* 2007; Mellor and Boorman, 1995). Abortion can occur in pregnant animals, and calves can be born with teratogenic defects (Roy, 2004). The severity of clinical disease and mortality rate is influenced by the breed and age of the animal as well as the virus strain that causes the infection (Mellor and Wittmann, 2002). In acute phases of BT, clinical signs in sheep are mainly associated with damage to microvascular endothelial cells (Singer *et al.,* 2001).

Those animals that do recover from BT may suffer from a number of long-lasting secondary effects, such as reduction in milk production, weight gain, wool break and temporary infertility (Wilson *et al.,* 2009).

2.6 Protein-specific humoral immune responses

BTV infection is marked by species and breed differences in clinical signs and immune responses (Neitz, 1948). VP2 induces virus neutralizing antibody responses in mice and ruminants (Huismans & Erasmus, 1981; Inumaru & Roy, 1987; Roy *et al.,* 1990). Since BTV serotype is determined using virus neutralization assays, it is considered to be the serotype-determining protein (Huismans & Erasmus, 1981). Neutralizing antibodies are detected approximately two weeks after natural infection and can persist for up to at least four to six years (Eschbaumer *et al.,* 2012). Presence of neutralizing antibodies is highly correlated with protection (Jeggo *et al.,* 1984b; Roy *et al.,* 1990; Oura *et al.,* 2009), but no precise minimum protective titer is defined, presumably because cell-mediated immune responses also play an important role. However, it has been indicated that BTV-8 neutralizing antibody titers of at least 1-1.5 $\log_{10}$ $TCID_{50}$ may be required for long-term protection in lambs (Oura *et al.,* 2010). In addition to VP2, it has been suggested that VP5 may play a role in inducing virus neutralizing antibodies, likely through a confirmatory association with VP2 (Roy *et al.,* 1990), and VP5 has additionally been shown to influence the specificity of neutralizing antibodies (Cowley & Gorman, 1989; Mertens *et al.,* 1989; DeMaula *et al.,* 2000).

However, recent experiments based on virus neutralizing antibody assays using reassortants between BTV-1 and BTV-8 have suggested that VP2 alone is responsible for determining BTV serotype (Shaw *et al.,* 2013) and for inducing neutralizing antibodies (Kochinger *et al.,* 2014).

Non-neutralizing antibodies against BTV are also induced by VP2, as well as by other VP and NS proteins. In particular, VP7 induces high titers of IgM antibodies in ruminants as early as 7-10 days following BTV infection (Zhou *et al.,* 2001; Bréard *et al.,* 2011) or IgM and IgG antibodies 7-21 days after vaccination with live or inactivated vaccines (Monaco *et al.,* 2004; Gethmann *et al.,* 2009; Oura *et al.,* 2009; Bréard *et al.,* 2011; Modumo & Venter, 2012).

The detection of VP7-specific antibodies indicates BTV infection of any serotype and IgG antibodies directed against VP7 can be detected for up to 10 or even 13 months following vaccination in some ruminants (Hultén *et al.,* 2013; Zanella *et al.,* 2013). As a result, VP7 is commonly used in diagnostic assays as a group-specific antigen (Zhou *et al.,* 2001; Hamblin, 2004; Mecham & Wilson, 2004; Anthony *et al.,* 2007).

BTV antisera have also been shown to recognize VP3, the other protein of the BTV inner capsid (Inumaru *et al.,* 1987), and like VP7, VP3 and the proteins of the transcription complex (VP1, VP4, VP6) have also been shown to be serologically reactive across BTV serotypes. Besides the outer capsid proteins, no other BTV protein is thought to induce or influence virus neutralizing antibody production as defined by standard BTV neutralization assays (Huismans & Erasmus, 1981; Kahlon *et al.,* 1983; Huismans & Els 1979; Inumaru & Roy, 1987). Despite being largely associated with the induction of cellular immunity, NS1, NS2, and NS3 have also been shown to induce humoral immune responses following BTV infection. The roles of these antibodies in viral clearance or protection are not known. In the sera of lambs and calves collected after vaccination, NS2-specific antibodies are regularly detected in serological assays, while antibody production to NS1 is less consistently identified (Richards *et al.,* 1988).

Additionally, NS1- and NS2-specific antibodies have been detected following natural infection in sheep or rams (Adkison *et al.,* 1987), and high titers of antibodies directed against NS1 and NS3 are also observed following BTV infection (Anderson *et al.,* 1993; López *et al.,* 2006; Barros *et al.,* 2009).

2.7 Protein-specific cellular immune responses

Since the mid-1960s, it has been suggested that cell-mediated immunity may play an important role in protection against BTV infection (Jochim *et al.,* 1965; Luedke & Jochim, 1968). However, it was not until the 1980s that the specific correlation between cellular immunity and BTV protection in sheep was more thoroughly examined (Jeggo & Wardley, 1982; Jeggo *et al.,* 1984a; Stott *et al.,* 1985).

Identification of the specific viral proteins that induce these responses continues to be performed using samples primarily from mice or sheep (Table 2). Both VP and NS proteins have been shown to induce some level of T cell responses. In general, the NS proteins have predominantly been associated with cross-serotype cellular immune responses (Andrew *et al.,* 1995; Jones *et al.,* 1996; Janardhana *et al.,* 1999). The duration of cellular immune responses following BTV vaccination or infection is not yet known.

Table 2. Selected reference list for protein-specific humoral and cellular immune responses induced by experimental or natural BTV vaccination or infection

	Humoral immune responses			**Cellular immune responses**	
	Mice/Rabbits	*Sheep*	*Cattle*	*Mice/Rabbits*	*Sheep*
VP2	(Huismans *et al.,* 1987; Inumaru & Roy, 1990; Franceschi *et al.,* 2011; Calvo-Pinilla *et al.,* 2012)	(Richards *et al.,* 1988; Odeón *et al.,* 1999; Oura *et al.,* 2009	Savini *et al.,* 2008; Eschbaumer *et al.,* 2012; Celma *et al.,* 2013)	CD4+ (Francesh *et al.,* 2011) CTL (Jones *et al.,* 1996	CD4+ (T helper) (Takamatsu *et al.,* 1990) CTL (Andrew *et al.,* 1995; Janardhana *et al.,* 1999)
VP5	(Huismans *et al.,* 1987; Calvo-Pinilla *et al.,* 2009a)	(Richards *et al.,* 1988; Wang *et al.,* 1995, 2013; Odeón *et al.,* 1999	(Odeón *et al.,* 1999)	-	CTL (Andrew *et al.,* 1995; Janardhana *et al.,* 1999)
VP7	(Calvo-Pinilla *et al.,* 2009a)	(Richards *et al.,* 1988; Wade-Evans *et al.,* 1997; Odeón *et al.,* 1999; Perrin *et al.,* 2007)	(Richards *et al.,* 1988; Barros *et al.,* 2009)	CD4+ (Rojas *et al.,* 2011) CTL (CD8+) (Rojas *et al.,* 2011	No CTL (Andrew *et al.,* 1995) Possible (Wade-Evans *et al.,* 1996) CTL (Janardhana *et al.,* 1999)
NS1	(Calvo-Pinilla *et al.,* 2012)	(Adkison *et al.,* 1988; Richards *et al.,* 1988; Anderson *et*	(Richards *et al.,* 1988	CD4+ (Rojas *et al.,* 2014) CTL (Jones *et al.,* 1996, 1997; Rojas	CD4+ (Rojas *et al.,* 2014) CTL (Andrew *et al.,* 1995; Janardhana *et al.,* 1999)

		al., 1993)		*et al.,* 2014)	
NS2	(Mecham *et al.,* 1986)	(Adkison *et al.,* 1988; Richards *et al.,* 1988)	(Richards *et al.,* 1988)	CTL (Jones *et al.,* 1996, 1997)	No CTL (Andrew *et al.,* 1995; Janardhana *et al.,* 1999)
NS3	-	(López *et al.,* 2006; Perrin *et al.,* 2007; Barros *et al.,* 2009)	(Barros *et al.,* 2009)	CTL (Jones *et al.,* 1996)	CTL(Andrew *et al.,* 1995) No CTL (Janardhana *et al.,* 1999)

Antibodies to VP2 (including virus-neutralizing antibodies) and VP7 have been reported in numerous studies, of which only a few references are provided here. Where no references are indicated, no relevant references exist.

The protein-specificity of T cell responses induced by BTV infection or vaccination in cattle is poorly characterized. However, VP2 and NS1 followed by VP5 and NS3 have been shown to be strong inducers of cytotoxic T cells in sheep. (Andrew *et al.,* 1995). In the same study, VP7 did not induce cytotoxic T lymphocytes(CTLs), and those that were induced by NS1, but not VP2, were shown to be reactive across serotypes. Both NS1 and VP2 have been shown to induce cross-serotype and serotype-specific T cell responses, including helper T cells, in other studies (Takamatsu *et al.,* 1990; Janardhana *et al.,* 1999; Rojas *et al.,* 2014).

VP7 has also been shown to provide protection in the absence of neutralizing antibodies, presumably through the induction of non-neutralizing serum antibodies or cell-mediated immune responses (WadeEvans *et al.,* 1996). This has also been shown for VP7 in combination with VP3 (Roy *et al.,* 1994; Stewart *et al.,* 2012).

Janardhana *et al.* observed that neither NS2 nor NS3 induced CTLs in sheep (Janardhana *et al.,* 1999). This is in contrast to Andrew's study (Andrew *et al.,* 1995) and an earlier study in mice, in which NS1, NS2, and NS3 induced the highest amount of CTL responses (Jones *et al.,* 1996). In fact, vaccine studies in BALB/c and CBA/Ca mice have demonstrated that NS2 alone can provide partial protection against BTV infection by inducing CTL production (Jones *et al.,* 1997).

In all of these studies, the protein-specific T cell responses have been variable among individuals and may be MHC (Major Histocompatibility Complex)-restricted (Jeggo *et al.,* 1985; Takamatsu & Jeggo, 1989). MHC molecules present specific antigens for recognition by T cells and the genes that encode them are highly polymorphic in cattle (Amills *et al.,* 1998; Ellis & Codner, 2012) and other species. MHC-restriction of BTV protein-specific T cell responses could potentially impact vaccination, particularly for vaccines with protection based on cellular immunity, by resulting in variable levels of protection due to MHC diversity.

2.8 Prevention and control

Traditional prevention and control measures for viral livestock diseases include the restriction of animal trade movements or quarantine of sick animals, optimization of zoosanitary and other biosecurity approaches, treatment when available, vaccination, and eradication or pre-emptive slaughter. For BTV, there is no specific treatment, and different approaches may be taken depending on whether the disease is endemic or epidemic in a particular region. Regardless of the control measure, reliable diagnostic tests and understanding of the epidemiological situation are essential to allow decision making bodies to make informed choices (Wierup, 2012).

2.8.1 Vaccines

The most effective means to protect animals against disease and to reduce spread of the virus after infection is vaccination. To combat the direct losses incurred due to BT outbreaks and to minimize the circulation of BTV, vaccination of livestock has been implemented by various countries according to their individual national policies, the serotype of the virus and the availability of vaccines (Caporale and Giovannini, 2010; Savini *et al.,* 2008).

Currently, there are two types of vaccines against BTV that are commercially available (Table 3) (i) modified live virus (MLV) vaccines, which are attenuated forms of BTV; and ii) inactivated vaccines, which are composed of whole killed BTV plus an adjuvant such as aluminum and/or saponins. Each vaccine type has advantages and disadvantages, as reviewed below, and therefore the development of novel BTV vaccines using new technologies is an expanding area of research.

A number of vaccines have been developed against BT. These include inactivated whole virus vaccines, live attenuated virus vaccines (modified live virus vaccines), recombinant vaccines and virus-like particle (VLP) vaccines. While these vaccines have various advantages and disadvantages, only attenuated virus vaccines and some inactivated vaccines are presently commercially available (Niedbalski, 2011; Noad and Roy, 2009; Osburn *et al.,* 1996; Savini *et al.,* 2008).

The most important reasons for BT vaccination are to prevent clinical disease, to reduce environmental spread of BTV, and to protect susceptible animals during movement between affected and BTV-free zones (Niedbalski, 2011). Ideally, a vaccine for BT should protect against more than one serotype, should not revert to virulence or recombine with circulating strains of the virus, and should not be able to replicate in the field (Roy *et al.,* 2009). It is also important that vaccines against BT are safe to use and allow for differentiation between naturally infected and vaccinated animals [DIVA, (Niedbalski, 2011)].

2.8.2 Live vaccines

Live vaccines hold a particular place in the history of BTV control. The first "attenuated" vaccine was generated by Theiler in 1908 through multiple passage of infectious blood in sheep (Theiler, 1908). The vaccine was routinely used for almost 40 years and was generally considered relatively effective in reducing the severity of disease in sheep. However, increasing numbers of complaints from farmers over insufficient protection and particularly severe reactions to this vaccine prompted investigations into immunological responses of vaccinated sheep to a variety of circulating BTV isolates (Neitz, 1948).

The subsequent discovery of several BTV strains and the lack of adequate cross-protection between heterologous serotypes prompted an investigation into alternative systems for BTV attenuation (Neitz, 1948). Theiler's vaccine was subsequently replaced by strains serially passaged in embryonated chicken eggs in the late 40s (Alexander *et al.,* 1947) followed by tissue-culture attenuated strains in the 60s. MLV generated by passage in eggs and in BHK-21 cells are still produced by Onderstepoort Biological Products in Pretoria (South Africa) and are widely used in Africa (Coetzee *et al.,* 2012).

However, with the increasing number of recognized serotypes, inclusion of multiple serotypes was necessary to achieve adequate protection against the majority of circulating viruses (Howell, 1960; Howell *et al.,* 1970). The current vaccine consists of three vials, each containing five different serotypes, segregated based on their *in vivo* replication efficiency (Coetzee *et al.,* 2012). MLV have also been used in Europe (Bulgaria, France, Italy, Portugal, and Spain) (Roy *et al.,* 2009).

2.8.3 Inactivated vaccines

Classcal inactivated vaccines are produced as killed whole virus, often using heat, ultraviolet radiation (UV), or chemical methods, including hydroxylamine and binary ethylenimine (Campbell, 1985; Di Emidio *et al.,* 2004; Ramakrishnan *et al.,* 2005, 2006; Savini *et al.,* 2007; Umeshappa *et al.,* 2010).

In India, few attempts have been made to develop inactivated BTV vaccines using BEI and hydroxylamine. BEI-inactivated BTV vaccine elicits both neutralizing and group-specific antibodies (Ramakrishnan *et al.,* 2006) whereas hydroxylamine-inactivated vaccine elicits group-specific antibodies (Ramakrishnan *et al.,* 2005). Both the inactivated vaccines show reduction in clinical signs and reduction in the duration of viremia. A pentavalent (BTV-1, -2, -10, -16 and -23) inactivated vaccine has been developed under the All India Net-Work Project –Bluetongue (AINP-BT) and commercialized (Reddy *et al.,* 2010).

Today, there are inactivated mono- and multivalent vaccines against BTV-1, -4, -8 and -9 as well, as summarized in Table 3. Compared to MLVs, inactivated vaccines are safer because they are not associated with viremia and do not allow the reassortment between field and vaccine strains. However, they are more expensive to produce and may be more costly to administer as they require two immunizations rather than one in order to provide a comparable duration of immunity (Rogan & Babiuk, 2005). Additionally, inactivated vaccines need to be formulated with an adjuvant so as to induce sufficient immune responses (Singh & O'Hagan, 2003).

Common adjuvants used in inactivated veterinary vaccines include aluminum hydroxide, saponins, and emulsions. Inactivated vaccines against BTV-8 have been evaluated in Europe under experimental and natural conditions regarding their safety and protective efficacy. In brief, after two immunizations, they have been shown to be safe and induce protective immunity against experimental clinical and virological read-outs of BTV-8 infection in sheep (Gethmann *et al.,* 2009; Hamers *et al.,* 2009a; Oura *et al.,* 2009; Bartram *et al.,* 2011; Bréard *et al.,* 2011; Moulin *et al.,* 2012; Pérez de Diego *et al.,* 2012) for at least one year (Hamers *et al.,* 2009b).

In particular, the immune responses induced by vaccination with commercially available inactivated vaccines includes neutralizing antibodies, serum antibodies, and CD8+ T cells (Umeshappa *et al.,* 2010; Pérez de Diego *et al.,* 2012). Some mild localized reactions have also been observed following their use (Hamers *et al.,* 2009a; Vetvac, 2014). Revaccination with inactivated vaccines is recommended after one year by manufacturers (Vetvac 2014).

However, due to the risks of teratogenicity, persistence in the field, under-attenuation or possible reversion to virulence, the use of MLV has been quite controversial (Dungu *et al.,* 2004; Monaco *et al.,* 2004; Savini *et al.,* 2010). Indeed, there has been a number of cases where immunisation with MLV strains has led to the development of clinical signs and viraemia in inoculated animals (Savini *et al.,* 2010; Veronesi *et al.,* 2010). One of the South African vaccine serotypes, which was shown to cause disease in European sheep breeds, was BTV-16 (Savini *et al.,* 2010). Indeed, in Italy, the vaccine had to be withdrawn from use and attempts have been made to produce a new attenuated vaccine, innocuous to the native breeds (Franchi *et al.,* 2008).

Table 3. List of commercially available BTV vaccines (adapted from The Center for Food Security and Public Health, 2014; Vetvac, 2014)

Vaccine name	Manufacturer	Target species	Antigen	Adjuvant	Countries of distribution
Blue Tongue Virus Vaccine	Veterinary Vaccines Production Centre (KARI, Kenya)	Goat, sheep	polyvalent BTV (attenuated)	None	Kenya
Bluetongue Vaccine	Onderstepoort Biological Products Ltd (South Africa)	Goat, sheep	polyvalent BTV (attenuated)	None	Namibia, South Africa
Bluetongue Vaccine	Colorado Serum Company (USA)	Goat, sheep	BTV-10 (attenuated)	None	USA, Canada
Bluevac BTV-1	CZ Veterinaria	Cattle, Sheep	BTV-1 (killed)	Aluminum hydroxide, saponin	Europe*
Bluevac BTV-4	S.A. (Spain)	Cattle, Sheep	BTV-4 (killed)	Aluminum hydroxide, saponin	
Bluevac BTV8			BTV-8 (killed)	Aluminum hydroxide, saponin	
Bluevac BTV1+4			BTV-1,-4 (killed)		
Bluevac BTV1+8			BTV-1,-8 (killed)		
Bluevac-10	PHL Associates Inc. (USA)	Sheep	BTV-10 (attenuated)		
Bluevac-11			BTV-11 (attenuated)		

Bluevac-17			BTV-17 (attenuated)	None	USA
Bluevax	Veterinary Vaccines Production Centre (KARI, Kenya)	Sheep	BTV	None	Kenya
BLUEVAX	Kenya Veterinary Vaccines Institute (Kenya)	Sheep	BTV-1,-2,-3,-4,-8,-12,134 (attenuated in embryonated chicken eggs)	None	Kenya
Bovilis BTV8	Merck Sharp & Dohme Ltd (MSD Animal Health, United Kingdom)	Cattle, sheep	BTV-8 (killed)	Aluminum hydroxide, saponin	Europe*
BTVPUR AlSap 1	Merial (France)	Cattle, sheep	BTV-1 (killed)	Aluminum hydroxide, saponin	Europe*
BTVPUR AlSap 8			BTV-8 (killed)	Aluminum hydroxide	Europe*
BTVPUR AlSap 1+8			BTV-1,-8 (killed)	Aluminum hydroxide	Europe*
BTVPUR AlSap 2+4		Sheep	BTV-2,-4 (killed)	Aluminum hydroxide	Europe*
Freeze dried monovalent Bluetongue vaccine	Central Veterinary Control and Research Institute (Turkey)	Sheep	BTV-4 (live)	None	Turkey
Syvazul 1	Laboratorios SYVA S.A. (Spain)	Cattle, sheep	BTV-1 (killed)	Oil	Spain, United Kingdom
Syvazul 1+8			BTV-1,-8 (killed)		
Syvazul 8			BTV-8 (killed)		
Syvazul-4		Sheep	BTV-4 (killed)		
Zulvac 1 Ovis	Zoetis/Pfizer (United Kingdom)	Sheep	BTV-1 (killed)	Aluminum hydroxide, saponin	United Kingdom
Zulvac 1+8 Ovis			BTV-1,-8 (killed)		
Zulvac 8 Ovis			BTV-8 (killed)		
Zulvac 8 Bovis		Cattle	BTV-8 (killed)		

*Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, The Netherlands, Poland, Portugal, Romania, Slovak Republic, Slovenia, Spain, Sweden, United Kingdom.

2.9 New vaccine designs

New vaccine designs rely on the same basic approach as classical vaccines: utilizing live or killed antigens. As discussed, classical vaccines against BTV have the advantage of working well to prevent and control virus spread, and therefore novel vaccines must match their efficacy. Additionally, the design of new vaccines should aim to improve upon the disadvantages of classical vaccines by enabling differentiating infected from vaccinated animals (DIVA) and by potentially targeting multiple serotypes of BTV. Many of the experimental vaccines targeting BTV have been listed in Table 4.

2.9.1 Recombinant subunit protein vaccines

Recombinant viral vector vaccines consist of a heterologous live attenuated virus that has been genetically modified to include genes encoding foreign antigens, so that those genes can be expressed within the host and subsequently induce protective immunity against the target virus. Often, these antigens are produced in high amount in host cells and therefore can induce strong immune responses (Calvo-Pinilla *et al.,* 2014). Antigen choice is especially important for inducing protective immunity, but other crucial considerations include recombinant vector stability, host range, expression and conformation of the antigen, duration of immunity, cost of production, and safety (Yokoyama *et al.,* 1997).

Potential pre-existing immunity and the location of the primary immune response to the viral vector itself can hinder the development of a protective immune response (Saxena *et al.,* 2013). Furthermore, since recombinant viral vectors are genetically modified, countries can be hesitant to implement their use. Capripox virus (Wade-Evans *et al.,* 1996; Perrin *et al.,* 2007), canarypox virus (Boone *et al.,* 2007), bovine herpes virus type 4 (Franceschi *et al.,* 2011), and vaccinia virus (Lobato *et al.,* 1997) vectors, among others, have all been used with varying levels of success as the basis of recombinant viral vector vaccines against BT (Table 4).

Additionally, disabled infectious single cycle (DISC) vaccines have been developed using reverse genetics technology, such that one essential gene product is missing from the produced virion and therefore the vaccine virus is only able to replicate for a single cycle in target cells. This type of vaccine may provide a safer alternative to MLVs, though more virus or several doses are likely needed for a DISC vaccine to be equally effective since the amplifying effect of MLVs is prevented under this design. Promising VP6-deficient DISC vaccines have been produced and shown to provide protection against experimental BTV challenge in sheep (Matsuo *et al.,* 2011; Celma *et al.,* 2013).

2.9.2 Virus-like particles (VLPs) as vaccines

Virus-like particle vaccines (VLPs) and subunit vaccines have also been shown to be promising vaccine candidates against BTV (Roy *et al.,* 1994; Stewart *et al.,* 2010, 2012). VLPs are structures that resemble the authentic conformation of native viruses and are formed when multiple subunits of protein complexes interact with each other in such a way that it results in self-assembly. BTV VLPs contain only the four structural proteins - VP2, VP3, VP5 and VP7 and mimic the structure of authentic virus particles. They are readily recognised by the immune system and present viral antigens in a confirmation that is more authentic than other subunit vaccines, making them excellent immunogens (Noad and Roy, 2003).

VLPs have been shown to be non-infectious and unable to replicate as they contain no viral genetic material (Rodriguez-Limas *et al.,* 2013). Since BTV VLPs do not contain the non-structural proteins or virus RNA, it is possible to distinguish between vaccinated and infected animals using molecular diagnostic techniques (Niedbalski, 2011). VLPs are excellent vaccine candidates as there is no risk of reversion to virulence or reassortment with wild virus strains as they contain only structural proteins (Roy *et al.,* 2009).

The repetitive, high density display of epitopes on VLPs renders them more immunogenic than subunit vaccines or chemically inactivated vaccines. The particulate nature of VLPs combined with epitope display enhance the humoral, cell-mediated and mucosal immune responses (Noad and Roy, 2003; Roy and Noad, 2008).

VLPs are excellent candidates for BTV vaccines, since both humoral and cellular immunity is important for protection against BTV (Calvo-Pinilla *et al.,* 2012). They are also safe to produce and handle and the host cells for their production do not necessarily come from mammalian sources, and therefore would not contain mammalian derived pathogens (Roy, 1996).

VLPs for BTV serotype 10 were first produced in insect cells using a baculovirus-based protein expression system; this system was shown to efficiently produce large amounts of proteins with the correct folding and assembly of large complexes (French *et al.,* 1990).

BTV VLPs are formed by the co-expression of the four major structural proteins of the virus (VP2, VP3, VP5 and VP7); these proteins self-assemble into triple-layered VLPs. Sheep vaccinated with the insect cell expressed BTV-10 VLPs developed neutralising antibodies and were protected against challenge with a homologous serotype, these studies showed that VLPs are both safe and effective to use as potential vaccines for BTV (Roy *et al.,* 1990; Stewart *et al.,* 2012). In other experiments, it was shown that low doses of VLPs were sufficient to give complete protection from virus challenge and none of the vaccinated animals developed clinical signs or viraemia.

Furthermore, sheep vaccinated with VLPs that were made separately from two different serotypes were shown to have complete protection against the vaccine serotypes as well as partial protection against related non-vaccine serotypes. It was also demonstrated that vaccination with VLPs provided protective efficacy lasting over 14 months (Noad and Roy, 2003; Roy *et al.,* 1994; Roy *et al.,* 2009).

Despite their efficacy, the cost of producing VLPs in insect cells is very high, which has hindered further development and commercialization of these vaccine candidates, alternative methods of production would be desirable to facilitate large-scale development of these vaccines. Recently BTV-8 VLPs were successfully expressed and assembled in *Nicotiana benthamiana* plants using a transient plant expression vector. The plant-expressed VLPs elicited an immune response in sheep providing protective immunity against challenge with a BTV-8 field isolate (Thuenemann *et al.,* 2013).

Adjuvants provide the advantage of allowing potent immune responses to be quickly induced and can help to direct an immune response to be humoral, cellular, or a combination of both (Petrovsky & Aguilar, 2004). Antigen choice, which is possible in subunit vaccine development, can also facilitate the induction and direction of immune responses to optimize a vaccine's protective efficacy, provided sufficient information is available to allow informed choices.

2.9.3 Differentiating Infected from Vaccinated Animals (DIVA)

DIVA is an increasingly important consideration for veterinary vaccine design because of the movement restrictions placed on BTV-positive ruminants during outbreaks (Bhanuprakash *et al.,* 2009). Countries face losing their disease-free status following widespread vaccination using conventional vaccines, which can have a devastating economic effect and in some cases has driven countries to slaughter vaccinated animals that could not be differentiated from infected animals, as in the case of the 2001 outbreak of foot-and-mouth disease in the Netherlands (Pluimers, 2004).

DIVA is also crucial for maintaining serological surveillance as a tool for monitoring changes in vaccine efficacy or local epidemiology, especially in *regions* with potential co-circulation of several strains or serotypes (Uttenthal *et al.,* 2009; Avellaneda *et al.,* 2010). Currently, there are no commercially available DIVA-compliant BTV vaccines. Some studies have suggested that NS1 (Anderson *et al.,* 1993) or NS3 (López *et al.,* 2006; Barros *et al.,* 2009) can be detected in infected, but not vaccinated, animals following the use of classical inactivated vaccines. However, since current vaccines contain whole virus and when inactivated may contain some of these NS proteins, there is a strong risk that false positives, especially after repeated vaccination, can complicate their use as DIVA targets. This has been observed with foot-and-mouth disease (Paton & Taylor, 2011). Therefore, new strategies must be employed to create companion DIVA tests for existing vaccines, or to create effective next generation DIVA vaccines based on existing diagnostic tests.

Table 4. Selected reference list for recent experimental BTV vaccines, including expression system, BTV proteins included in vaccine formulations, target species, and resulting clinical and virological protection against BTV challenge resulting in varying severity

Reference	Expression system	Proteins (serotype)	Species	Clinical protectin	Virological protection
Killed vaccines					
(Roy *et al.,* 1990)	Sf9-baculovirus	VP2 (50 µg) (BTV-10). VP2 (100, 200 µg) (BTV-10). VP2,VP5 (BTV-10) . VP1,VP2,VP5-7, NS13, (BTV-10); VP3 (BTV-17)	Sheep	Partial Full Full Full	Partial Full Full Full
(Roy *et al.,* 1994; Stewart *et al.,* 2012, p 2)	Sf9-baculovirus	Virus-like particles (VLPs): VP2, VP5, VP3, VP7 (BTV-1, -2, -8, -13, 17)	Sheep	Full	Full
(Stewart *et al.,* 2012)	Sf9-baculovirus	Core-like particles (CLPs): VP3, VP	Sheep	Partial	Partial
(Jabbar *et al.,* 2013)	Bacterial	VP2, VP5, VP7 (BTV-8)	IFNAR (-/-) mice	Partial	Partial
(Mohd Jaafar *et al.,* 2014)	C41 (DE3) E. col	VP2, VP5 (BTV-4)	IFNAR (-/-) mice	Full	Partial
Live vaccines					
(Wade-Evans *et al.,* 1996)	Capripox virus	VP7 (BTV-1)	Lambs	Partial	Not tested
(Lobato *et al.,* 1997)	Vaccinia virus	VP2 (BTV-1) VP2, VP5 (BTV-1)	Sheep	Partial	Partial
(Boone *et al.,* 2007)	Canarypox virus	VP2, VP5 (BTV-17)	Sheep	Full	Full
(Perrin *et al.,* 2007)	Capripox virus	VP2, VP7, NS1, NS3 (BTV-2)	Sheep	Partial	Partial

(Calvo-Pinilla *et al.,* 2009b, 2012	Modified Vaccinia Ankara virus + DNA	(BTV-4)	IFNAR (-/-) mice	Partial	Full
(Franceschi *et al.,* 2011	BoHV-4*	VP2 (BTV-8)	IFNAR (-/-) mice	Partial	Partial
Ma *et al.,* 2012)	EHV-1**	VP2(BTV-8) VP2, VP5 (BTV-8)	IFNAR (-/-) mice	Partial Partial	None Full
(Kochinger *et al.,* 2014)	Vesicular stomatitis virus (single-cycle)	VP2 (BTV-8) VP5 (BTV-8) VP2, VP5 (BTV-8)	Sheep	Partial None Full	Partial None Full
(van Gennip *et al.,* 2012	Reassortants (reverse genetics)	BTV-6 backbone + VP2, VP5 BTV-1. BTV-6 backbone + VP2, VP5 (BTV-8). BTV-6	Sheep	Partial Partial Partial	Full Full Full
(Feenstra *et al.,* 2014)	Reassortans with NS3/NS3A knockout mutation (reverse genetics)	BTV-1backbone + VP2 (BTV-8) BTV-6 backbone + VP2 (BTV-8) BTV-8	Sheep	Partial Partial Partial	Partial Full Partial

*Bovine herpes virus type 4; **equine herpes virus type 1; ***disabled infectious single cycle.

2.9.4 Plant expression systems

Over the last 20 years, plant-based expression of heterologous proteins has attracted much interest as it has many advantages over other eukaryotic expression systems. These include high biomass, ease of scale up, cost effectiveness and a low risk of contamination (Lai and Chen, 2012; Sainsbury and Lomonossoff, 2008). Furthermore, plant-based expression systems are safe to use as they do not contain the human and animal pathogens that are potentially an issue when using mammalian cell-based production systems (Fischer *et al.,* 2012; Pogue *et al.,* 2010; Rybicki, 2010; Xu *et al.,* 2012).

A wide variety of plants has been utilized for the expression of human and animal vaccines, with the hepatitis B surface antigen expressed in transgenic tobacco being one of the first VLP candidate vaccines (Mason *et al.,* 1992; Rybicki, 2010). Ever since 1992, well-characterized viruses such as human immunodeficiency virus, human papilloma virus, influenza virus, foot and mouth disease virus and rotavirus, to name a few, have been successfully investigated for the formation of VLPs in plants (Scotti and Rybicki, 2013).

CHAPTER III

MATERIALS AND METHODS

3.1 General laboratory reagents and materials

The required media and reagents were prepared using molecular grade chemicals. The reagents and labware were availed from suppliers including Bangalore Genei (Bangalore), HiMedia (Mumbai), Merck (Mumbai), Sigma Aldrich, Qiagen and Invitrogen (USA). All media, broth and aqueous solutions were prepared using double glass distilled water and sterilized by autoclaving at 121°C for 15 minutes or by membrane filtration using 0.22 µm membrane filter (Millipore Corporation, Bedford, MA, USA). Common reagents used in different procedures were prepared as per standard procedures. Their compositions are provided in the Annexure.

3.1.1 Glassware

Glassware such as test tubes, flasks, pipettes etc. manufactured by Borosil, and Schott Duran brand were used throughout the study. Glassware were soaked in neutral detergent (Labolene) overnight and then scrubbed and washed thoroughly under running tap water. Then they were washed with de-ionized water followed with single distilled water and finally rinsed with double distilled water, air dried, packed and sterilized in hot air oven at 160°C for 2 hours.

3.1.2 Plasticware

Sterile nuclease-free 0.2 mL, 0.6 mL, 1.5 mL and 2 mL microfuge tubes from Axygen and 15 mL and 50 mL centrifuge tubes of Tarsons, 0.01 mL, 0.1 mL 0.2 mL and 1.0 mL disposable tips from Axygen were used for the work. Sterile 96 well microtiter plate of Nunc was used for enzyme-linked immuno sorbent assay.(ELISA).

The microfuge tubes, micropipette tips and PCR tubes were sterilized by autoclaving at 121^{o}C for 15 minutes.

3.1.3 Equipment

The equipment used in the study are listed below

1. Laminar Flow Cabinet
2. Centrifuges
    - Centrifuge 5415D/R- Eppendorf-Germany
    - Cooling centrifuge- Sigma 3K30
    - Centrifuge 5810R- Eppendorff-Germany
    - Cooling centrifuge- REMI
3. Binocular Inverted Microscope
4. PCR Thermocycler-Hi-Media Prima- Duo
5. ELISA Reader, BioTek U.S.A
6. Incubators
    - CO_2 Incubator
    - C-24 KC Classic benchtop shaker Incubator (New Brunswick Scientific)
7. U.V transilluminator- GeNei Bangalore – India
8. Gel Doc Gene Flash-Syngene Bioimagine
9. Spectrophotometer -ThermoFischer Scientific
10. NanoDrop Lite Spectrophotometer -Thermo Scientific
11. Water Bath-BS11 Lab companion
12. pH meter-Eutech
13. Horizontal electrophoresis apparatus and powerpack-BioRad- UK
14. SDS-PAGE Apparatus-Bio-Rad Laboratories, Inc, UA
15. Weighing balance-Citizen

16. Sonicator-Sonics Vibrocell
17. Dry Bath-Genei Bangalore- India
18. VoretexGenei-2-Scientific Industries
19. Western blot apparatus-BioRad-UK
20. Micropippetes of various volumes- Eppendorf Germany
21. Gel rocker-Genei Bangalore- India
22. Autoclave- LabTech
23. Hot air oven-LabTech
24. Vaccutainer with clot activator-BD Vaccutainer SST™II Advance

3.1.4 Molecular Biology Kits

PrimeScript™ 1st strand cDNA Synthesis Kit- TaKaRa BIO INC.

QIAquick PCR Purification Kit-QIAGEN Germany

QIAprep Spin Miniprep Kit- QIAGEN Germany

Ni-NTA Fast Start Kit – QIAGEN Germany

3.1.5 Buffers and Reagents

Detailed formulations of all the reagents and buffers used in the study are given in Appendix I. Chemicals used for molecular work in the present study were of molecular grade from TaKaRa, HiMedia, New England BioLabs, Sigma (USA) and Life Technologies.

3.2 Identification of conserved epitopes in VP2 and VP5

Data sets of complete sequences for segments 2 (encoding VP2) and segment 6 (encoding VP5) of the BTV genome were compiled from GenBank (Capri *et al.,* 2010) and aligned using the MUSCLE program (Edgar 2004) and then adjusted manually using MEGA 5.2.1 alignment editing software (Tamura *et al.,* 2011).

3.3 Selection pressure analysis on VP2 and VP5

Selection pressures acting on individual codons of VP2 and VP5 were determined using SLAC (**S**ingle-**L**ikelihood **A**ncestor **C**ounting) platform at www.datamonkey.org which uses a combination of maximum-likelihood (ML) and counting approaches to infer nonsynonymous (*dN*) and synonymous (*dS*) substitution rates on a per-site basis for a given coding alignment and corresponding phylogeny (Kosakovsky *et al.,* 2006; Kosakovsky *et al.,* 2005*;* Delprot *et al.,* 2010; Suzuki & Gojobori 1999).

3.4 Immunogenicity of cVP2

B cell epitopes and T cell (MHC I and MHC II) epitopes of cVP2 were predicted by "*IEDB analysis resource v 2.5"* using consensus sequence of cVP2 that was generated using *Jalview* (Wang *et al.,* 2008; Kim *et al.,* 2012; Zhang *et al.,* 1999; Lundegaard *et al.,* 2008; Sidney *et al.,* 2008; Hoof *et al.,* 2009; Nielsen & Andreatta 2016).

3.5 3-D Protein structure homology modelling

Homology modelling of protein structures of VP2, cVP2, and VP5 were performed using SWISS-MODEL (*ProMod3 Version 1.0.2.*) at www.expasy.org which is a fully automated web-based protein structure homology modelling expert system.

3.6 Cell lines

Baby hamster kidney (BHK-21), Vero cell lines kindly provided by Ella Foundation, Shameerpet, Hyderabad, were propagated and maintained at the Department of Veterinary Microbiology, C.V.Sc., Rajendranagar.

3.6.1 Propagation and maintenance of BHK-21 cell line

BHK-21 cells were propagated and maintained in tissue culture flasks with growth medium (GIBCO^{TM} Minimum Essential Medium, LOT. No.1708563) with 5% fetal bovine serum (FBS) (HiMedia). After formation of confluent monolayer, the growth medium was decanted completely and washed with 1X PBS. After washing, 0.1% trypsin was added and incubated at 37^{o}C for 3-5 minutes for the cells to detach from the flask. The cells were dislodged by gentle tapping of the bottle, and growth medium with 5% fetal bovine serum was then added. The detached cells were flushed in growth medium and cell count was carried out under inverted microscope using hemocytometer by trypan blue exclusion assay.

3.6.2 Cell count- trypan blue exclusion assay

Cell suspension was mixed thoroughly to take a 50 µL of sample. This cell sample was added to 450 µL of 0.4 % trypan blue solution and mixed gently. Neubauer chamber with glass cover was placed on the microscope stage. Then 20 µL of mixture was loaded into the chamber of hemocytometer slide, by capillary action. Live cells were counted by 10X objective, in the central 1 mm^2 area of the grid. The cell concentration was calculated using the formula given below.

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

Dilution factor = 10

c – Cell concentration (cells /mL)

n – Number of cells in the area

3.6.3 Cell seeding

Cells were seeded at a concentration of 2×10^4 cells per mL into tissue culture flasks and incubated at 37°C in 5% CO_2 tension until complete monolayer was formed.

3.6.4 Infection of BHK-21 cells with bluetongue virus serotype-16

BHK-21 cell culture adapted bluetongue virus serotype-16 that was isolated from BT outbreaks during 2016 from Andhra Pradesh, and maintained at the Department of Microbiology, C.V.Sc., Rajendranagar was used in the present study. At 80-90% confluence of the monolayer in tissue culture flask, growth medium was discarded and washed with 1X PBS. Then, 2 mL of MEM with 1% FBS was added along with 20 µL of stock virus and incubated at 37°C in 5% CO_2 incubator with intermittent swaying. After 30 minutes of virus adsorption, 3 mL of 1% MEM with FBS was added and sealed with paraffin film and kept in CO_2 incubator along with control flask in which only maintenance medium was added without virus. Then, infected bottles were subjected to microscopic examination at every 12-18 h interval in comparison with control flask for CPE in BHK-21 cells. The cytopathetic effect (CPE) is characterized by vacuole formation, rounding and clumping of cells and eventually complete thinning of the monolayer.

3.6.5 Titration of the virus

Titre of putative BTV-16 serotype was determined in terms of tissue culture infective dose 50 ($TCID_{50}$) in 96 well tissue culture plate. Cells were seeded at the rate of $1x10^4$ per well in MEM with 5% FBS and incubated in CO_2 incubator at 37°C. At 80-90% confluence, cells were infected with 100 µL of 10^{-1} to 10^{-8} serially diluted virus in 1% growth medium, each dilution in a separate row in replicates of eight along with two cell control rows and incubated in CO_2 incubator (Table 5). Plates were observed at

12-18 h interval for characteristic CPE under inverted microscope and final readings were taken on 5th day of infection. Virus titer was calculated according to Reed and Munch method (1938).

Table 5. Infection of 96-well plate for $TCID_{50}$ calculation

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

CC-cell control

3.6.6 Protocol for crystal violet staining

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

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

3.6.7 Freeze thawing of cell culture fluid

At 90% detachment of monolayer in infected flasks, the cells were freeze thawed two times. Freeze/thaw involved rapid freezing at -70ºC for 20 min and rapid thawing to 37ºC in a water bath.

3.7 Extraction of viral RNA

1. Five mL of freeze/thawed infected BHK-21 cell culture harvest containing virus was centrifuged at 4000 rpm for 15 min at 4°C to obtain cell pellet (Centrifuge 5810 R, Eppendorf).
2. To the pellet, 750 µL of TRIZOL® reagent (Ref: 15596018; Ambion®) was added and vortexed for a minute followed by incubation at room temperature for 5 min.
3. Chloroform 250 µL was added to the above, vortexed and incubated at room temperature for 10 minutes.
4. The above samples were centrifuged at 13200 rpm for 15 min in cooling centrifuge.
5. Aqueous phase was transferred to nuclease free microcentrifuge tube; interphase and lower phase were discarded.
6. RNA was precipitated by adding isopropanol and 0.8 M sodium citrate/1.2 M NaCl, half volume of the aqueous phase each, mixed by gentle inversion.
7. The tubes were incubated at -20°C overnight, centrifuged at 13200 rpm for 15 min. and the supernatant was discarded.
8. The pellet was washed with 0.5 mL of 75% ethanol by centrifuging at 13200 rpm for 5 min.
9. The supernatant was discarded and the pellet was dried on kimwipe.

10. The RNA was resuspended in 30 µL of nuclease-free water by pipetting up and down a few times.

3.7.1 Agarose gel electrophoresis

Gel electrophoresis was carried out for confirmation of segmented double stranded RNA pattern of the virus by comparison of migration distances with 1Kb DNA ladder (Cat.No.N3232S; New England Biolabs).

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

2. After letting it cool down to 50ºC, ethidium bromide (EtBr) (E8751-10G; SigmaAldrich) was added to a final concentration of 0.5 µg/mL. The content was swirled gently to mix the reagents.

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

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

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

6. Electrophoresis was carried out at 75 volts for 1 hour and 30 minutes.

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

3.8 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed in two steps. First cDNA synthesis was carried out and the synthesized cDNA was used for PCR.

3.8.1 Reverse transcription

The reaction was carried out using PrimeScript™ 1st strand cDNA Synthesis kit (Cat No.6110A; TaKaRa). A 20µL of BTV-16 RNA was converted to 40 µL of cDNA, in following steps.

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

2) The following mixture was prepared in microfuge tube.

Reagent	**Volume**
Random hexamers	2 µL
dNTP Mixture	2 µL
Template RNA	20 µL
RNase free dH2O	6 µL
Total	30 µL

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

4) The following reaction mixture was prepared.

Reagent	Volume
Template RNA Primer Mixture	30 µL
5X PrimeScripit Buffer	8 µL
RNase Inhibitor	1 µL
PrimeScript RTase	1 µL
Total	40 µL

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

25ºC	10 min
42ºC	60 min
70ºC	10 min (for enzyme inactivation)
4ºC	∞

6) The cDNA obtained was stored at -20ºC (Sanyo Biomedical Freezer).

3.9 Virus Isolates

Isolates of BTV strains (BTV-1, -2, -4, -5, -9, -10, -12, -16, -21, -23, and -24 were adapted to BHK-21 cell line till CPE was moticed. RNA was extracted from the cell culture fluid, confirmed by typing primers and these eleven serotypes are maintained in Department of Veterinary Microbiology.

3.10 Designing of primers

The primers were designed in accordance to the vector map in which they were cloned. The details of the individual primers and their designing are given below.

3.10.1 Primers for cVP2

Forward Primer-cVP2

A leader sequence consisting of 6 extra base pairs on the 5' end of the primer was added to assist with restriction enzyme digestion, followed by Restriction Site: *Bam*HI (GGATCC) and then an extra nucleotide G was added basing on the pRSET-B map in order to prevent mispairing and ensure that the gene was in frame. BTV-16 cVP2 sequence was starting with AGA and so a start codon (ATG) was added which was followed by the hybridization sequence that binds to the sequence to be amplified.

Reverse primer-cVP2

For the Reverse Primer, the reverse complement of the sequence was used. Reverse primer contained the final 18bases of the ORF, the stop codon, restriction site *Eco*RI (GAATTC) and then 6 extra nucleotides *i.e.*, leader sequence to improve restriction enzyme digestion. Then the reverse-complement of the sequence that could successfully amplify the ORF was added. The reverse-complement was generated by using the software *The Sequence Manipulation Suit.*

3.10.2 Primers for VP5 and NS1

Failure in the expression of the VP5 and NS1 in pRSET-B led to the alternate selection of a larger primary vector pET-21a (+).

pET21a (+) was obtained from National Institute of Animal Biotechnology (NIAB) and was used for cloning VP5 and NS1; a new set of primers having the restriction sites of *Bam*HI and *Hind*III was used for both the proteins. Expression failure of VP5 and NS1 in pET-21a (+) led to selection of new restriction sites *Nhe*I and *Xho*I, primers and were designed accordingly. The details of all the primers designed with their respective restriction sites and vector details are given in Table 6.

However, even this procedure ended up in expression failure. Hence, to overcome codon bias in the sequence of VP5 and NS1, codon optimized gene sequences were synthesized (Genscript). New set of primers were then designed for the optimized sequence that had His-Tag with restriction site of *Nco*I and *Hind*III for VP5 and *Nde*I, *Hind*III for NS1. The details of the primers designed for codon optimized sequence are given below in Table 7.

Table 6. Oligonucleotide sequences for cloning cVP2, VP5 and NS1 into pRSET-B, and pET21a (+) vectors

SEROTYPE	**PRIMERS**	**RE/VECTOR**
BTV-16/VP2	F: 5’TAAGCA***GGATCC*****G**ATG**CGTTTCTATGTGTTGCTAAT**3’ R:5’TGCTTA***GAATTC*****TCAGTCAAAGAGGTTAACGCGCC3’**	***Bam*HI** ***EcoRI*** **pRSET-*B***
BTV-16/VP5	F: 5’TAAGCA***GGATCC*****G**ATG**GGTAAAATCATCAAATC**3’ R:5’TGCTTA***AAGCTT*****CTCACGCGTTTTTTAGGAAGA**3’	***Bam*HI** ***Hind*III** **pRSET-*B***
BTV-16/VP5	F: 5’TAAGCA***GGATCC***ATG**GGTAAAATCATCAAATC**3’ R: 5’TGCTTA***AAGCTT*****CGCGTTTTTTAGGAAGAGCG**3’	***Bam*HI** ***Hind*III** **pET-21a (+)**
BTV-16/NS1	F:5’TAAGCA***GGATCC***ATG**GAGCGCTTTTTGAGAAAA**3’ R:5’TGCTTA***AAGCTT*****ATACTCCATCCACATCTGAGA**3’	***Bam*HI** ***Hind*III** **pET-21a (+)**
BTV-16/VP5	F:5’CATGTAGC***GCTAGC*****GGTAAAATCATCAAATCACT**3’ R:5’CATGTAGC***CTCGAG*****CGCGTTTTTTAGGAAGAGCG**3’	***Nhe*I** ***Xho*I** **pET-21a (+)**
BTV-16/NS1	F:5’CATGTAGC***GCTAGC*****GAGCGCTTTTTGAGAAAAT**3’ R:5’CATGTAGC***CTCGAG*****ATACTCCATCCACATCTGAG**3’	***Nhe*I** ***Xho*I** **pET-21a (+)**
BTV-16/VP5	F:5’*CCATG* **GGAAAGATAATAAAATCACTAAGTAGGTTCG**3’ R:5’*AAGCTT***TCATTACGCATTTTTCAGGAACAGCGGAACA3’**	***Nco*I** ***Hind*III** **pET-21a (+)**
BTV-16/NS1	F: 5’*CATATG***GAACGCTTTCTGCGCAAAT**3’ R:5’ *AAGCTT***TCATTAGTATTCCATCCACA**3’	***Nde*I** ***Hind*III** **pET-21a (+)**

Underlined nucleotide sequence is restriction enzyme recognition sequence, and italicized sequence indicates restriction enzyme specific sites. Sequence in bold indicates gene recognition sequence.

Table 7. Oligonucleotide sequences of optimized sequences for cloning VP5 and NS1 into pET28b (+)

SEROTYPE	**PRIMERS**	**Restriction Enzyme**
BTV-16/VP5	F:5’***CCATGC*****GGAAAGATAATAAAATCACTAAGTAGGTTCG**3’ R:5’***AAGCTT*****TCATTACGCATTTTTCAGGAACAGCGGAACA**3’	***Nco*I** ***Hind*III**
BTV-16/NS1	F: 5’**CATATGGAACGCTTTCTGCGCAAAT**3’ R:5’ ***AAGCTT*****TCATTAGTATTCCATCCACA**3’	***Nde*I** ***Hind*III**

Italicized sequence indicates restriction enzyme specific sites. Sequence in bold indicates gene recognition sequence.

3.11 Polymerase Chain Reaction

The optimized primers were used for amplification of the cVP2, VP5 and NS1 genes by PCR using the respective designed primers as mentioned in Table 5 and Table 6.

3.11.1 Reagents

Reagents used for PCR consisted of 10x *Taq* buffer A with 15 mM $MgCl_2$ (Genei^{TM}), 10 mM dNTPs (Genei^{TM}), *Taq* polymerase (3 U/µL, Genei^{TM}), forward and reverse primers, and molecular biology grade water (HiMedia).

3.11.2 Other materials

Microfuge 1.5 mL and 0.2 mL PCR tubes (Axygen), 1 Kb and 100 bp DNA ladder (100 U/µL, Genei^{TM}), ethidium bromide (10 mg/mL, Sigma-Aldrich), DNA gel loading dye 6x (Genei^{TM}), TAE buffer (pH 8.3), agarose (HiMedia) and submerged horizontal electrophoresis cell and PowerPac^{TM} basic power supply (Bio-Rad) were used.

3.11.3 Protocol

PCR was carried out using a master mix for a 25 µL reaction with the primers described before. The reaction mixture and thermal cycling conditions used were as follows for 25 µL PCR reaction:

1. The master mix was prepared as per required reaction and gently vortexd, short spinned and distributed 22.5 µL into each 0.2 mL PCR tubes followed by addition of 2.0 µL of template DNA.
2. The tube was loaded onto an Eppendorf thermal cycler and the reaction was carried out as given in Table 8.

Template	2.0 µL
Taq Buffer 10x	2.5 µL
dNTPs 10 mM	1.0 µL
4Forward primer (10 µM)	2.5 µL
Reverse primer (10 µM)	2.5 µL
Taq polymerase	1.0 µL
Nuclease free water	13.5 µL
Total reaction volume	25.0 µL

Table 8. PCR cycle conditions for amplification of cVP2, VP5 and NS1 genes

Conditions	**cVP2**	**VP5**	**NS1**	
Initial denaturation	94°C - 05 min	94°C - 05 min	94°C - 05 min	
Denaturation	94°C - 30 sec	94°C - 30 sec	94°C - 30 sec	**35 Cycles**
Annealing	55°C - 60 sec	56°C - 1.5 min	58°C - 1.5 min	
Extension	72°C - 60 sec	72°C - 60 sec	72°C - 60 sec	
Final extension	72°C - 10 min	72°C - 10 min	72°C - 10 min	
Hold	4° C	4° C	4° C	

3.11.4 Preparation of agarose gel

Agarose gels were made with 1 % (w/v) agarose (MB002-500G; HiMedia) in 1x TAE containing ethidium bromide. Samples were prepared by mixing sample with DNA running buffer to a final concentration of 1x and 5 µL was loaded per well. Gels were run in a tank containing 1x TAE for 30 min at 80 V. Bands were visualized by UV using a Gene flash, Syngene bio imager.

3.12 Vectors

pRSET-B was used for cVP2, and pET28b (+) was used for VP5 and NS1. The maps of the vectors are given in Figure (10 and 11). The vector (plasmids) was grown in 10mL LB supplemented with relevant antibiotic overnight. Antibiotic concentrations used are shown in the Table 8. The plasmids were isolated and purified by QIA prep Spin Miniprep Kit- QIAGEN Germany. In cases where not sufficient DNA was obtained the alkaline lysis method was used.

Table 9. Concentrations of antibiotics used in this study

Abbreviation	**Antibiotic**	**Working concentration (µg/mL)**
Amp	Ampicillin	100
Cm	Chloramphenicol	34
Kan	Kanamycin	50

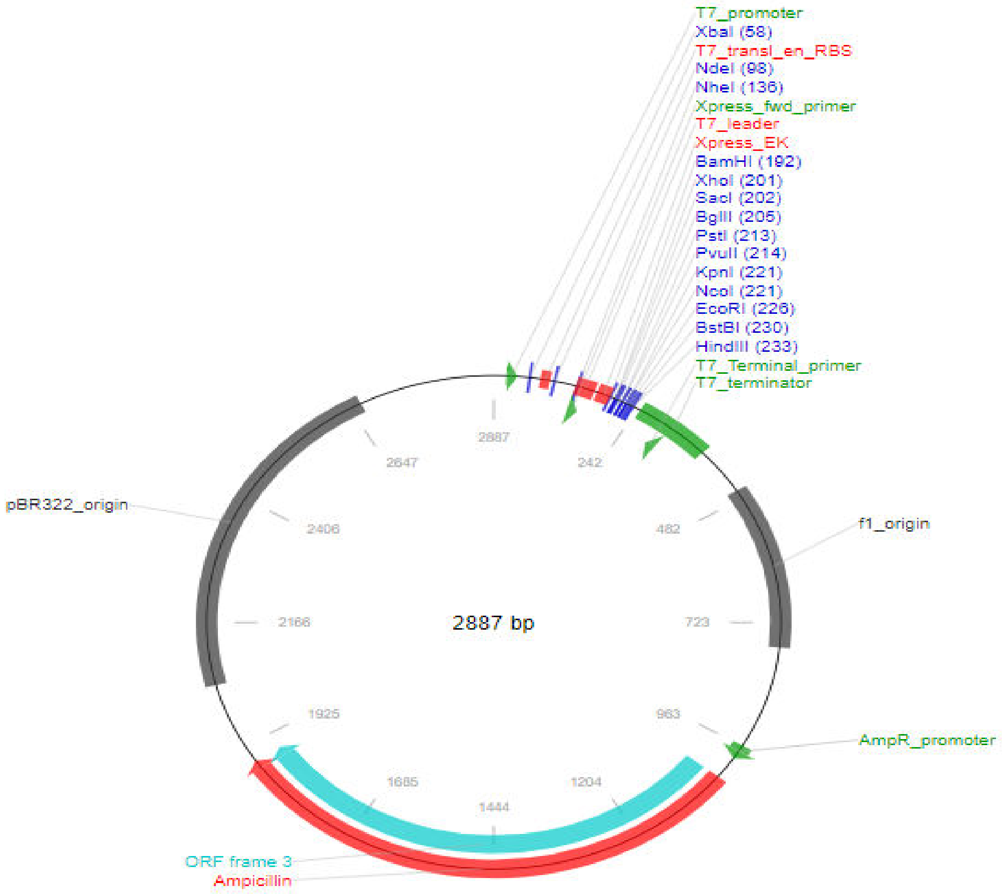
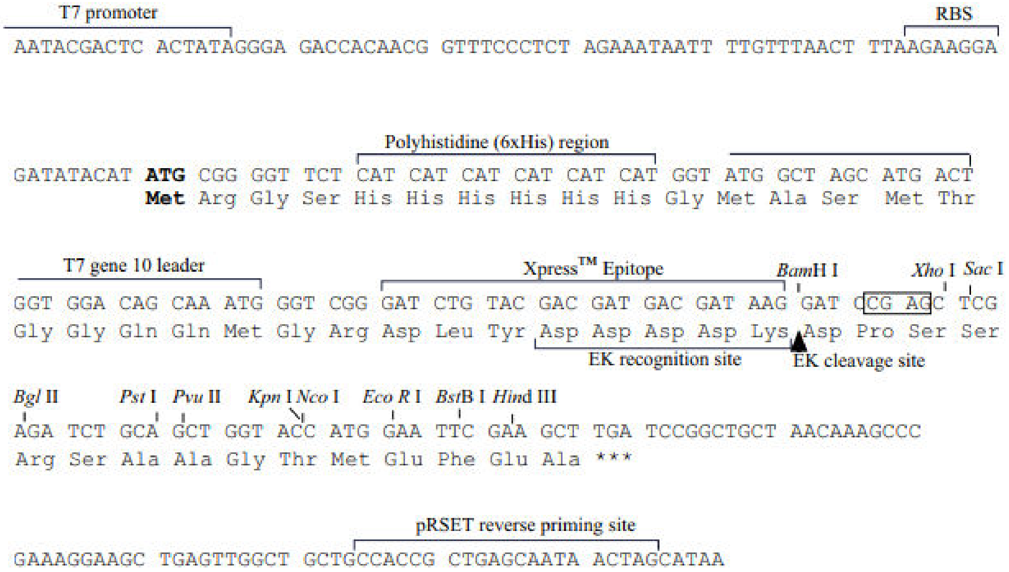


Fig. 10: DNA sequence of **multiple cloning site** (MCS) region- pRSET-B



```
   T7 promoter                                                                           RBS
AATACGACTC ACTATAGGGA GACCACAACG GTTTCCCTCT AGAAATAATT TTGTTTAACT TTAAGAAGGA

                                   Polyhistidine (6xHis) region
GATATACAT ATG CGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT
          Met Arg Gly Ser His His His His His His Gly Met Ala Ser  Met Thr

   T7 gene 10 leader                                  Xpress™ Epitope          BamH I      Xho I Sac I
GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GAT CCG AGC TCG
Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp Pro Ser Ser
                                          EK recognition site  EK cleavage site

Bgl II     Pst I  Pvu II   Kpn I Nco I   Eco R I  BstB I  Hind III
AGA TCT GCA GCT GGT ACC ATG GAA TTC GAA GCT TGA TCCGGCTGCT AACAAAGCCC
Arg Ser Ala Ala Gly Thr Met Glu Phe Glu Ala ***

                                 pRSET reverse priming site
GAAAGGAAGC TGAGTTGGCT GCTGCCACCG CTGAGCAATA ACTAGCATAA
```

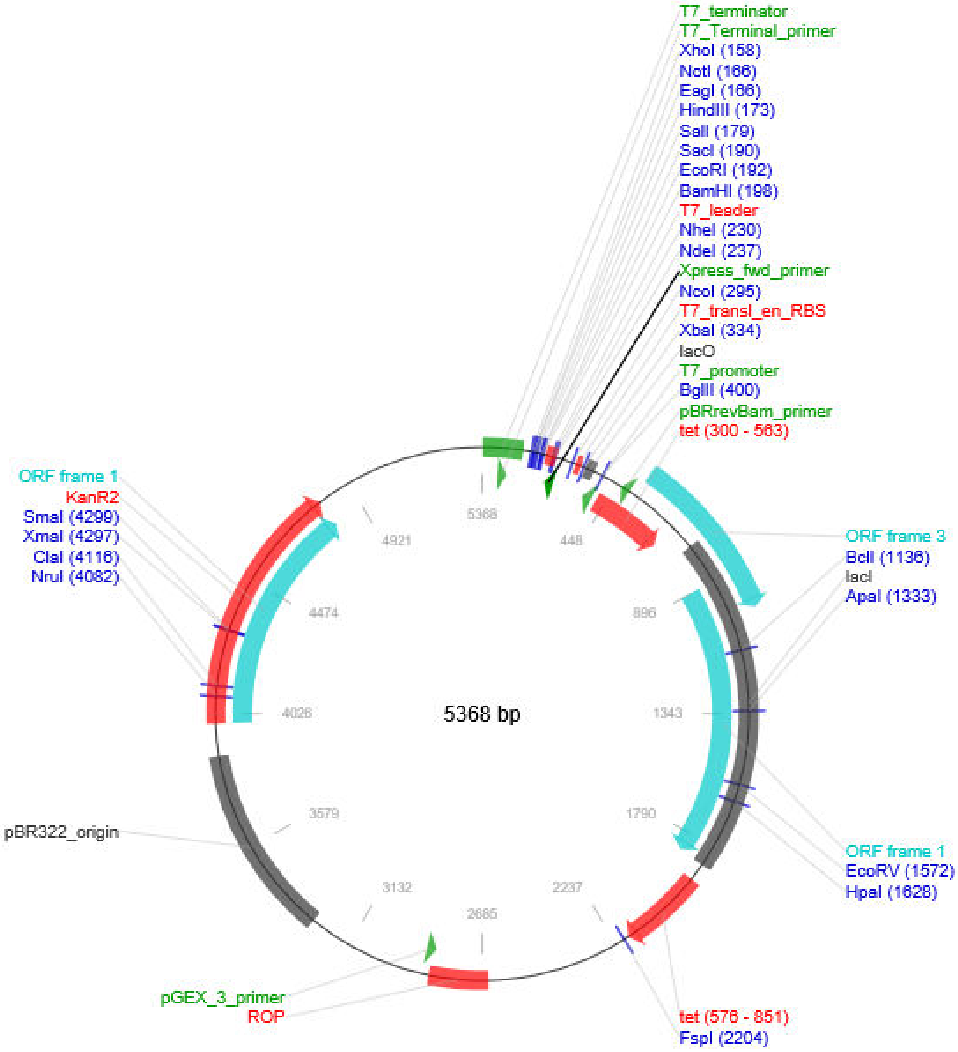
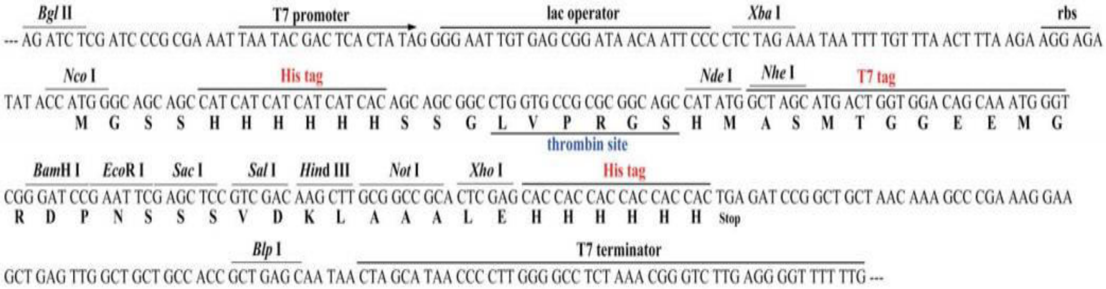


Fig. 11: DNA sequence of **multiple cloning site** (MCS) region- pET-28b (+)



--- AG ATC TCG ATC CCG CGA AAT TAA TAC GAC TCA CTA TAG GGG AAT TGT GAG CGG ATA ACA ATT CCC CTC TAG AAA TAA TTT TGT TTA ACT TTA AGA AGG AGA

TAT ACC ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG CCG CGC GGC AGC CAT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT
M G S S H H H H H H S S G L V P R G S H M A S M T G G E E M G

CGG GAT CCG AAT TCG AGC TCC GTC GAC AAG CTT GCG GCC GCA CTC GAG CAC CAC CAC CAC CAC CAC TGA GAT CCG GCT GCT AAC AAA GCC CGA AAG GAA
R D P N S S S V D K L A A A L E H H H H H H Stop

GCT GAG TTG GCT GCT GCC ACC GCT GAG CAA TAA CTA GCA TAA CCC CTT GGG GCC TCT AAA CGG GTC TTG AGG GGT TTT TTG ---

3.13 Restriction Digestion

` Restriction digestion of plasmids and insert for cloning was performed in 30 µL of solution containing added reagents in the order of water, 1X NEB enzyme buffer, 1 µg of DNA (vector, plasmid) and one unit of the restriction enzyme. All the contents were mixed gently by tapping. Reactions were incubated at 37°C for 3 h and analyzed by agarose gel electrophoresis. Enzymes were inactivated by heating as appropriate. For short term storage the product for short term storage the product was stored at 4°C. After analysis by agarose gel electrophoresis the product was purified by Qiagen PCR cleanup. The digested products were then used for ligation. The complete details of the vectors, restriction enzymes and the conditions used are given in Table 10.

Table 10. Details of the restriction enzymes with buffers used and inactivation temperatures

Insert	Vector	Restriction Enzymes	SITES	BUFFER	CONDITIONS
cVP2	**pRSET-B**	***BamHI***	GGATCC	CutSmart Buffer	37°C – 4 h 60°C – 20 min
		***Eco*RI**	GAATTC		
VP5	**pET-28b (+)**	***Nco*I**	CCATGG		37°C – 4 h 80°C – 20 min
		***Hind*III**	AAGCTT		
NS1	**pET-28b (+)**	***Nde*I**	CATATG		37°C – 4 h 80°C – 20 min
		***Hind*III**	AAGCTT		

3.14 Ligation

` Fragments to be ligated were double digested to generate sticky ends. Restriction digested fragments were ligated together in 20 µL solution containing: 1 x NEB T4 ligase buffer, T4 DNA ligase, vector and insert at 1:3 molar concentration calculated by using NEBiocalculator (Table 10). The reaction was incubated for 18 h at

16^oC followed by transformation into competent *E. coli* DH5α cells. Antibiotic resistant colonies were isolated and checked by colony PCR.

Table 11. Setup of ligation reaction

COMPONENT	REACTION (20µL)
T4 DNA Ligase Buffer (10X)*	2 µL
Vector : Insert	1:3
Nuclease-free water	to 20 µL
T4 DNA Ligase	1 mL

3.15 Preparation of *E. coli* DH5α competent cells

The competent cells were prepared using calcium chloride method as describe by Sambrook and Russell 2011.

1. *E. coli* DH5α cells (50 µL) from glycerol stock maintained at Directorate of PoultryResearch (ICAR-DPR) was inoculated into 5 mL of LB broth and was incubated overnight at 37^oC in a shaker incubator at 180 rpm until the culture reached an OD of 0.5 at 600 nm.

2. One mL of the overnight growth culture was inoculated into 100 mL of LB broth (1:100) and incubated at 37^oC in a shaker incubator at 180 rpm for 2-3 h till the bacterial growth attained log phase ($O.D_{600}$ of 0.3-0.4).

3. The culture was chilled on ice and the cells were harvested by centrifuging at 4000 rpm for 10 min at 4^oC in a cooling centrifuge.

4. The cell pellet was resuspended in 25 mL of ice cold solution of 100 mM $CaCl_2$ and chilled on ice for 30 min.

5. The cells were recovered by centrifugation as above and finally the pellet was resuspended in 1.75 mL 100 mM of ice-cold $CaCl_2$ and 0.75 mL of sterile glycerol.
6. The suspension was gently mixed and aliquoted at 100 µL volumes into pre-chilled 1.5 mL microcentrifuge tubes and stored at -80°C for further use.

3.16 Transformation of plasmids containing cVP2, VP5 and NS1 into DH5α cells

1. DH5α competent *E. coli* cells were thawed on ice for 10 minutes, until the last ice crystals disappeared.
2. Ligated products ware added to the cell mixture. Tube was carefully flicked 4–5 times to mix cells and DNA.
3. The mixture was placed on ice for 30 minutes.
4. Heat shock was given at exactly 42°C for 20 seconds, and placed on ice for 5 minutes.
5. LB broth (250 µL) was added into the mixture.
6. The mixture was placed at 37°C for 60 minutes in shaker incubator (250 rpm).
7. Then the cells were mixed thoroughly by flicking the tube and inverting,

Plating was done with three different volumes of 50 µL, 100 µL and 200 µL by spreading it onto a antibiotic selection plate and incubated overnight at 37°C.

3.17 Screening of recombinant clones of cVP2, VP5 and NS1

On the bottom of each petri dish, the colonies to be tested were marked. Using sterile toothpick, individual colonies were picked up and dipped into each reaction tube with appropriate labels. To create a stock of each individual colony, the toothpick was dipped into 3 mL of growth medium with appropriate antibiotics and cultured overnight. Gene-specific primers (cVP2, VP5 and NS1) were used for the particular clones using a 2 µL broth culture as a template. The mastermix and the PCR conditions used for the gene amplifications are mentioned in Table 4. The amplified products were subjected to gel electrophoresis with 100 bp, 1000 bp standard DNA molecular marker. The positive clones were subjected to plasmid isolation and stored at -80°C with 15% glycerol for further use.

3.18 Extraction of recombinant plasmid DNA

Plasmid DNA of the positive clones was extracted using QIAGEN® Plasmid Purification kit.

1. 100 µL of broth culture containing the positive clone was inoculated into 10 mL of LB broth.
2. The broth was inoculated overnight at 37°C in a shaker incubator at 180 rpm.
3. The culture was centrifuged at 6000*g* for 15 min at 4°C and the supernatant was discarded.
4. The pellet was resuspended in 250µL Buffer P1 with added RNase A. the pellet was resuspended completely by vortexing and pipetting.
5. Next, 250 µL of buffer P2 was added to the above and mixed by inverting the tubes 2-3 times.

6. To the above 350 µL of buffer P3 was added and mixed immediately by inverting the tube 10 times.
7. The mixture was centrifuged at 13200 rpm for 10 min; the supernatant was transferred to the column provided in the kit.
8. The column was centrifuged at 13000 rpm for 1 min and the flow through was discarded.
9. PE buffer 750 µL was then added to the column and centrifuged at 13200 rpm for 1 min; the centrifugation was repeated to remove the traces of ethanol.
10. The column was then transferred to fresh 1.5 mL microcentrifuge tube and 30 µL of nuclease free water was added carefully through the center of the column.
11. The column was allowed to stand for 5 min and centrifuged at 13200 rpm for 1 min.
12. The concentration of the plasmid was determined using a NanoDrop Spectrophotometer and the plasmid was stored at -20°C for further use.

3.19 DNA purity and concentration

The DNA purity in terms of concentration and quality was measured with the help of NanoDrop by taking the OD readings at 260 and 280nm. The good quality sample with OD_{260}:OD_{280} ranging from 1.8-2.0 was used further.

3.20 Sequencing

To confirm the identity of the rescued plasmid with insert, the plasmid with the inserts of cVP2, VP5 and NS1 were sent for Xcelris Labs Ltd, Gujarat. The sequences were obtained using Sangers sequencing. Sequencing was done using T7 promoter and T7 terminator primers of respective vectors into which they were cloned. The chromatograms received after sequencing were analyzed using MEGA. The sequence reading errors if any were edited and assembled using the BioEdit software and MEGA alignment module. The consensus sequence was obtained upon editing the forward and reverse sequences. Following sequence confirmation that the gene of interest was in frame, protein expression was initiated.

3.21 Transformation into expression hosts

Bl21 (DE3) *E. coli* host expression system was employed for the expression for cVP2, VP5 and NS1. Transformation protocol was the same as transformation of DH5α cells.

3.22 Protein Expression

Protein expression was carried out by IPTG induction for all the three recombinant proteins. However VP5, NS1 failed to show significant expression. Hence, autoinduction was employed for these two recombinant proteins.

IPTG Induction

For initial trial expressions 10 mL of cell culture were grown in LB with 100 µg/mL ampicillin at $37^{o}C$, in a shaker incubator until the OD_{600} reached 0.4-0.6, at which point IPTG was added at a concentration of 0.5 mM. To check expression, cells were spun down, lysed using lysis buffer and spun to remove insoluble debris. The insoluble pellet was re-suspended in an equal volume of lysis buffer. Total cell extract as well as soluble and insoluble fractions were taken to assess expression by Coomassie blue staining.

For large scale production, 1-5 L of liquid medium (with antibiotic) was inoculated with a freshly grown colony or 10 mL of freshly grown culture and incubated at 37^{o}C until OD_{600} reached 0.4–0.6 and IPTG was added to a concentration of 0.5 mM. after induction with IPTG cVP2 was grown for overnight and culture was collected at different time points 2 h, 4 h, 6 h, and overnight to check for the perfect induction. Once grown cells are spun down at 6,000 *g* for 20 min at 4^{o} C and frozen at -80^{o} C until needed.

Autoinduction

Autoinduction method was used for expression of VP5 and NS1. The autoinduction medium (ZYP-5052-SP) was prepared by using 1% Soy peptone, 0.5% yeast extract, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.05% glucose and 0.2% α-lactose. All the above solutions were autoclaved separately at 121°C for 15 minutes. The medium for autoinduction was prepared by mixing the above stock solutions aseptically. The preparation of these reagents and compositions are given in detail in Appendix. The expression protocol for autoinduction involved inoculation of the primary culture to freshly prepared autoinduction medium with kanamycin 50 µg/mL in a sterile flask under aseptic conditions. For pre-culture, 2 mL of the LB medium was inoculated with a single colony picked directly from the antibiotic selection LB plate, and then cultivated at 37^{o}C with shaking at 200 rpm for 6 h. then 1µL of the primary culture was inoculated to the freshly prepared autoinduction medium (2 mL) and incubated in a rotator shaker at 37^{o}C overnight. For the main cultivation, five seperate 200 mL cultures of autoinduction medium containing kanamycin 50 µg/mL were inoculated with 100 µL of the primary culture and grown at 37^{o}C overnight in a shaker incubater.

The cultures resulting from IPTG and autoinduction were centrifuged at 4000 rpm for 10 min and the cells were used for analysis.

3.23 Protein solubility

The culture before induction i.e. culture at OD_{600} of 0.4 served as uninduced culture. The sample after induction was centrifuged and pellet was used for further use. The pellet without addition of lysis buffer was considered as cell lysate.

1. To the pellet, lysis buffer (50 mM Tris pH 8.0, 10 mM, NaCl, 10 mM Imidazole, 1% glycerol and TritonX-100 0.1%) was added at a rate of 10 mL for 1 gram of pellet.
2. The sample was mixed by vortexing and sonicated at 30% amplitude, 10 cycles 15 s pulse with intervals of 30 s each on ice.
3. After sonication, the sample was centrifuged at 10000 rpm for 10 min at 4^{o}C.
4. Following centrifugation, the supernatant and the pellet were separated.
5. The pellet was redissolved in 500 µL of fresh lysis buffer, and this served as a pellet fraction.

3.24 SDS polyacrylamide gel electrophoresis

The pellet and the supernatant were collected separately and analyzed by using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmili (1970) followed by Coomassie Brilliant Blue R250 staining. The process was as follows.

1. The glass plates were thoroughly cleaned with absolute ethanol.
2. The gasket was secured and both the plates were clamped together in a slab gel cast (Bio-Rad Germany).
3. Resolving gel (Table 11) was freshly prepared and poured between the glass plates.
4. The gel was then allowed to polymerize at room temperature and the gel top was covered with deionized water such that the gel dries.

5. Succeeding polymerization of gel, the overlaid water was removed and the gel was washed with water to remove un-polymerized gel if any.
6. Stacking gel (Table 12) was poured till the brim over the polymerized gel. Comb was immediately placed between the plates with no air getting trapped between the comb and the gel.
7. The glass plate sandwich bearing polymerized gel was removed from the gel caster and was assembled into a vertical electrophoresis apparatus containing freshly prepared 1X Tris-Glycine running buffer. Combs were then gently removed from the gel and the wells were flushed with running buffer.

3.24.1 Preparation of samples and loading of samples

1. The sample was prepared by combining equal volumes of protein sample and Lamelli 2X treatment buffers in a tube and the tube was heated in a dry-bath at 95^{o}C for 10 min followed by centrifugation to pellet the cell debris.
2. Protein samples (20 µL) together with the protein marker (Medium Range-GeNei) were loaded into the wells.
3. The electrodes were placed to their respective sockets of buffer tank and the proteins were electrophorosed in 1X tank buffer at constant voltage till they entered the resolving gel.
4. Once they have entered the resolving gel, the voltage was increased and the run was stopped when the tracking dye reached the bottom of the gel.
5. The gel was removed from plates, rinsed with water and the resolving portion of the gel was subjected to staining.

3.24.2 Staining and destaining of gels

The gels were stained in 0.1% Coomassie Brilliant Blue R250, 40% (v/v) methanol, 10% (v/v) glacial acetic acid for 1hr. They were then destained using 20% (v/v) methanol, 10% (v/v) glacial acetic acid until the background of the gel was clear and protein bands were visible. Gel was visualized for resolved protein and compared with refence protein marker – Medium Range GeNei Bangalore.

Table 12. Compositions used for preparation of Resolving gel and Stacking gel

S.No	Reagents	Resolving Gel	Stacking Gel
1.	Water	1.9 mL	1.4 mL
2.	Tris	1.3 mL	250 µL
3.	Acrylamide30%	1.7 mL	330 µL
4.	SDS10%	50 µL	20 µL
5.	APs10%	50 µL	20 µL
6.	TEMED	2.5 µL	2.5 µL

Resolving gel-1.5 M Tris (pH-8.8); Stacking gel-1 M Tris- (pH-6.8)

3.25 Western blot

1. Initially, the proteins were subjected to (SDS-PAGE) was casted and electrophored in the manner described above.
2. Then, the gel was removed from the plate, washed gently with water and equilibrated in transfer buffer (Appendix I) for 15 min.
3. Meanwhile, PVDF (0.45µm, polyvinyledene fluoride) membrane and Whatsmanns filter paper were cut to the same as the gel circumference.
4. The PVDF membrane was initially immersed in 100% methanol for 15 sec and then in transfer buffer, while the filters were flooded directly in to the transfer buffer. This was followed by setting up of the transfer cassette.
5. The gel cassette was assembled by placing a fiber pad on top of the black side of the cassette, a piece of filter paper on top of the pad and pre-

equilibrated gel on top of the filter paper. Meanwhile, drenching was done with transfer buffer such that it (pad, paper and gel) did not get dried up.

6. Then a pre-equilibrated membrane was placed carefully on the drenched gel such that no air bubble got trapped under the gel. And on this membrane, a second piece of wetted fiber pad followed by wetted filter paper was placed.
7. This assembled cassette was closed, locked and placed back in to the western blotting tank (Bio-Rad) containing transfer buffer up to the fill line with the latch side up.
8. The tank top was covered with lid with color-coded cables (on lid) attached to the electrode cards of the same color.
9. They (electrodes) were then connected to a power supply and blotting was carried out (100V for 1 h).
10. The cassette was taken out and the gel-membrane sandwich was disassembled.
11. The PVDF membrane was washed gently in double distilled water such that no residual gel piece or sample adhered to membrane.
12. This blotted membrane was then submerged in 5% skim milk powder (SMP) for blocking the non-specific binding sites.
13. The skim milk powder solution was decanted and the membrane was washed with PBS containing 0.1% Tween-20.
14. The membrane was incubated with anti-His monoclonal antibodies (Sigma-Aldrich, Bengaluru, India) at a dilution of 1:15000 in 3% SMP in PBS at 4^{o}C overnight.
15. Next day, the membrane was washed (3 times) with the TBST for 5 min (incubate) for each wash (Appendix I) in order to get rid of unattached antibody (primary).

16. Then, after third wash, the membrane was exposed to 1:10000 diluted secondary antibody (Goat-antimouse IgG) for 60 min and was subjected to constant continuous rocking.
17. After incubation, three times washing was done with TBST for 5 min for each wash to drain out unbound secondary antibody.
18. Finally, the membrane was incubated (5-30 min) in TMB substrate solution till the bands appeared.
19. The TMB reaction was stopped by washing the membrane with water and letting it dry. The band was visualized for detection of target protein.
20. Membranes were incubated with ECL reagent (Thermo Scientific, Bengaluru, India), and His-tagged proteins were detected using X-ray film.

3.26 Protein purification

Following confirmation of the proteins on the western blot, the proteins were subjected to purification.

1. *E. coli* BL21 (DE3) cells expressing target proteins (cVP2, VP5 and NS1) were chosen for one liter expression and purification. After induction with 0.5 mM IPTG at 37°C for 8h, and autoinduced cultures of VP5 and NS1 cells were lysed with 50mM Tris pH8 10mM NaCl, 10 mM Imidazole, 1% glycerol and 0.1% Triton X-100.
2. Lysis buffer (10 mL) was added to 1g of pellet and mixed thoroughly by vortexing.
3. The pellet after treatment with the above was suspended for sonication at 30% amplitude, of 10 cycles 15 s pulse with 30 s each on ice.
4. The above was then centrifuged at 10000 rpm for 30 min at 4°C.

5. The supernatant after centrifugation was loaded onto the Ni column and allowed to flow completely into the gel bed.
6. The flow through fractions were retained and small aliquots of about 5 µL were used for analyzing on SDS-PAGE.
7. The column was washed with 50 mL of wash buffer.
8. The wash fractions were also retained to assess if any protein eluted in wash buffer.
9. The protein was eluted with a stepwise gradient of imidazole and 0.5 mL was collected as the first fraction and the rest as 1 mL fractions.
10. The absorbance was checked at 280 nm. Peak fractions were pooled and again absorbance was checked at A280 nm of the pool.
11. The purity of the eluted His-tag fusion protein was checked by SDS-PAGE and desalted by dialysis against desired buffer.

3.27 Dialysis

1. Required length of dialysis tubing was removed from the packaging tube with an additional ~1 inch (2.54 cm) for tube closure was pulled out.
2. The tubing was cut at a 90^o angle using scissor.
3. Approximately 0.25 inch at one end of the tubing was sealed using a thread.
4. Samples were added into the open end carefully and this end was also sealed as described above.
5. The seals were inspected to ensure ends are secured properly and there is no leakage.
6. Using an appropriate sized container, the filled dialysis tubing was floated in dialysis buffer.

3.27.1 Dialysis of cVP2

The elution fractions that had good purity and an OD $_{280}$ greater than 0.5 were pooled and loaded into the dialysis tubing as described above. The above solution was dialyzed against 0.5 M L-Arginine and 1X PBS for a period of 6 h with the change in buffer every 2 h. after dialysis.

3.27.2 Dialysis of VP5

The fractions corresponding to good purity were pooled and refolded by dialyzing against buffer 50 mM Tris-HCl, 150 mM NaCl, 10% Glycerol, 0.5 M L-Arginine, pH 8.0. The dialysis was performed with a 14 kDa cut-off dialysis membrane for 4 hours and changimg fresh buffer for additional 16 hours. After dialysis, the sample was centrifuged at 13000 rpm for 30 min and filtered through a 0.22 µm membrane.

3.27.3 Dialysis of NS1

Pooled fractions of the protein that had good purity and refolding was done by dialyzing against buffer 1×PBS, 10% glycerol, 0.2% SDS, pH 7.4. The dialysis was performed in 14 kDa cut-off dialysis membrane for 4 hours and changing fresh buffer for additional 16 hours. After dialysis, the sample was centrifuged at 13000 rpm for 30 min and filtered through a 0.22 µm membrane. The three protein samples after dialysis were again run on SDS-PAGE to check the purity of the protein and then quantified by Bradford's method of protein estimation.

3.28 Procedure for Bradford's method of Protein Estimation.

1. Standards BSA (0.5 mg/mL) and test sample was prepared in duplicates.
2. Adjusted the volume to 0.2 mL with distilled water.
3. Bradford's reagent (3 mL) was added and mixed thoroughly. Incubated at room temperature for 10 minutes.
4. Optical density on spectrophotometer at 595 nm was recorded.
5. Calibration or standard curve was constructed by plotting average optical density reading on 'Y' axis against standard protein concentration (in µg) on 'X' axis.
6. The 'x' value was recorded from the graph corresponding to the optical density reading for the test sample.
7. Calculated the concentration of protein in the test sample using the following formula: Protein concentration in test sample = x/v mg/mL.

Where x = Value from graph in µg v = Volume of sample in µL.

Using a standard curve, concentration of protein in the test sample was determined.

3.29 Recombinant Subunit Vaccine

The protein (cVP2, VP5 and NS1) concentration in the purified suspension was adjusted to 25 µg/mL and was blended with Montanide ISA in 1:1 ratio and homogenized thoroughly. The mixture was used as inocula.

3.30 Sterility check

The sterility of the vaccines was confirmed by seeding 0.1 mL of the protein on to nutrient agar in duplicate of which one was placed at 24°C and another at 37°C for two days to check for any microbial growth

3.31 Immunogenicity studies

3.31.1 Mice

Immunization studies were carried out with 36 BALB/c mice. Animals were divided into six groups (Group 1 to Group 6) with six animals in each group (Table 12). Group 6 containing 6 animals was kept as control and received plain Montanide. Test animals were injected with 25 µg/mL of the purified protein mixed with equal quantity of Montanide subcutaneously at the back of the neck. The commercially available pentavalent vaccine (RAKSHA-BLU, Indian Immunologicals) containing BTV-1, -2, -10, -16 and -23 were used to immunize the Group 5 animals. Animals were boosted twice with the same antigen concentrations at days 14, 28 and were monitored till day 40. All the animals in the study were examined daily for the presence of reactions if any till day 40.

Table 13. Protocol for immunization of mice with recombinant proteins

Group	**No. of animals**	**Day 0**	**Day 14**	**Day 28**	**Day 41**
Group 1 (cVP2)	6	25 µg	25 µg	25 µg	Blood Collection
Group 2 (VP5)	6	25 µg	25 µg	25 µg	Blood Collection
Group 3 (NS1)	6	25 µg	25 µg	25 µg	Blood Collection
Group 4 (cVP2, VP5 NS1) ~25 µg each	6	(cVP2,VP5 NS1)~25 µg each	(cVP2,VP5 NS1)~25 µg each	(cVP2,VP5 NS1)~25 µg each	Blood Collection
Group 5	6	100 µL of pentavalent vaccine	100 µL of pentavalent vaccine	100 µL of pentavalent vaccine	Blood Collection
Group 6 (Control)	6	Montanide	Montanide	Montanide	Blood Collection

3.31.2 Animal Inoculations

Syringe was filled with appropriate amount of inocula and then the animal was restrained. The area of inoculation was cleaned with alcohol swab. The needle was inserted at the base of the skin fold between thumb and opposing finger. The inocula were administered in a steady fluid motion.

3.31.3 Blood Collection-Intracardiac Puncture

Mice were anaesthetized with isopropyl alcohol. Needle was inserted at the base of sternum at a 20° angle under or just left to the xyphoid process, and then the syringe was aspirated slowly. Around 1 mL of the blood was collected from each mouse.

3.31.4 Processing of the sample

After collection of the whole blood sample in the BD vaccutainers the samples were allowed to clot by leaving it undisturbed for 30 min at room temperature. Clot was removed by centrifugation at 4000 rpm for 15 min at 4°C. The resulting supernatant (serum) was transferred immediately to a clean micro-centrifuge tube and stored at -80°C till use.

3.32 Standardization of Indirect-Enzyme Linked Immunosorbent Assay (I-ELISA) with recombinant antigens

Indirect ELISA was standardized by checkerboard titration method. The antigen was diluted in carbonate bicarbonate buffer pH 9.6 and used for coating ELISA plates. The optimum concentration of antigen and antibody to be used in ELISA was determined by checkerboard titration using different concentrations of antigen against different dilutions of 40 day serum samples. The dilution of the antigen which showed maximum absorbance reading and started to maintain almost stationary phase was taken as the optimum. The absorbance readings were taken at 450 nm in ELISA reader.

3.32.1 Determination of antibody levels by ELISA

1. Polyvinyl microtitration plates were coated with 100 µL of the optimized antigen that was diluted with 0.05 M carbonate buffer, pH 9.6, and incubated overnight at 4°C to allow antigen to absorb passively to the ELISA plate.
2. After incubation, antigen was removed by emptying the wells and washing. This was done by adding with 200 µL of phosphate buffered saline with tween (PBST) in wells and incubating for 5 min with gentle rocking

followed by emptying of the plates. The process was repeated thrice; 100 µL of blocking buffer was added to each well and incubated for 60 min at 37°C.

3. The plates were washed with (PBS-T) three times. Sample was tested in duplicate in serum diluent buffer and plates were incubated for 90 min at 37°C.
4. The plates were washed with PBST for three times. 100 µL of goat anti-mouse IgG-horseradish peroxidase(HRP) (1:200) diluted in dilution buffer was added to all wells and incubated for 90min at 37° C.
5. The plates were washed with PBS-T 3 times. Substrate tetramethyl benzidine/hydrogen peroxide (TMB) of 50 µL was added to all wells and incubated at room temperature for 15 min.
6. Finally, 50 µL of 1N sulphuric acid was added to all wells as a stopping solution. The plates were read at 450 nm in micro plate ELISA reader.

3.32.2 I-ELISA for detection of field samples

The procedure was the same from step1 to step3 as above except that the conjugate was anti-sheep IgG (whole molecule)–peroxidase antibody produced in donkey (Sigma-Aldrich). Anti-sheep IgG conjugate was diluted at 1:1000 ratio with dilution buffer. Diluted conjugate (100 µL) was added to each well and incubated at room temperature for one hour. Buffer substrate solution was prepared by dissolving the contents of buffer substrate capsule in 100 mL of deionized water. Two mg of OPD (1 mg/2.5 mL) tablet is dissolved in 5 mL buffer substrate solution. After preparing the OPD buffer substrate solution, the wells were washed five times with washing buffer. Then 100 µL of substrate OPD solution was added to all the wells and the plate was incubated for 10 minutes in dark. After 10 minutes, 50µL of stop reagent (3 N sulfuric acid) was quickly added to all the wells and OD values for each well were taken at 490 nm wavelength using ELISA microplate reader.

3.33 NEUTRALLIZATION STUDIES

Neutrallization studies were carried out using the plaque purified BTV- 1, -2, -4, -5, -9, -10, -12, -16, -21 -23 and -24 serotypes at a concentration of 100 $TCID_{50}$ incubated with equal amount of increasing dilution of serum. 96-well plates were seeded with Vero cells at the concentration of 1×10^4 cells per well in 5% MEM and incubated at 37^oC with 5% $CO_{2.}$ On second day, after observing monolayer in 96-well plate, serum raised against cVP2, VP5 and NS1 was serially diluted (1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640 and 1/1280) and 50 µL each of the diluted serum was incubated with equal quantity of 100 $TCID_{50}$/50µL virus in deep well plate for 1 hour at 37^oC. Hundred µL of this mix was used to infect each well in triplicate. Plates were observed under inverted microscope at 12 h interval for CPE and the final titer was taken on day five. The neutralization titre was determined as the dilution of serum giving a 50% neutralization endpoint. SNT antibody titres were expressed as $\log_{10}$ reciprocal of the highest positive serum dilution.

CHAPTER-IV

RESULTS

4.1 Identification of conserved epitopes in VP2 and VP5 and their homology modelling

A compilation was prepared for the available 29 serotypes of bluetongue virus for segment 2 that codes for VP2, segment 6 that codes for VP5 protein, and segment 5 that codes for NS1 from NCBI GenBank. MEGA analysis revealed conserved regions among different serotypes for VP2 and VP5 (Fig. 12 & 14) and NS1 is much conserved throughout the gene sequence. In the Seg-2 alignment, a number of gaps due to insertions and deletions among the different serotypes were observed whereas VP5 nucleotide and protein sequences were rather conserved. The amino acid sequence alignment showed that, though VP2 is highly variable, several regions are relatively more conserved between serotypes. According to the present study analysis, most conserved region of VP2 is evident from 338-383 aa (Fig. 13). Interestingly, 3D homology modeling of VP2 protein of different serotypes of BTV revealed different structures whereas cVP2 region of those serotypes revealed similar protein structure (Fig. 15). Being rather conserved, VP5 of different BTV serotypes revealed similar protein structure on homology modelling (Fig. 15).

4.1.2 Selection pressure analysis on VP2 and VP5

To better understand the evolutionary dynamics, we sought to determine the selection pressure acting on individual codons of VP2 and VP5. The global estimate of *dN/dS* was 0.16 for VP2 and 0.058 for VP5, indicating strong purifying selection. Moreover, high number of negatively selected codons was identified in both (850/988 for VP2 and 506/528 for VP5). Even more interesting observation was that all the codons in the putative conserved region of VP2 (cVP2) are under strong negative selection.

4.1.3 Immunogenicity of cVP2

Two MHCI, two MHCII binding, and three B cell epitopes were predicted with significant percentile ranks suggesting a strong immunogenicity potential of cVP2 (Table 14).

```
Species/Abbrv               *  *  * * *    * *  ********   * *  *** *   *
1. KP696573 BTV1 (VP2 IND) RFYILLMIAASDTFNTRIWWSNPYPCLRGTLMASETKLGDVYSMIR
2. KP696603 BTV2 (VP2IND)  RFYVLLAIAASDTYNSRIWWSNPYPCLRGTLIAAETKLGDVYFTLR
3. KF560418 BTV4 (VP2IND)  RFYALIAIAATDTQRGRVWRTNPYPCLRGAIIASECELGDVYYTLR
4. KP696633 BTV9 (VP2IND)  RFYALLLICATDAYSRRIWRSNPYPCLRGTLIAAECVLGDPYKTLR
5. KP339245 BTV10 (VP2IND) RFYALIAIAATDTQQGRVWRTNPYPCLRGALIAAECELGDVYFTLR
6. KC662613 BTV12 (VP2IND) KFYALLAIAATDSYRWRIWWSNPYPCLRGTIIACEMELGDVYKTLR
7. KP339225 BTV16 (VP2IND) RFYVLLMIAASDTNNGRVWWSNPYPCLRGALIASECKLGDVYHKLR
8. KP339235 BTV21 (VP2IND) RFFVLIMIAASDTYNSRIWWTNPYPCLRGALIASETSLGDVYWTLR
9. KP696691 BTV23 (VP2IND) RFYVLIMIAASDTYKGRIWWSNPYPCLRGSLIAAEAQLGDVYNTMR
```

Fig. 12: Clustal W multiple nucleotide sequence alignment of VP2 segment of different BTV serotypes (obtained from GenBank)

Alignment of the VP2 segment showing similar color across different serotypes indicates homologous (conserved) region and different color indicates partial or nonhomologous region.

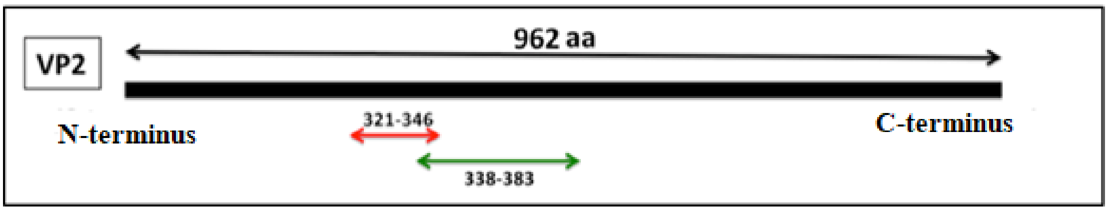


Fig. 13: VP2 protein architecture

Shown here is the overlap of conserved region of VP2 (cVP2) in green with the putative neutralising epitope region (red).

```
Species/Abbrv            * *  * *********** * ***** ***** * *  *  ** ******** * ** ** **** *   **  *
1. KF664128 BTV1 (VP5INI EIERDGMQEEAIQEIAGMTADVLEAASEEVPLIGAGMATAVATGRAIEGAYKLKKVINALSGIDLTHLRTPKIEPSVV
2. JQ904068 BTV2 (VP5INI EIERDGMQEEAIQEIAGMTADVLEAASEEVPLIGAGMATAVATGRAIEGAYKLKKVINALSGIDLTHLRTPKIEPSVV
3. KF560422 BTV4 (VP5INI EIERDGMQEEAIQEIAGMTADVLEAASEEVPLIGAGMATAVATGRAIEGAYKLKKVINALSGIDLSHMRSPKIEPTII
4. KP339249 BTV10 (VP5II EIERDGMQEEAIQEIAGMTADVLEAASEEVPLIGAGM?TAVATGRAIEGAYKLKKVINALSGIDLSHMRSPKIEPTII
5. KC662617 BTV12 (VP5II EVEKEGLQEEAIQEIAGMSAEVLEAAAEEVPLFGAGVATSIATARAIEGAYKIKAVISALTGIDLTHLNVPKVQPKTL
6. KF664138 BTV16 (VP5IN EIERDGMQEEAIQEIAGMTADVLEAASEEVPLVGSGMATAIATGRAIEGAYKLKKVINALSGIDLSHLRTPKIEPTMV
7. KP339239 BTV21 (VP5II EIERDGMQEEAIQEIAGMTADVLEAASEEVPLVGSGMATAIATGRAIEGAYKLKKVINALSGIDLSHLRTPKIEPTMV
8. KP696685 BTV23 (VP5II EVERDGMQEEAIQEIAGMTADVLEAASEEVPLIGAGMATAVATGRAIEGAYKLKKVINALSGIDLTHLRTPKIEPTVV
```

Fig. 14: Clustal W multiple nucleotide sequence alignment of VP5 segment of different BTV serotypes (obtained from Gene Bank)

Alignment of the VP5 segment showing similar color across different serotypes indicates homologous (conserved) region and different color indicates partial or nonhomologous region.

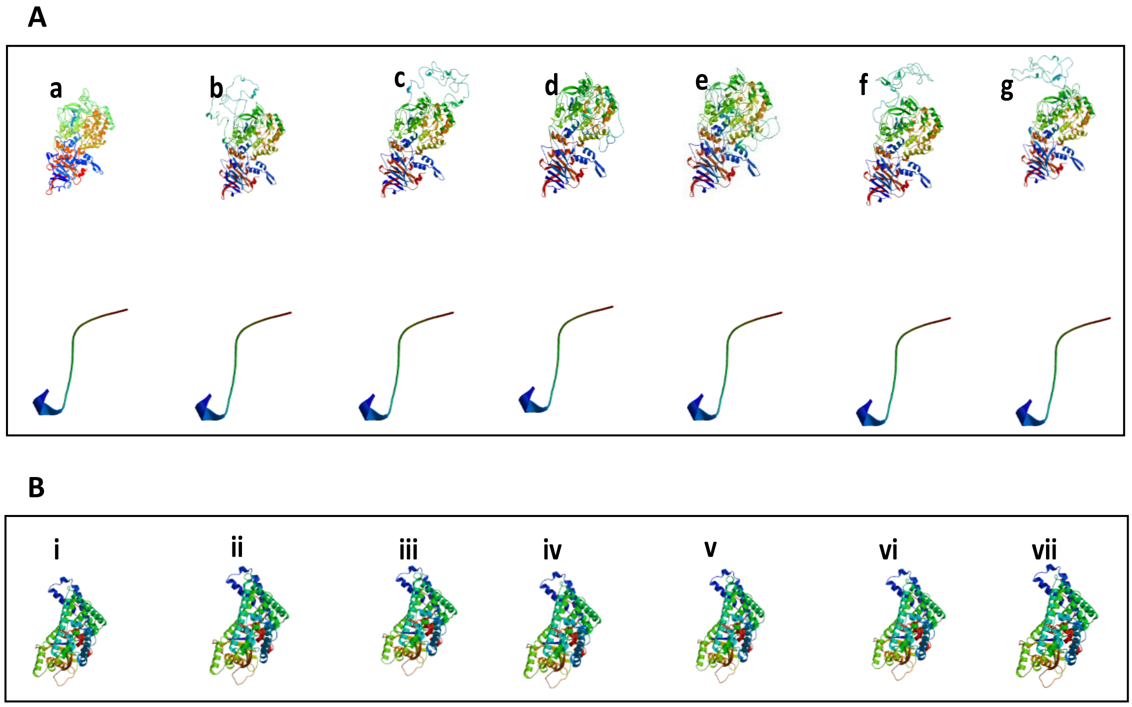


Fig. 15: Similar 3-D protein structure of cVP2 among different BTV serotypes

EXPASY Swiss Model generated homology 3D models of proteins VP2 (top panel of A), cVP2 (bottom panel of A), and VP5 (B) belonging to BTV serotypes 1, 2, 4, 10, 16, 21, and 23 as depicted in a, b, c, d, e, f, g and i, ii, iii, iv, v, vi, and vii, respectively. Variation in homlogy models of VP2 can be seen among BTV serotypes, whereas similar homology structures can be seen in cVP2 and VP5 of different BTV serotypes.

Table 14. Putative antigenic epitopes of conserved VP2 region (cVP2)

	Position in cVP2 (338-383 aa)	**Sequence**
B cell epitope	355-364	IWWSNPYPCL
	373-381	CELGDVYKT
	347-353	ATDTYNG
MHC I epitope	357-368	WSNPYPCLRGAL
	358-368	SNPYPCLRGAL
MHC II epitope	338-352	RFYALIMIAATDTYN
	339-353	FYALIMIAATDTYNG

4.2 Propagation and cultivation of BTV in BHK-21 cell line

BHK-21 cells that showed a confluency of 70-80% within 36 h of subculture (Fig. 16A) were used for infection with BTV serotypes-1, -2, -4, -5, -9, -10, -12, -16, -21, -23 and -24. After 3 days of incubation, a CPE, characterized by retraction of the cell membrane, rounding and clumping of the nuclear chromatin, was observed, followed by detachment of cells from the monolayer a day after the observation of the CPE (Fig. 16B). After detachment of the monolayer, the virus fluid was sealed and stored at 4^{o}C until use in the form of infected tissue culture fluid.

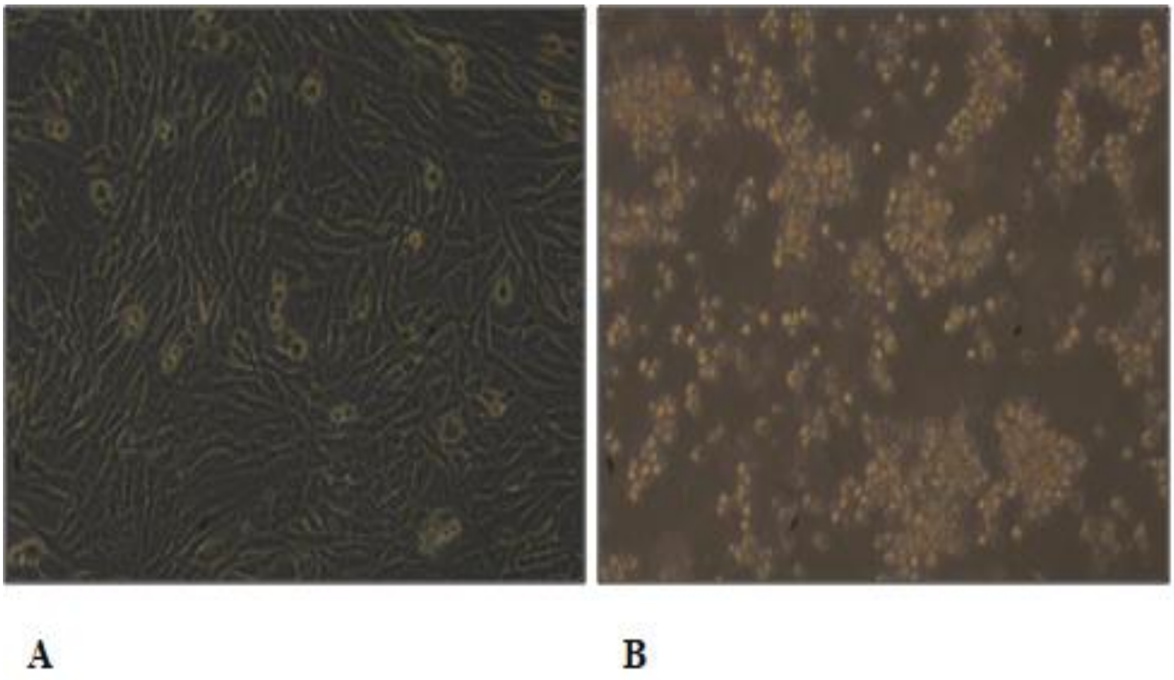


Fig. 16: Propagation of BTV-16 in BHK-21 cell line:

16A. Uninfected BHK-21 cell line showing adherent fibroblast monolayer.
16B. BTV-16 infected BHK-21 cells showing cytopathic effect characterized by rounding and degeneration of the infected cells.

4.3 RNA Extraction and RT PCR

RNA extracted from BTV-16 infected BHK-21 cell culture fluid by Trizol method showed clear segmented pattern of dsRNA genome with clearly resolved eight segments and two unresolved segments when analyzed on 1.5% agarose gel (Fig. 17A). The isolated RNA was used to synthesize cDNA followed by PCR with cVP2, VP5 and NS1 gene-specific primers and PCR products were visualized on agarose gel electrophoresis. The amplified products had a size of 265 bp (cVP2), 1580 bp (VP5) and 1660 bp (NS1) (Fig. 17B, 17C and 17D).

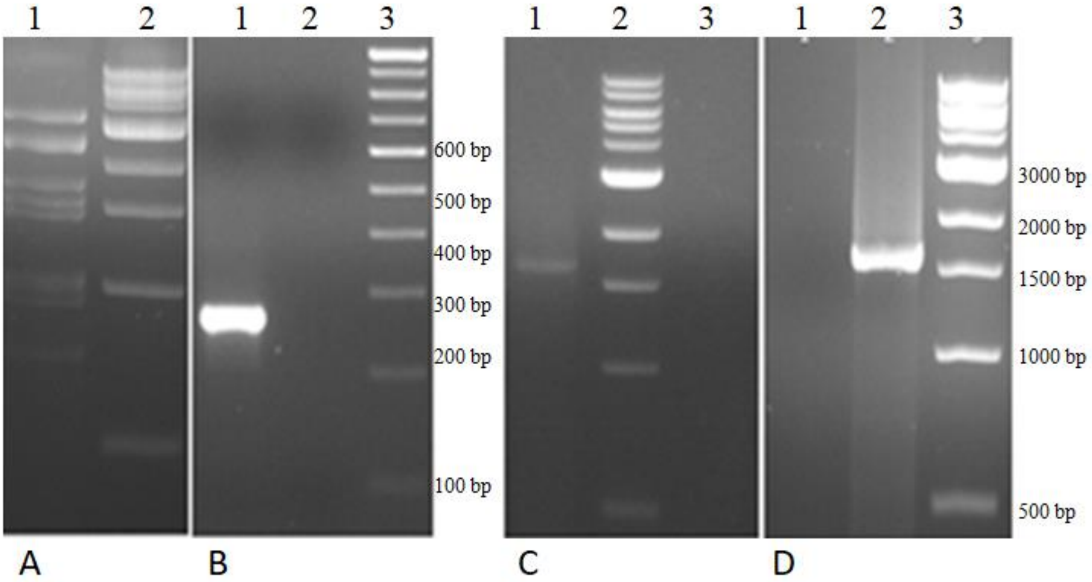


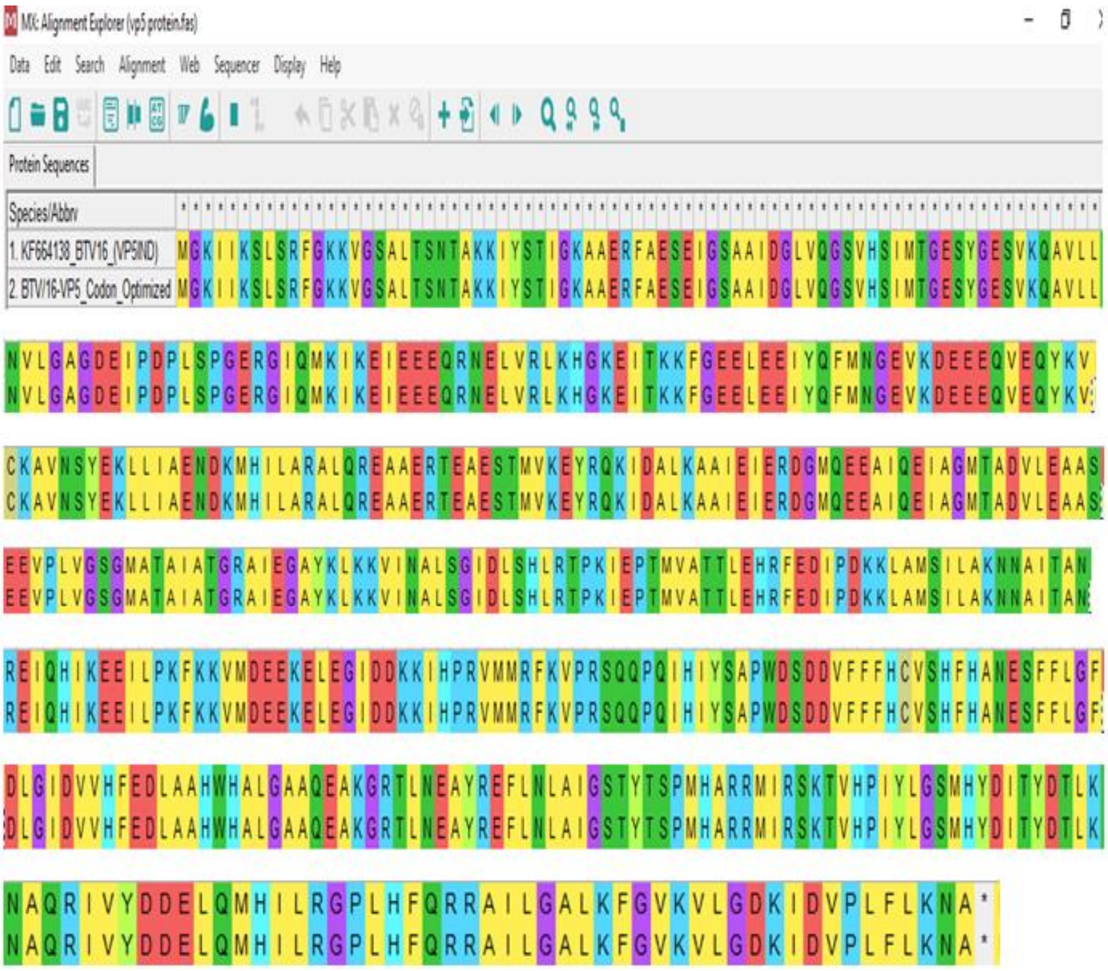
Fig. 17: Amplification of cVP2, VP5, and NS1:

17A: Lane 1: Double-stranded ten segmented pattern of BTV-16 RNA; Lane 2: Molecular weight marker.
17B: Lane 1: Amplification of cVP2 (265 bp); Lane 2: Negative control; Lane 3: 100 bp DNA ladder.
17C: Lane 1: Amplification of VP5 (1610 bp); Lane 2: 1 kbp DNA ladder Lane 3: Negative control.
17D: Lane 1: Negative control; Lane2: Amplification of NS1 (1671 bp); Lane 3: 1 kbp DNA ladder.

4.4 Cloning and Transformation

The amplified cVP2 was cloned into pRSET-B vector. However, VP5 and NS1 could not be expressed upon cloning in different bacterial vectors. Since the failure could have been due to codon bias for protein expression in bacterial system, these two genes were codon optimized to be cloned and expressed in BL21 (DE3) host system. With this, the gene sequence was altered for efficient codon usage by *E. coli's* transcription machinery but the protein sequence remained unchanged (Fig. 18 & 19).

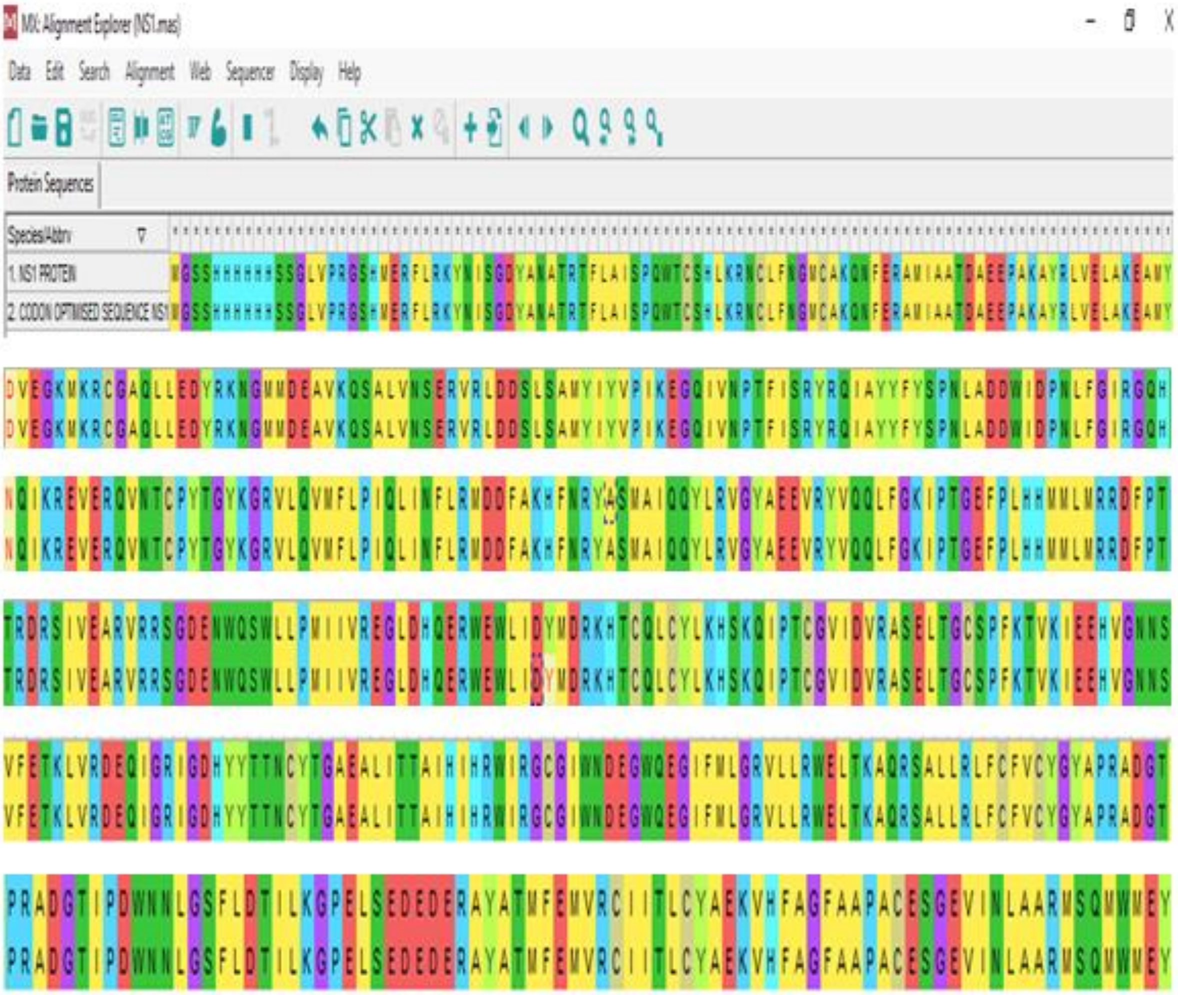
The codon optimized genes were used to amplify products for cloning using primers as mentioned in the materials and methods. The PCR products (cVP2/VP5/NS1) and vectors were purified by using QIAGEN purification kit and purified products were used for restriction digestion. The insert and vector were digested with the respective restriction enzymes as given in Table 9. The digested insert and vector were purified with QIAGEN PCR purification kit and used for ligation. The purified insert and vector were quantified and used for ligation with T4 DNA ligase (16^oC overnight) with 1:3 molar ratio of vector:insert calculated by using NEBiocalculator. After ligation of insert and vector, the ligated products were transformed into DH5α competent cells. The products were transformed by heat shock treatment and colonies were formed on LB plate with ampicillin (100 µg/mL) for cVP2 and kanamycin(30 µg/mL) for VP5 and NS1 (Fig. 20a, 21a & 22a).



```
1. KF664138_BTV16_(VP5IND)         MGKIIKSLSRFGKKVGSALTSNTAKKIYSTIGKAAERFAESEIGSAAIDGLVQGSVHSIMTGESYGESVKQAVLL
2. BTV/16-VP5_Codon_Optimized      MGKIIKSLSRFGKKVGSALTSNTAKKIYSTIGKAAERFAESEIGSAAIDGLVQGSVHSIMTGESYGESVKQAVLL

NVLGAGDEIPDPLSPGERGIQMKIKEIEEEQRNELVRLKHGKEITKKFGEELEEIYQFMNGEVKDEEEQVEQYKV
NVLGAGDEIPDPLSPGERGIQMKIKEIEEEQRNELVRLKHGKEITKKFGEELEEIYQFMNGEVKDEEEQVEQYKV

CKAVNSYEKLLIAENDKMHILARALQREAAERTEAESTMVKEYRQKIDALKAAIEIERDGMQEEAIQEIAGMTADVLEAAS
CKAVNSYEKLLIAENDKMHILARALQREAAERTEAESTMVKEYRQKIDALKAAIEIERDGMQEEAIQEIAGMTADVLEAAS

EEVPLVGSGMATAIATGRAIEGAYKLKKVINALSGIDLSHLRTPKIEPTMVATTLEHRFEDIPDKKLAMSILAKNNAITAN
EEVPLVGSGMATAIATGRAIEGAYKLKKVINALSGIDLSHLRTPKIEPTMVATTLEHRFEDIPDKKLAMSILAKNNAITAN

REIQHIKEEILPKFKKVMDEEKELEGIDDKKIHPRVMMRFKVPRSQQPQIHIYSAPWDSDDVFFFHCVSHFHANESFFLGF
REIQHIKEEILPKFKKVMDEEKELEGIDDKKIHPRVMMRFKVPRSQQPQIHIYSAPWDSDDVFFFHCVSHFHANESFFLGF

DLGIDVVHFEDLAAHWHALGAAQEAKGRTLNEAYREFLNLAIGSTYTSPMHARRMIRSKTVHPIYLGSMHYDITYDTLK
DLGIDVVHFEDLAAHWHALGAAQEAKGRTLNEAYREFLNLAIGSTYTSPMHARRMIRSKTVHPIYLGSMHYDITYDTLK

NAQRIVYDDELQMHILRGPLHFQRRAILGALKFGVKVLGDKIDVPLFLKNA*
NAQRIVYDDELQMHILRGPLHFQRRAILGALKFGVKVLGDKIDVPLFLKNA*
```

Fig. 18: Amino acid sequence alignment of VP5 protein from BTV-16 serotype and codon optimized amino acid sequence after translation

Alignment showing complete homology (similar color) between BTV-16 VP5 protein and the codon optimized translated sequence.



```
1. NS1 PROTEIN                      MGSSHHHHHHSSGLVPRGSHMERFLRKYNISGDYANATRTFLAISPQWTCSHLKRNCLFNGMCAKQNFERAMIAATDAEEPAKAYRLVELAKEAMY
2. CODON OPTIMISED SEQUENCE NS1     MGSSHHHHHHSSGLVPRGSHMERFLRKYNISGDYANATRTFLAISPQWTCSHLKRNCLFNGMCAKQNFERAMIAATDAEEPAKAYRLVELAKEAMY

DVEGKMKRCGAQLLEDYRKNGMMDEAVKQSALVNSERVRLDDSLSAMYIYVPIKEGQIVNPTFISRYRQIAYYFYSPNLADDWIDPNLFGIRGQH
DVEGKMKRCGAQLLEDYRKNGMMDEAVKQSALVNSERVRLDDSLSAMYIYVPIKEGQIVNPTFISRYRQIAYYFYSPNLADDWIDPNLFGIRGQH

NQIKREVERQVNTCPYTGYKGRVLQVMFLPIQLINFLRMDDFAKHFNRYASMAIQQYLRVGYAEEVRYVQQLFGKIPTGEFPLHHMMLMRRDFPT
NQIKREVERQVNTCPYTGYKGRVLQVMFLPIQLINFLRMDDFAKHFNRYASMAIQQYLRVGYAEEVRYVQQLFGKIPTGEFPLHHMMLMRRDFPT

TRDRSIVEARVRRSGDENWQSWLLPMIIVREGLDHQERWEWLIDYMDRKHTCQLCYLKHSKQIPTCGVIDVRASELTGCSPFKTVKIEEHVGNNS
TRDRSIVEARVRRSGDENWQSWLLPMIIVREGLDHQERWEWLIDYMDRKHTCQLCYLKHSKQIPTCGVIDVRASELTGCSPFKTVKIEEHVGNNS

VFETKLVRDEQIGRIGDHYYTTNCYTGAEALITTAIHIHRWIRGCGIWNDEGWQEGIFMLGRVLLRWELTKAQRSALLRLFCFVCYGYAPRADGT
VFETKLVRDEQIGRIGDHYYTTNCYTGAEALITTAIHIHRWIRGCGIWNDEGWQEGIFMLGRVLLRWELTKAQRSALLRLFCFVCYGYAPRADGT

PRADGTIPDWNNLGSFLDTILKGPELSEDEDERAYATMFEMVRCIITLCYAEKVHFAGFAAPACESGEVINLAARMSQMWMEY
PRADGTIPDWNNLGSFLDTILKGPELSEDEDERAYATMFEMVRCIITLCYAEKVHFAGFAAPACESGEVINLAARMSQMWMEY
```

Fig. 19: Amino acid sequence alignment of NS1 protein from BTV-16 serotype and Codon optimized amino acid sequence after translation

Alignment showing complete homology (similar color) between BTV-16 NS1 protein and the codon optimized translated sequence.

4.5 Colony PCR

To confirm the presence of insert and to make a stock of each individual colony, individual colonies were picked with a sterile toothpick, which was then dipped into 3 mL growth medium with appropriate antibiotics and cultured overnight. Gene-specific primers (cVP2, VP5 and NS1) were used for the particular clones using 2 µL broth culture as a template. The amplified products were subjected to gel electrophoresis with 100 bp, 1000 bp standard DNA molecular marker. The positive clones having amplicon sizes of 265 bp, 1610 bp and 1671 bp for cVP2, VP5 and NS1, respectively (Fig. 20b, 21b &22b) were subjected for plasmid isolation and further stored at -80^{0}C with 15% glycerol for further use.

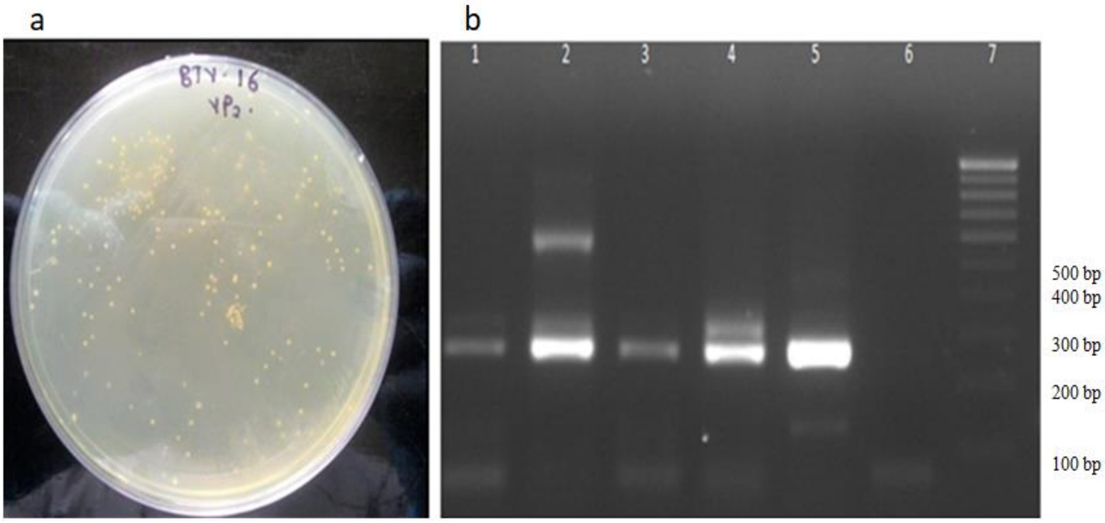


Fig: 20. Transformation of cVP2 in DH5α

Fig. 20a: Recombinant colonies cVP2 upon transformation in DH5α cells
LB plate with ampicillin showing transformed colonies of cVP2.
Fig. 20b: Colony PCR of the transformed clones cVP2
Lanes 1, 2, 3, 4=Amplified products of cVP2 at 265 bp; Lane 5= Positive Control; Lane 6= Negative control; Lane 7=100 bp Ladder

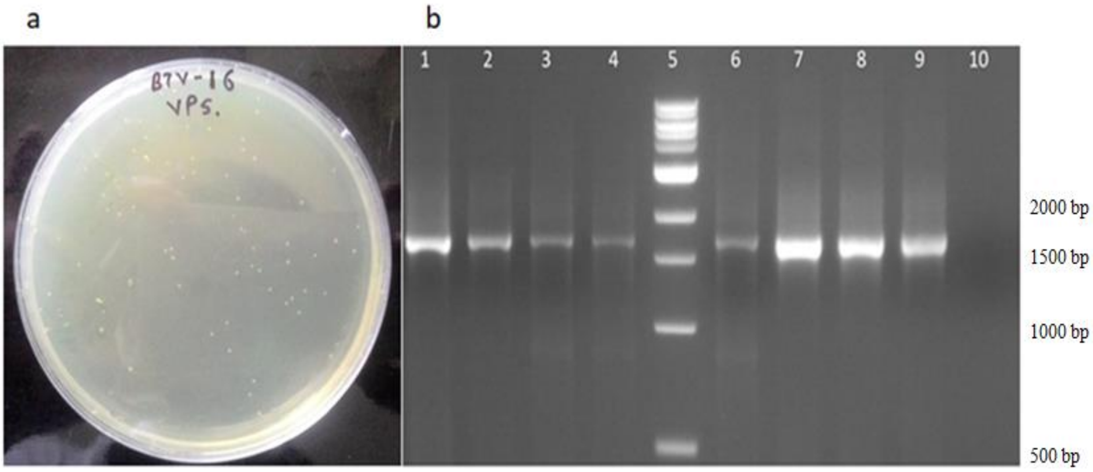


Fig: 21. Transformation of VP5 in DH5α

Fig. 21a: Recombinant colonies VP5 upon transformation in DH5α cells
LB plate with ampicillin showing transformed colonies of VP5.
Fig. 21b: Colony PCR of the transformed clones VP5
Lanes 1, 2, 3, 4, 6, 7, 8 =Amplified products of VP5 at 1610bp; Lane 5=1 Kb Ladder; Lane 9=Positive Control; Lane 10=Negative Control.

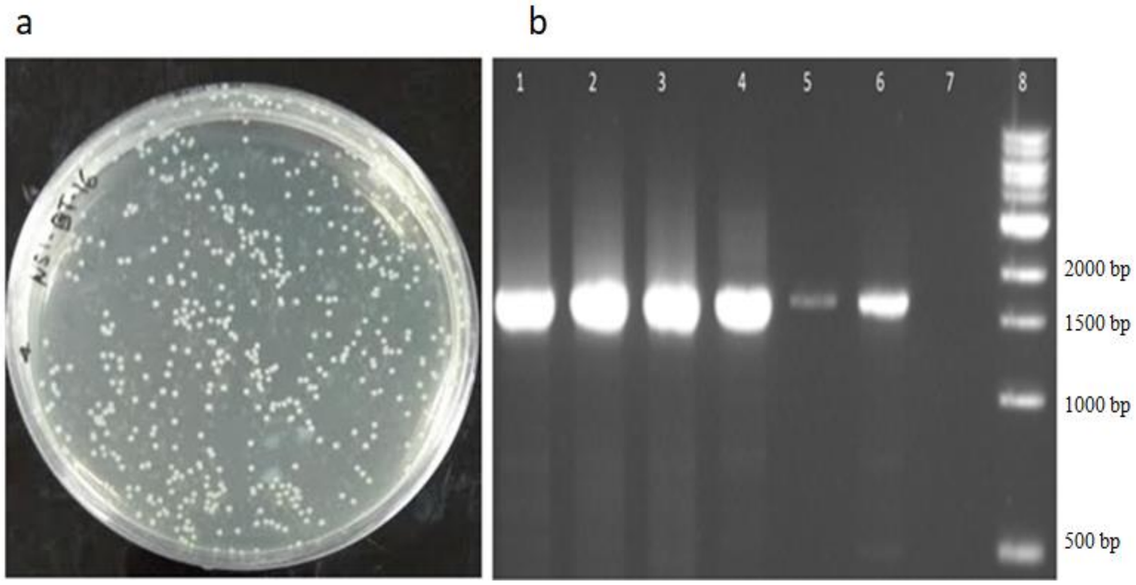


Fig. 22: Transformation of NS1 in DH5α

Fig. 22a: Recombinant colonies NS1 upon transformation in DH5α cells
LB plate with ampicillin showing transformed colonies of NS1.
Fig. 22b: Colony PCR of the transformed clones NS1
Lanes 1, 2, 3, 4, 5= Amplified products of NS1at 1671 bp; Lane 6 =Positive control; Lane 7= Negative control; Lane 8= 1 Kb Ladder.

4.5.1 Confirmation of transformants by restriction digestion and sequence analysis

The positive clones of cVP2, VP5 and NS1 genes in colony PCR were inoculated into LB broth with ampicillin 100 µg/mL for cVP2 and kanamycin 50 µg/mL for VP5 and NS1. The plasmids were isolated from overnight culture with plasmid isolation kit (QIAGEN). The isolated plasmids were first confirmed for orientation and mutations at restriction site by releasing the gene insert from the plasmid by restriction digestion, and further sent for sequencing to check for orientation of the insert and any mutations. The digested product of cVP2, VP5 and NS1 with their respective restriction enzymes showed a release of 265 bp, 1610 bp and 1671 bp products, respectively, as compared to the empty vector control that had no release of the products (Fig. 23), indicating positive transformants. Upon sequencing with universal primers (T7 forward and T7 reverse), analysis revealed that the constructs were in frame with the poly-histidine tag with no mutations within the gene of interest. These plasmids with gene inserts were used for transformation into expression host *E. coli* BL21 (DE3) strain for protein expression.

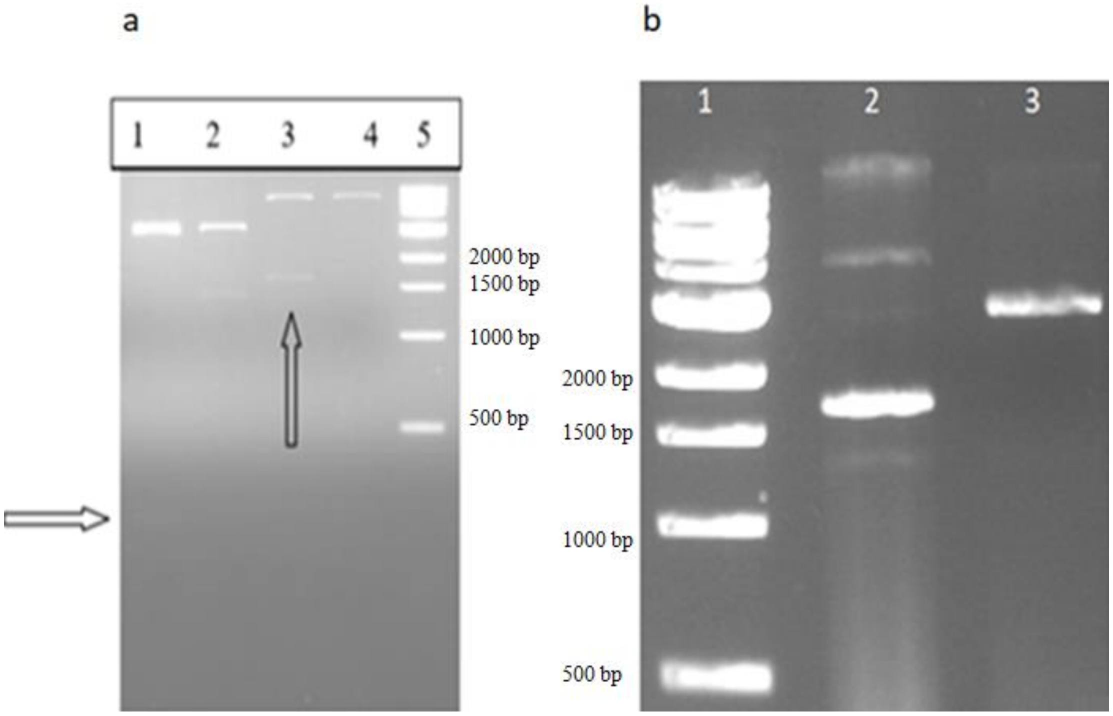


Fig. 23: Restriction digestion and release of insert genes (cVP2, VP5 and NS1)

a. Arrows pointing in lanes 1 and 3 indicate released cVP2 and VP5 from plasmids pRSET-B and pET28b (+), by digestion with *Bam*HI, *Eco*RI and *Nco*I, *Hind*III, respectively. Lanes 2 and 4 are plasmid alone controls without the insert. Lane 5 is 1 Kb DNA ladder.

b. Bottom band in lane 2 indicates release of NS1 from pET28b (+) plasmid by digestion with *Nde*I, *Hind*III and lane 3 indicates linearized pet28b (+) after digestion with *Nde*I, *Hind*III. Lane 1 is 1 Kb DNA ladder.

4.6 Transformation into expression host

The recombinant plasmids (construct) containing cVP2, VP5 and NS1 that were found to be in frame by sequencing were transformed into the expression vector BL21 (DE3) *E. coli* cells. Recombinant clones were obtained on LB media plates at 37^{o}C containing 100 µg/mL of ampicillin and kanamycin 50 µg/mL, respectively. Transformed clones were observed on selective antibiotic plates for cVP2, VP5 and NS1 after 24 h of incubation at 37^{o}C (Fig. 24a, 25a & 26a). The transformed clones were confirmed by colony PCR.

4.7 Colony PCR

Four to five transformed clones were used for screening for the presence of insert by colony PCR using gene-specific primers. PCR reaction was set up with their respective optimum conditions for amplification. The amplified products were compared to their respective positive controls with the expected product sizes of 265 bp for cVP2, 1660 bp for VP5 and 1671 bp for NS1 (Fig. 24b, 25b and 26b). The clones positive by colony PCR were grown in large quantities, 5 mL of which was made a glycerol stock and the overnight culture was used for inducing protein expression.

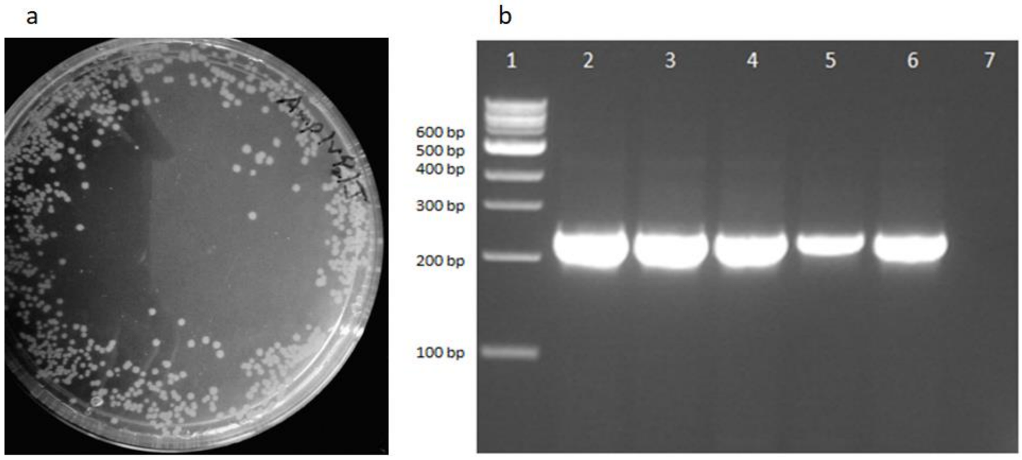


Fig. 24: Transformation of cVP2 to BL21 (DE3)

Fig. 24a: Recombinant colonies of cVP2 after transformation in expression host-BL21 (DE3): LB plate with ampicillin showing transformed colonies of cVP2
Fig. 24b: Colony PCR of the transformed clones cVP2 in BL21 (DE3) host cells. Lane 1=100 bp Ladder; Lane 2, 3, 4, 5=Amplified products of cVP2 at 265 bp; Lane 6=Positive control; Lane 7= Negative control.

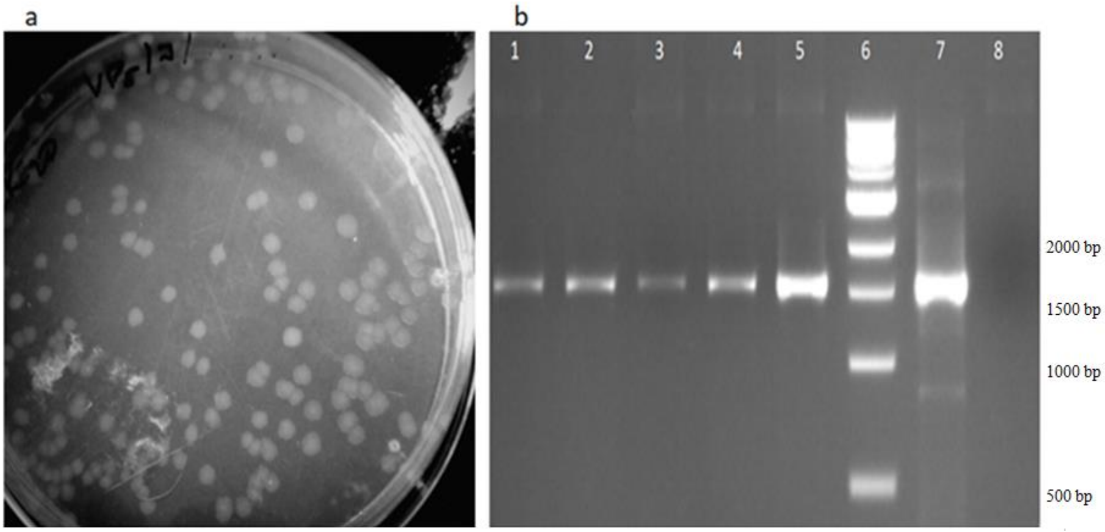


Fig. 25: Transformation of VP5 to BL21 (DE3)

Fig. 25a: Recombinant colonies of VP5 after transformation in expression host-BL21 (DE3): LB plate with ampicillin showing transformed colonies of VP5
Fig. 25b: Colony PCR of the transformed clones VP5 in BL21 (DE3) host cells. Lane 1, 2, 3, 4, 5=Amplified products of VP5 at 1610bp; Lane 6=100 bp Ladder; Lane 7=Positive control; Lane 8= Negative control.

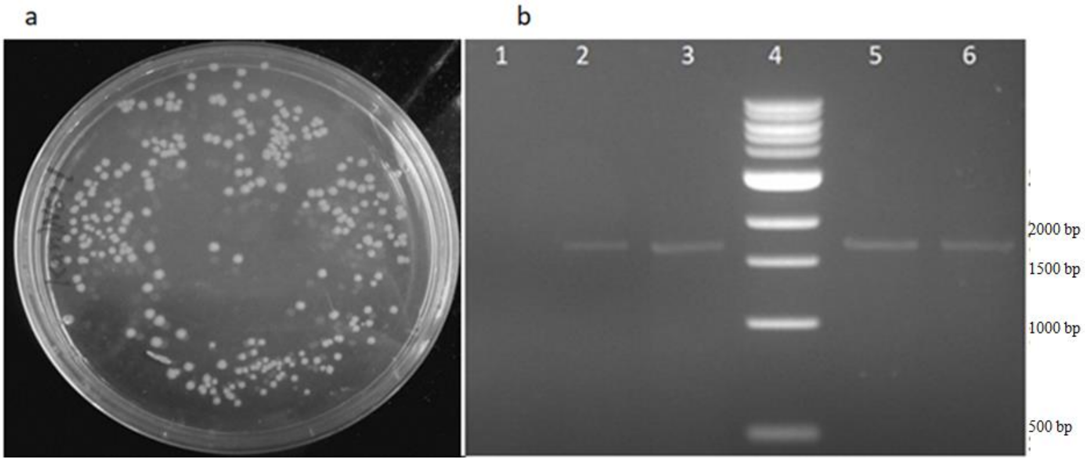


Fig. 26: Transformation of NS1 to BL21 (DE3)

Fig. 26a: Recombinant colonies of NS1 after transformation in expression host-BL21 (DE3): LB plate with ampicillin showing transformed colonies of NS1
Fig. 26b: Colony PCR of the transformed clones NS1 in BL21 (DE3) host cells. Lane 3, 5 and 6=Amplified products of NS1 at 1671 bp; Lane 2=Positive control; Lane 1= Negative control; Lane 4=1000 bp Ladder.

4.8 Recombinant protein expression and purification

Protein production was IPTG dependent for cVP2, and autoinduction for VP5 and NS1.

4.8.1 Expression of the recombinant proteins cVP2, VP5 and NS1

Starter cultures of BL21(DE3) harboring the pRSET-B cVP2 plasmid was inoculated into 2 L of LB medium (with selective antibiotic) at 37°C until OD_{600} reached 0.4–0.6 and IPTG added to a concentration of 0.5 mM. Induced cultures were collected every two hours to check for the detectable expression point. For VP5 and NS1 the BL21 (DE3) colonies with inserts VP5 and NS1, were grown in LB with kanamycin 50 µg/mL and were induced into autoinduction medium for overnight at 37°C overnight in rotary shaker.

After completion of the induction, one mL of the cVP2 culture was collected at different time points and the autoinduced VP5 and NS1 cultures were pelleted and resuspended in 1X SDS-PAGE loading buffer, boiled at 95°C for 10 min, subjected to 10% denaturing SDS-PAGE and stained with Coomassie brilliant blue R-250.

Coomassie staining revealed cVP2 expression with overnight induction showing overexpressed band at 9.35 KDa as compared to the uninduced culture (Fig. 27). For VP5 and NS1, the autoinduced cultures showed expressed proteins as compared to the control pET-28b (+) not containing insert at 59.8 KDa for VP5 (Fig. 28) and 61.2 KDa for NS1 (Fig. 29). The molecular sizes of these proteins were consistent with the predicted molecular masses.

The SDS-PAGE gels were then blotted to PVDF membranes for western blot analysis. Upon blotting, the presence of histidine tagged proteins was established by using anti-histidine antibodies that was detected in a chromogenic reaction on nitrocellulose membrane. Upon incubation of the membrane with ECL reagent and development of X-ray film for cVP2 (Fig. 30) showing a band at 9.35 KDa, and upon incubation of the membrane with ECL reagent and development of X-ray film for VP5 and NS1 (Fig.31 and 32) indicating the presence of the proteins at 59.8 KDa and 61.2 KDa, respectively.

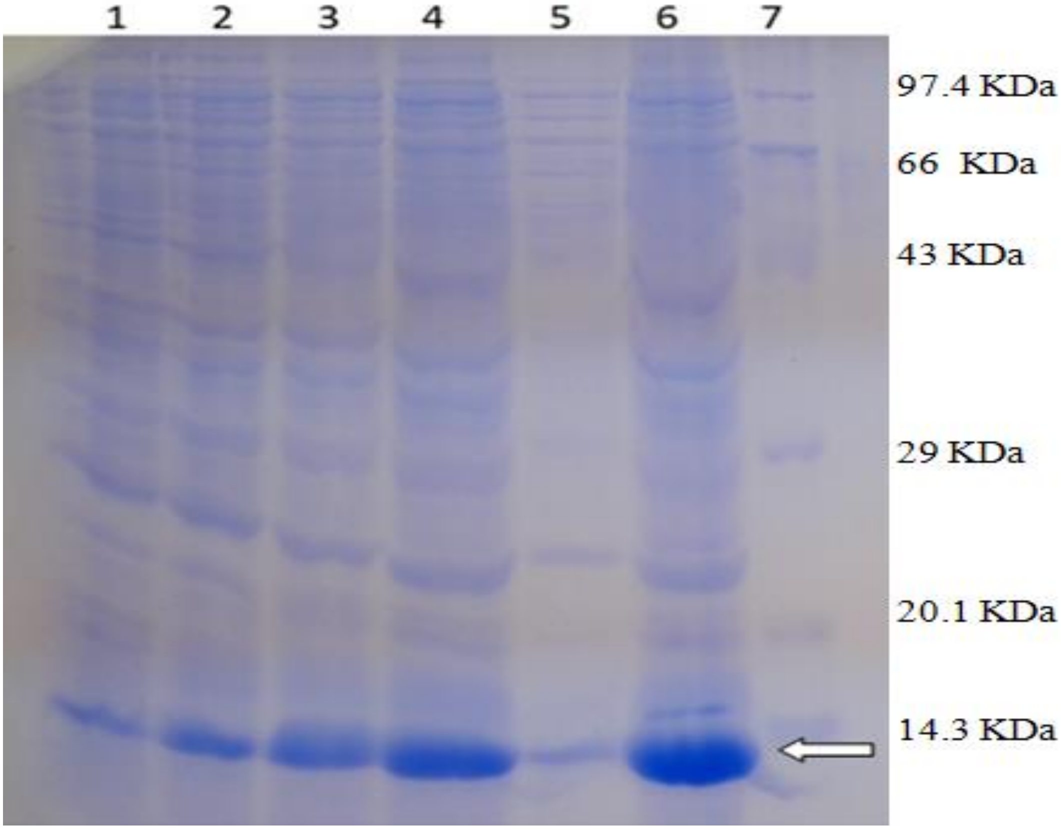


Fig. 27: Detection of cVP2 expression in BL21 (DE3) *E. coli*.

SDS-PAGE illustrating expression of cVP2 at different time points post-induction with IPTG.

Lane 1: Cell lysate without induction
Lane 2: Cell lysate with induction for 2 h at 37°C
Lane 3: Cell lysate with induction for 4 h at 37°C
Lane 4: Cell lysate with induction for 6 h at 37°C
Lane 5: Cell lysate with induction for 6 h at 4°C
Lane 6: Cell lysate with induction for overnight at 37°C
Lane 7: Protein marker, Medium range

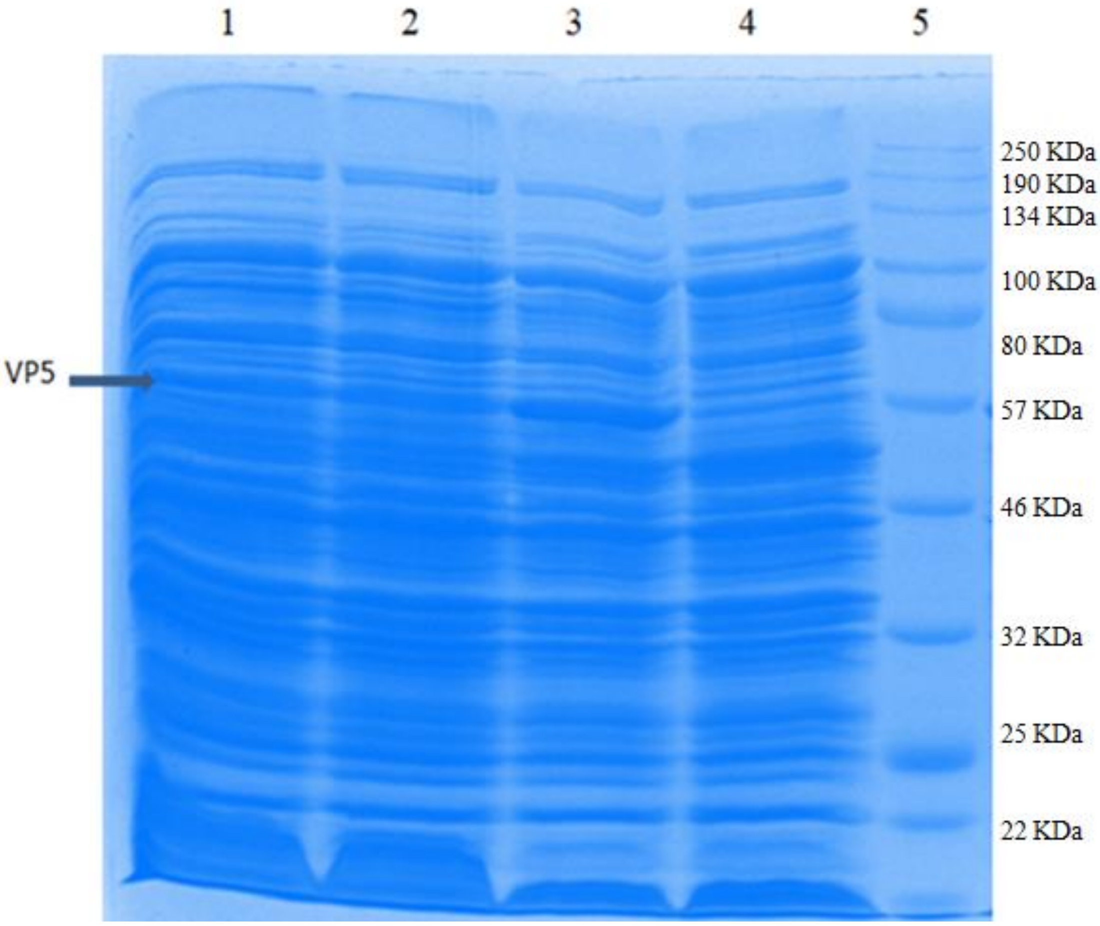


Fig. 28: Detection of VP5 protein expression

Specific protein expression of VP5 was observed by Coomassie blue staining in comparison with pET-28b (+) cell lysate.
Lane 1, 2 and 3: VP5 expressed colonies
Lane 4: pET-28b (+) cell lysate
Lane 5: Molecular weight marker (Color Prestained Protein Standard, NEB, Cat. no-P7712L)

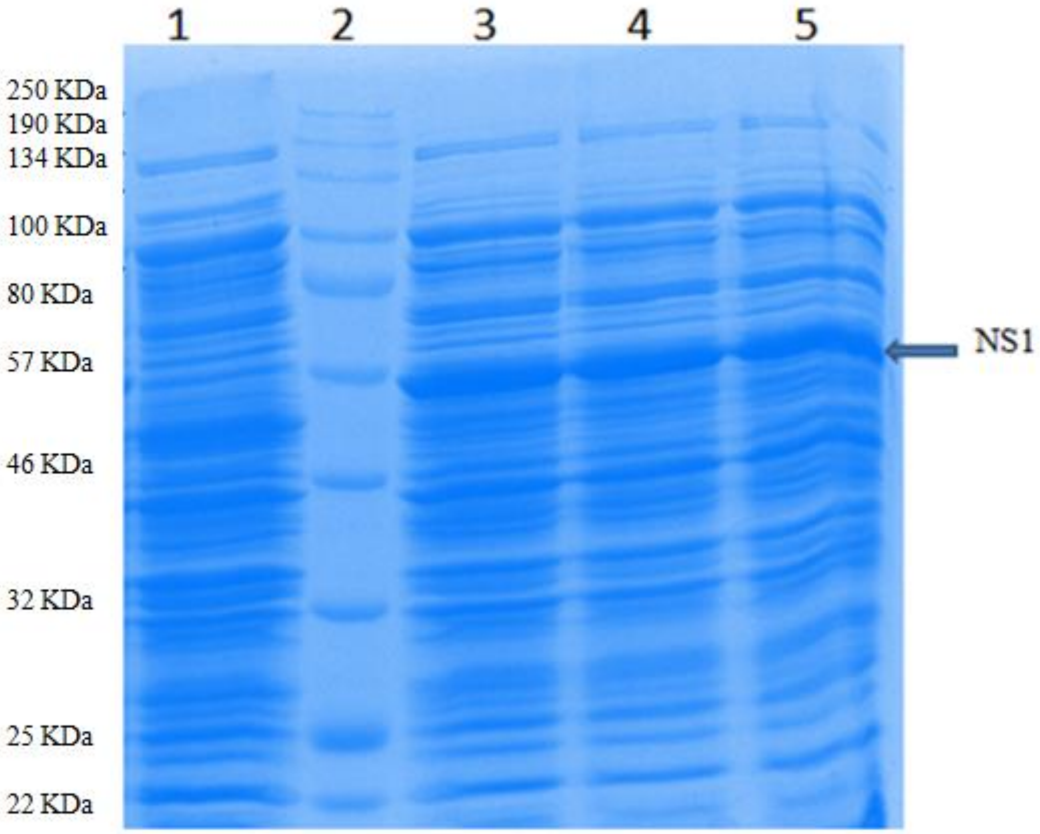


Fig. 29: Detection of NS1 protein expression

Specific protein expression of NS1 was observed by Coomassie blue staining in comparison with pET-28b (+) cell lysate
Lane 1: pET-28b (+) cell lysate
Lane 2: Molecular weight marker (Color Prestained Protein Standard, NEB, Cat. no-P7712L)
Lane 3, 4 and 5: NS1expressed colonies

⇧

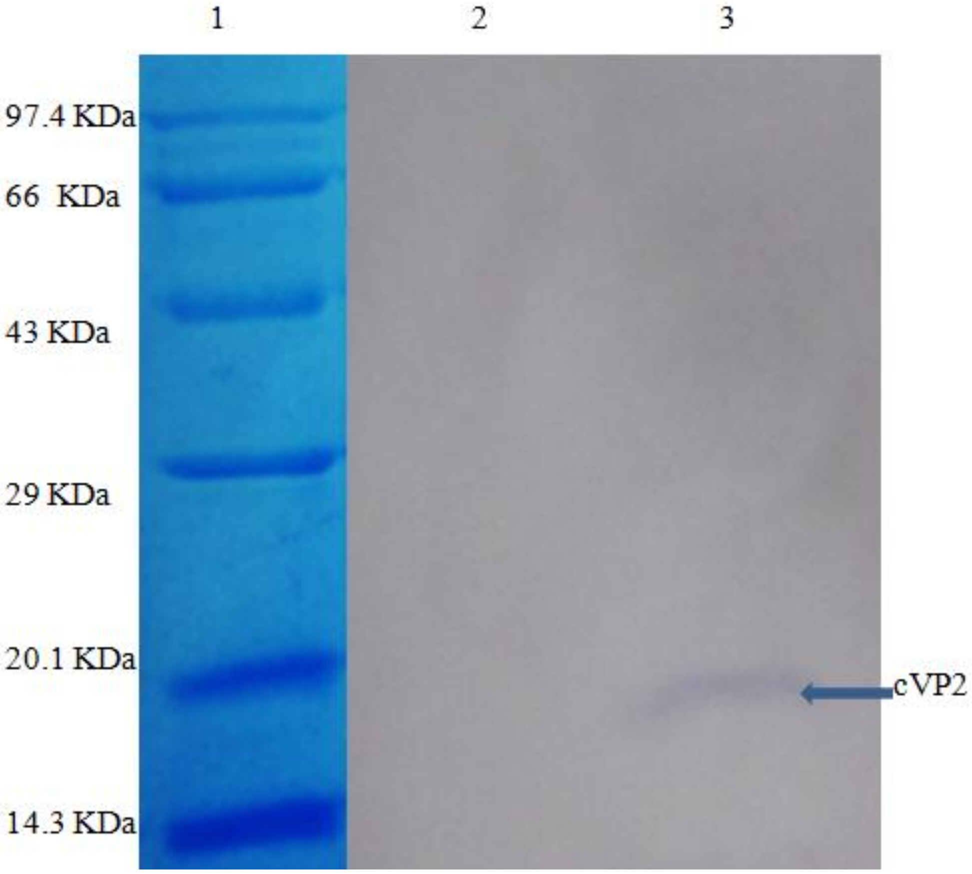


Fig. 30: Confirmation of the tagged protein cVP2 by Western blot

Western blot showing the evidence of cVP2 protein at 10 KDa and absence of the band in the control (uninduced culture).
Lane 1: Protein Marker Medium range
Lane 2: Uninduced culture
Lane 3: cVP2 cell extract after induction with IPTG showing a band at approximate molecular weight of 9.35 KDa.

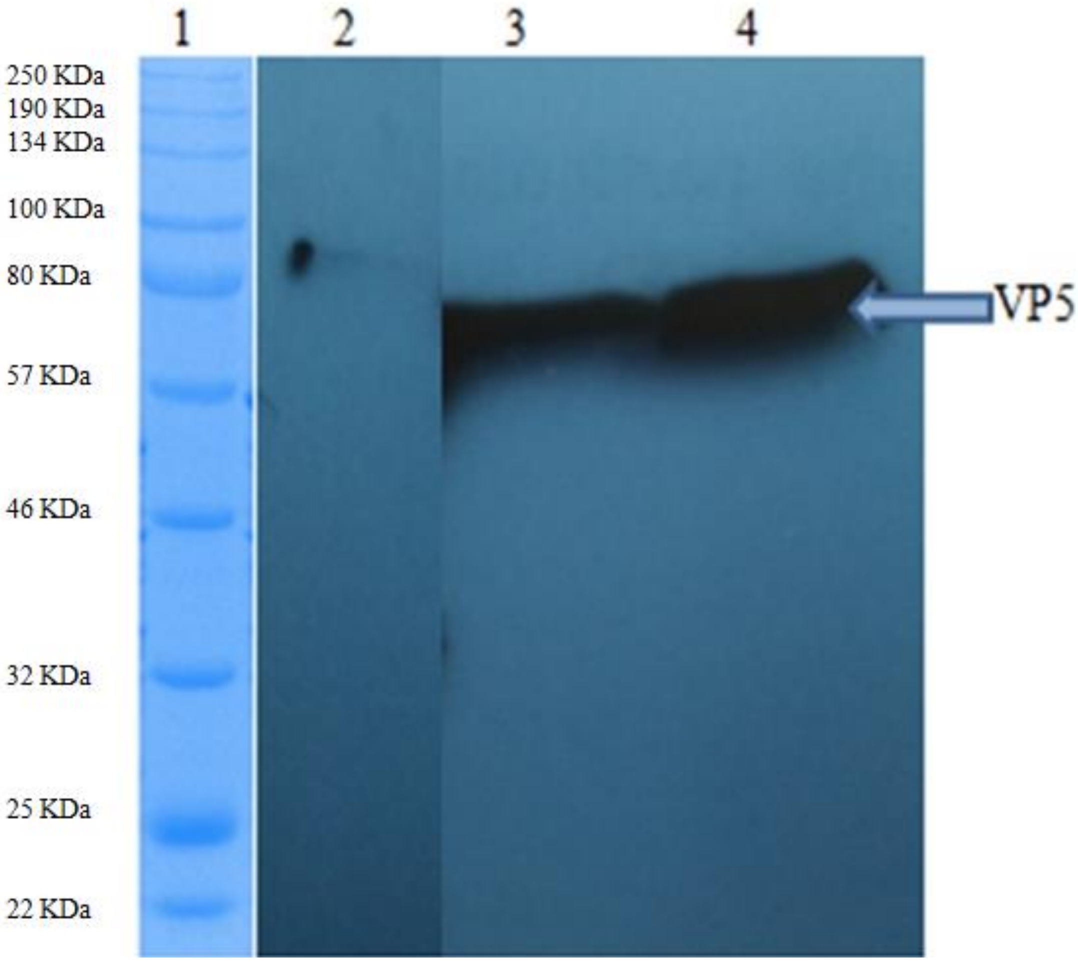


Fig. 31: Confirmation of the tagged protein VP5 by Western blot

The expressed clones of VP5 were subjected to SDS-PAGE, and the His-tagged proteins were detected using anti-His antibodies
Lane 1: Molecular weight marker (Color Prestained Protein Standard, NEB, Cat. no-P7712L).
Lane 2: pET-28b (+) Vector control
Lane 3 and 4: Expressed colonies of VP5 showing a band at approximate molecular weight of 59.8 KDa.

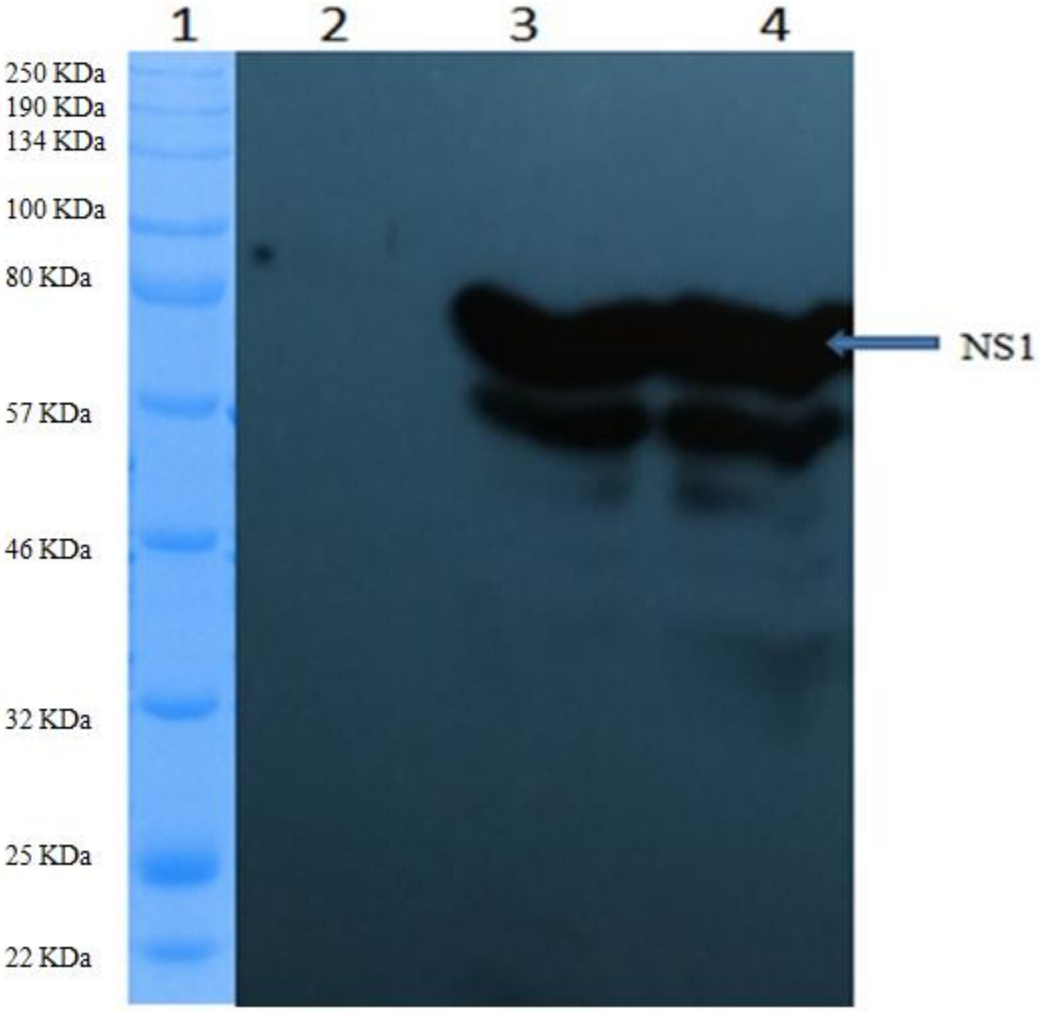


Fig. 32: Confirmation of the tagged protein NS1 by Western blot

The expressed clones of NS1 were subjected to SDS-PAGE, and the His-tagged proteins were detected using anti-His antibodies.
Lane1: Molecular weight marker (Color Prestained Protein Standard, NEB, Cat. no-P7712L)
Lane 2: pET-28b (+) Vector control
Lane 3 and 4: Expressed colonies of NS1 showing a band at approximate molecular weight of 61.2 KDa.

4.8.2 Protein solubility

Ten mL of autoinduced cultures were pelleted and resuspended in 10 mL of lysis buffer and cells were sonicated and lysate was pelleted by centrifugation. The supernatant and pellet were collected separately and analyzed on SDS-PAGE. Fractionation of the bacterial lysate showed that all the three recombinant proteins were in IBs (inclusion bodies) (Fig. 33, 34 and 35), i.e., in insoluable form.

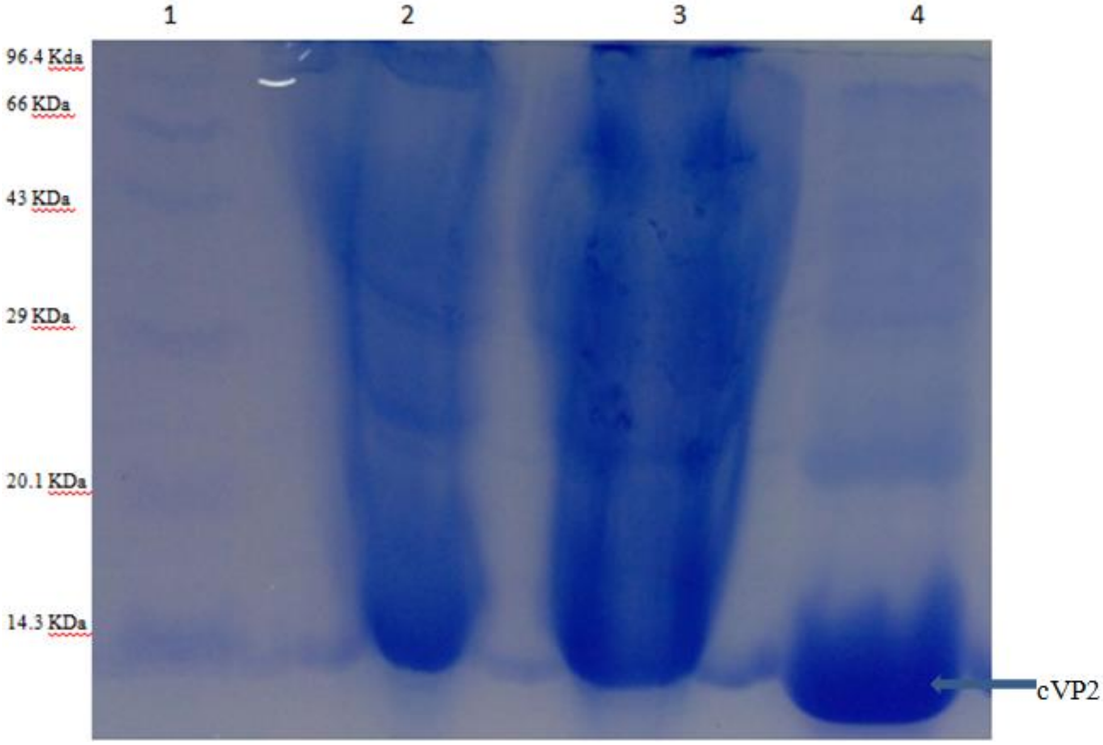


Fig. 33: Detection of solubility of the expressed recombinant protein cVP2

The total cell lysate of the induced cVP2 and the supernatant and the pellet fractions were subjected to Coomassie staining for detection of the solubility. SDS-PAGE analysis revealed cVP2 in the inclusion bodies at approximate molecular weight of 9.35 KDa.

Lane 1: Protein Marker Medium range
Lane 2: Cell lysate
Lane 3: Supernatant of cVP2 after sonication
Lane 4: Pellet of cVP2 after sonication

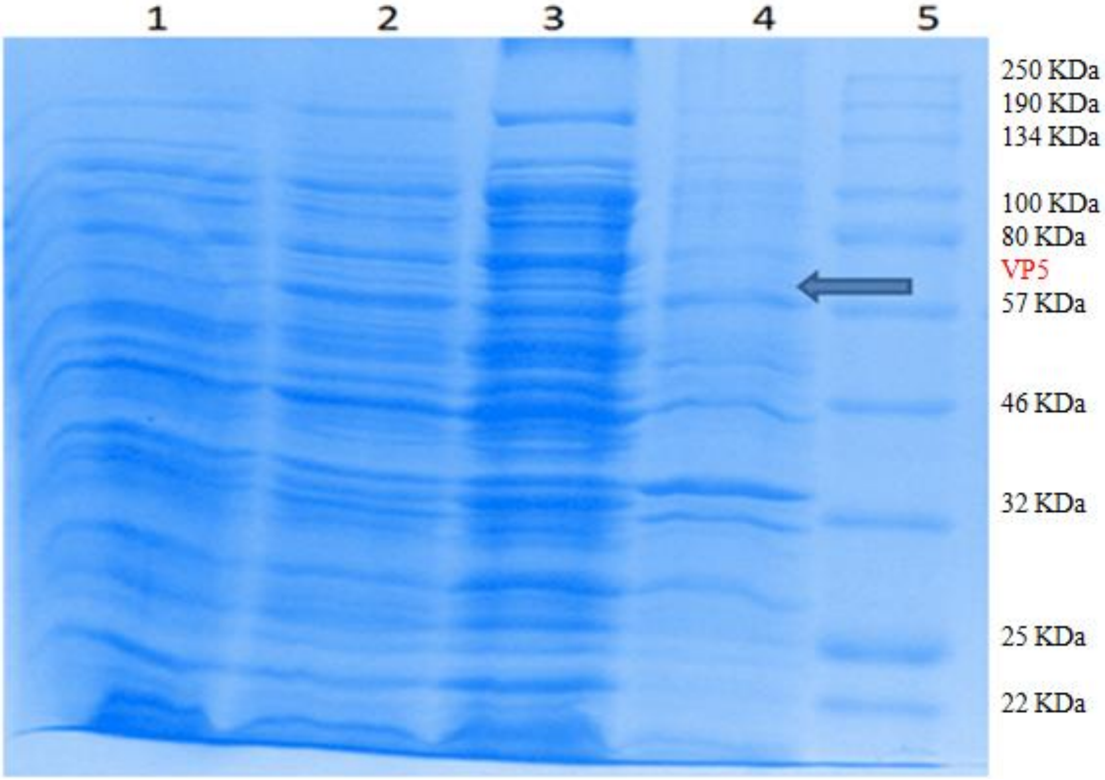


Fig. 34: Detection of solubility of the expressed recombinant protein VP5

Induced proteins (VP5) were subjected to reducing SDS-PAGE and visualized by Coomassie blue staining. Specific protein expression at approximate molecular weight of 59.8 KDa was observed by comparison with pET-28b (+) *E. coli* lysate.
Lane 1: pET-28b (+) vector control
Lane 2: Total cell lysate after induction
Lane 3: Supernatant after sonication
Lane 4: Pellet after sonication
Lane 5: Molecular weight marker (Color Prestained Protein Standard, NEB, Cat. no-P7712L).

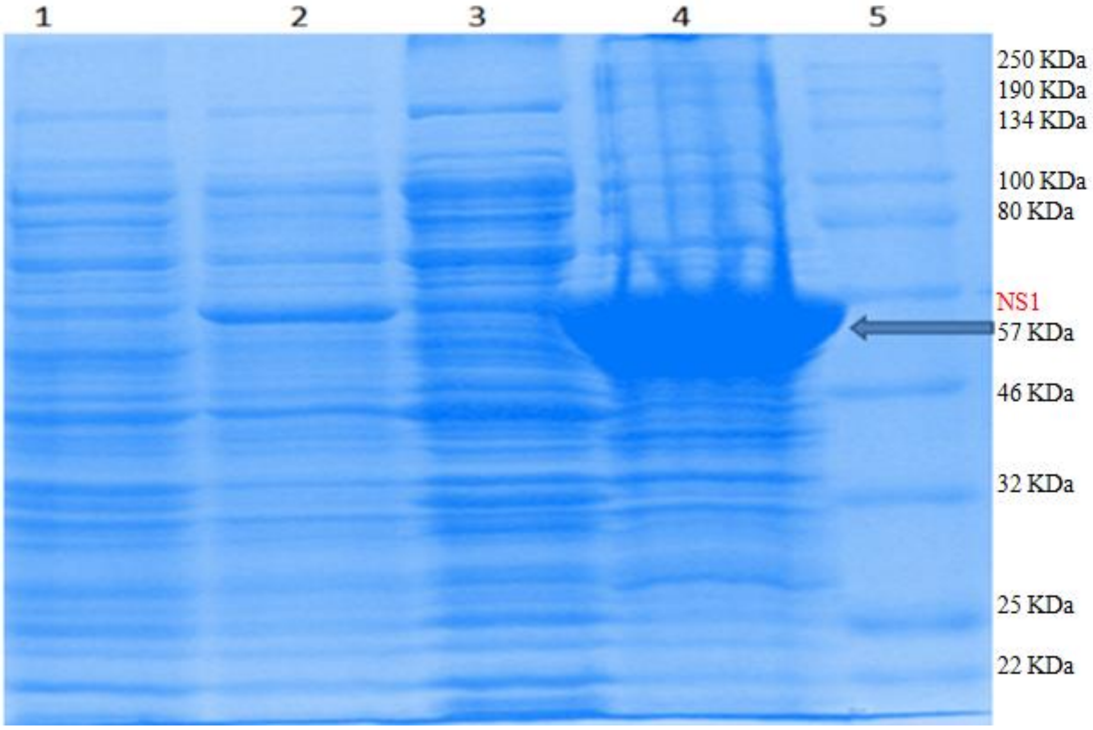


Fig. 35: Detection of solubility of the expressed recombinant protein NS1

Induced proteins (NS1) were subjected to reducing SDS-PAGE and visualized by Coomassie blue staining. Specific protein expression of NS1 at approximate molecular weight of 61.2 KDa can be observed by comparison with pET-28b (+) *E. coli* lysate.
Lane 1: pET-28b (+) vector control
Lane 2: Total cell lysate after induction
Lane 3: Supernatant after sonication
Lane 4: Pellet after sonication Lane5: Molecular weight marker (Color Prestained Protein Standard, NEB, Cat. no-P7712L).

4.8.3 Purification of the recombinant proteins cVP2, VP5 and NS1

Sonicated lysate was clarified by centrifugation and was used to load Ni-NTA column. The three recombinant proteins were purified from the inclusion bodies and had apparent molecular sizes of 9.35 KDa, 59.8 KDa and 66.6 KDa for cVP2, VP5 and NS1, respectively. The purity of the eluted His tag fusion protein was estimated by densitometric analysis of the Coomassie blue stained SDS-PAGE gel and was found to be 90%, 80% and 85% for cVP2, VP5 and NS1, respectively (Fig. 36). Purified proteins were desalted by dialysis against desired buffer and were quantified by Bradford’s method of protein estimation with bovine serum albumin (BSA) as standard. The protein concentrations were found be 1.8 mg/mL, 1.1 mg/mL and 0.45 mg/ml for cVP2, VP5 and NS1 respectively with BSA as a standard.

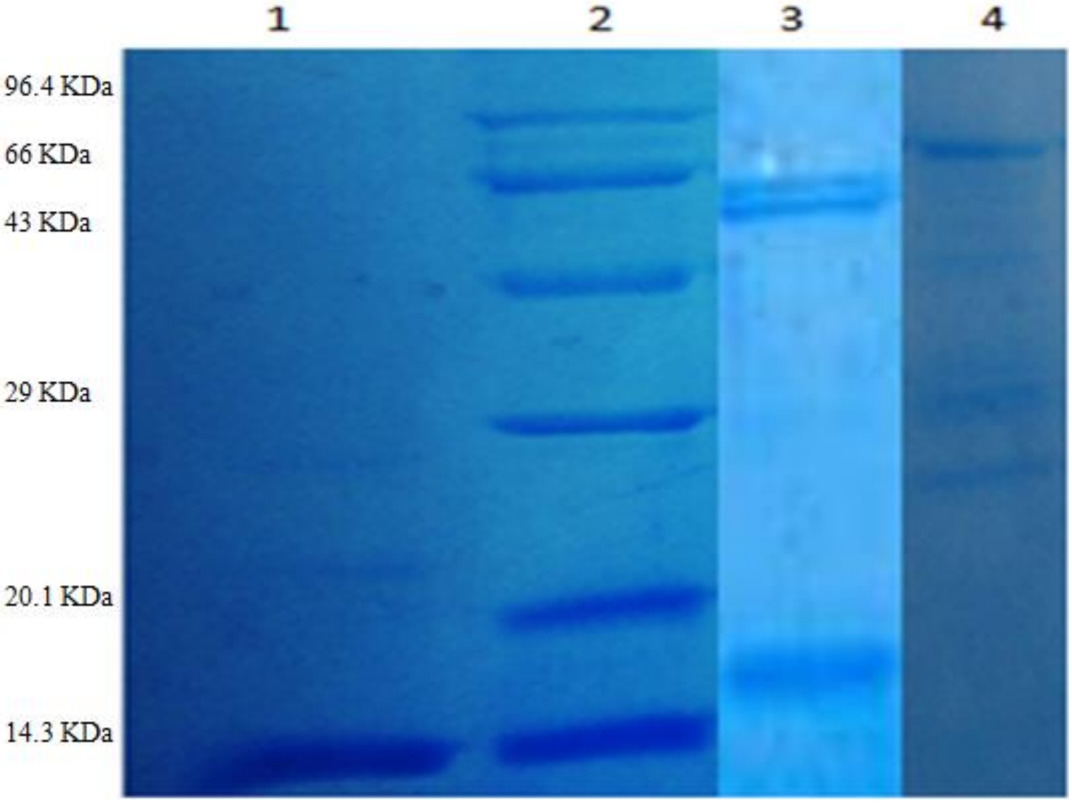


Fig. 36: SDS-PAGE analysis of purified cVP2, VP5 and NS1

The proteins cVP2, VP5 and NS1 after purification by Ni-NTA column were loaded on to the SDS-PAGE gel and stained by Coomassie blue staining. Specific proteins corresponding to their desired molecular weights were observed.
Lane 1: purified cVP2 (9.35 KDa); Lane 2: Molecular weight marker;
Lane 3: purified VP5 (59.8 KDa); Lane 4: purified NS1 (61.2 KDa).

4.9 Immunogenicity studies in mice

4.9.1 Sterility check

Nutrient agar, Sabourauds dextrose agar were inoculated with whole cVP2, VP5 and NS1 with Montanide as adjuvant, No growth of bacteria or fungi was observed during incubation period for 7 days at 4^{o}C and 37^{o}C.

4.9.2 Evaluation of immunogenicity of the recombinant proteins cVP2, VP5 and NS1 in mice

In order to determine the immunogenicity of the purified recombinant proteins BALB/c mice were immunized by dividing the animals into six groups (G1 to G6) with six animals in each group. Immunization schedule was followed as described in materials and methods with subcutaneous injection at the back of the neck (Fig. 37). All animals remained healthy throughout the study and no adverse reactions were seen. Randomly two animals from each group were autopsied and no internal changes in any of the organ were notified (Fig. 38). The serum from each group was used to evaluate the humoral antibody response by I-ELISA and Serum Neutralisation Test (SNT).

Fig. 37: Subcutaneous injection of the BALB/c mice at the back of the neck

Mice were restrained in position on the top of the cage cover and around 100 µL of the protein were injected subcutaneously at the back of the neck.

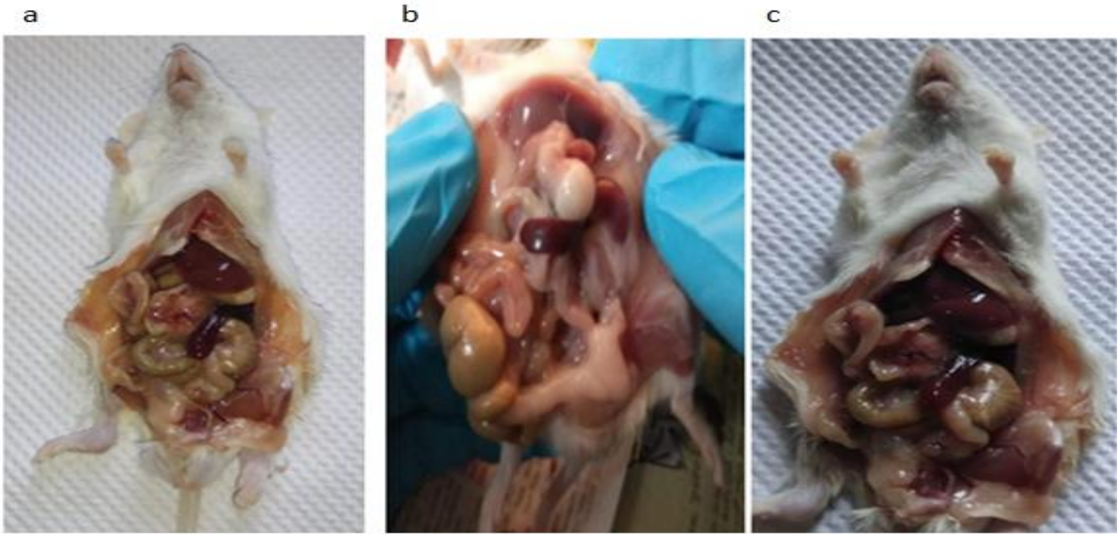


Fig. 38: Autopsy of mice after 40 days of immunization studies

38a. Postmortem of mouse (study group) showing absence of lesions after 40 days of immunization.
38b. Postmortem of Group 5 (commercial vaccine) showing absence of lesions after 40 days of immunization.
38c. Postmortem of control group showing absence of lesions after 40 days of immunization.

4.10 Determination of working standards for Indirect-Enzyme Linked Immunosorbent Assay (I-ELISA) with recombinant antigens

Working concentration of each component of indirect ELISA was assessed by checkerboard titration. The OD values with respect to various dilutions of recombinant proteins (cVP2 18, 9, 4.5, 2.25, 1.12 and 0.56 µg/well, VP5 11, 5.5, 2.75, 1.37 and 0.68 µg/well, NS1 4.5, 2.25, 1.12, 0.56, 0.28, 0.14 µg/well) against different dilutions of antibodies (cVP2, VP5 and NS1) developed in mice using goat anti-mouse IgG conjugate 1:15000 was recorded. It was observed that 4.5, 2.75, 1.12 µg of recombinant antigen cVP2, VP5, NS1 per well and 1/20 dilution of anti cVP2, VP5 and NS1 antibodies showed maximum OD values which almost plateaued. Results showed that the mice developed antibodies against to the given recombinant protein (Fig. 39). The ability of these antibodies to neutralize different BTV serotypes was assessed by serum neutralization test.

4.11 Recombinant NS1 as a tool to detect the field isolates

The recombinant NS1 purified protein was coated with various dilutions of NS1 4.5, 2.25, 1.12, 0.56, 0.28, 0.14 µg/well against 1/20 of diluted known BTV positive field serum sample using anti-sheep IgG-peroxidase and OPD as substrate. Results showed that the NS1 recombinant protein reacted with field samples that are known to be positive for BTV (Fig. 40).

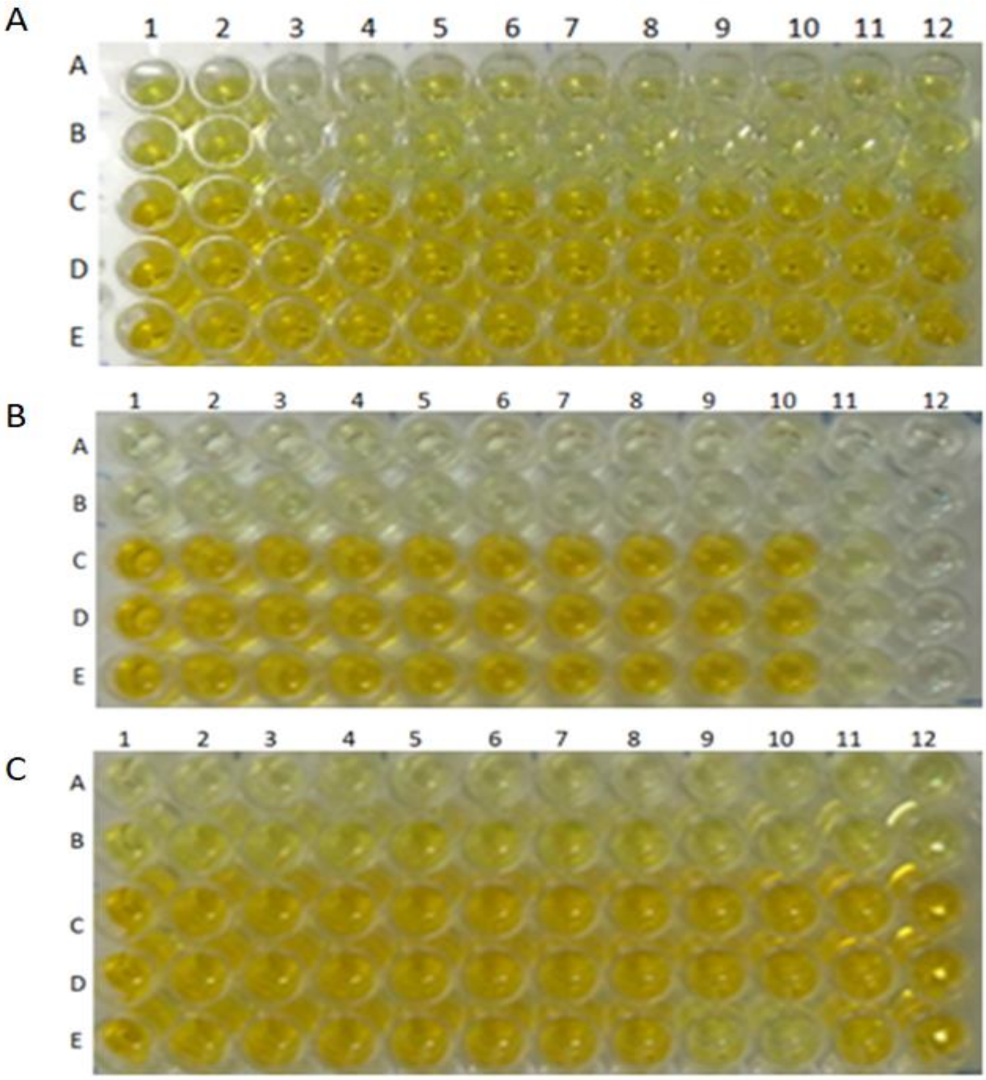


Fig. 39: Standardization of Indirect ELISA for checking the immunogenicity of cVP2, VP5 and NS1 in mice

A		1	2	3	4	5	6	7	8	9	10	11	12
		18µg		9µg		4.5µg		2.25µg		1.12µg		0.56µg	
A	Blank	0.312	0.320	0.264	0.155	0.157	0.027	0.018	0.072	0.082	0.020	0.011	0.002
B	N.C	0.526	0.533	0.249	0.245	**0.104**	**0.106**	0.085	0.182	0.058	0.026	0.089	0.098
C	1/20	2.688	2.691	2.502	2.403	**2.512**	**2.560**	2.284	2.275	2.347	2.340	2.022	2.285
D	1/100	2.598	2.500	2.359	2.212	2.288	2.408	2.334	2.436	2.297	2.159	1.964	2.153
E	1/200	2.522	2.436	2.468	2.182	2.349	2.339	2.182	2.300	2.289	2.375	2.178	2.384

B		1	2	3	4	5	6	7	8	9	10	EMPTY
		11 µg		5.5µg		2.75µg		1.37µg		0.68µg		
A	Blank	0.337	0.233	0.278	0.164	0.141	0.026	0.012	0.074	0.189	0.041	
B	N.C	0.564	0.575	0.256	0.265	**0.121**	**0.132**	0.024	0.024	0.222	0.044	
C	1/20	2.701	2.738	2.651	2.524	**2.570**	**2.626**	2.413	2.408	2.463	2.468	
D	1/100	2.600	2.558	2.513	2.348	2.419	2.495	2.438	2.562	2.406	2.283	
E	1/200	2.664	2.534	2.498	2.221	2.427	2.410	2.295	2.385	2.345	2.450	

C		1	2	3	4	5	6	7	8	9	10	11	12
		4.5 µg		2.25µg		1.12µg		0.56µg		0.28µg		0.14µg	
A	Blank	0.053	0.076	0.005	0.047	0.028	0.015	0.012	0.142	0.010	0.036	0.143	0.068
B	N.C	0.514	0.530	0.383	0.354	**0.129**	**0.145**	0.068	0.025	0.013	0.089	0.028	0.009
C	1/20	2.790	2.718	2.690	2.677	**2.649**	**2.626**	2.531	2.561	2.558	2.741	2.638	2.746
D	1/100	2.759	2.708	2.654	2.712	2.654	2.684	2.582	2.617	2.615	2.663	2.824	2.392
E	1/200	2.706	2.673	2.779	2.852	2.627	2.684	2.657	2.628	0.432	0.205	2.219	2.543

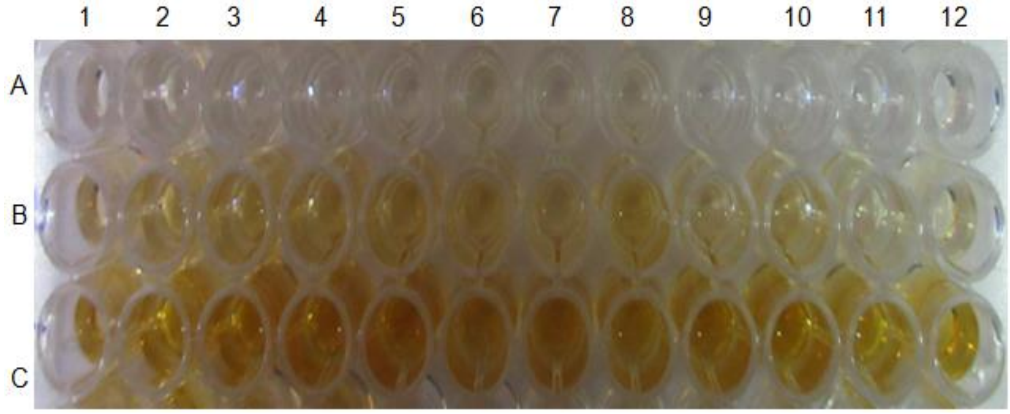


Fig. 40: Indirect ELISA with field samples

Columns 1- 12 were coated with varying concentrations of NS1 (1-2: 4.5 µg/well, 3-4: 2.25 µg/well, 5-6: 1.125 µg/well, 7-8: 0.56 µg/well, 9-10: 0.28 µg/well, 11-12: 0.14 µg/well). A1 to A12: Blank; B1 to B12: Negative control(NC); C1-C12: commercial VP7 ELISA kit BTV positive sera samples which are also found to be positive in the current study.

		1	2	3	4	5	6	7	8	9	10	11	12
		4.5 µg		2.25µg		1.12µg		0.56µg		0.28µg		0.14µg	
A	Blank	0.052	0.053	0.053	0.057	0.054	0.053	0.057	0.054	0.057	0.058	0.054	0.056
B	N.C	0.584	0.580	0.394	0.377	**0.137**	**0.147**	0.086	0.062	0.031	0.098	0.027	0.019
C	Positive Sera Samples	2.885	2.890	2.582	2.489	2.455	2.458	2.498	2.482	2.368	2.359	2.285	2.234

4.12 TITRATION OF THE VIRUS

$TCID_{50}$ was calculated for available types (BTV-1, -2, - 4, - 5, - 9, -10, -12, -16,- 21,- 23, and -24) which were used in serum neutralization tests. Titers for BTV-1, -2,- 4,- 5,- 9, -10, -12, -16,- 21,- 23, and -24 are given in Table 15.

Table 15. TCID50 of BTV isolates to be used in the study

BTV-serotype	$TCID_{50}$ Calculated /mL
BTV-1	$10^{4.74}$
BTV-2	$10^{5.36}$
BTV-4	$10^{4.39}$
BTV-5	$10^{6.78}$
BTV-9	$10^{5.74}$
BTV-10	$10^{4.31}$
BTV-12	$10^{6.11}$
BTV-16	$10^{6.1}$
BTV-21	$10^{5.5}$
BTV-23	$10^{4.6}$
BTV-24	$10^{3.5}$

4.13 Neutralization assays of antibodies raised to the recombinant proteins

The virus neutralizing activities of the antiserum raised to cVP2, VP5 and NS1 proteins were investigated by monitoring CPE in monolayers of Vero cells cultures infected with BTV serotypes (BTV-1, -2, -4, -5, -9, -10, -12, -16, -21, -23 and -24) against different dilutions of the serum (1/10 to 1/1280). The cytopathic effect was assessed on the fifth day post incubation by staining. The wells exhibiting CPE were scored as (X) indicating absence of neutralization and the wells without CPE were scored as (✓) indicating neutralization (Table 16).

Neutralization analysis showed that anti-cVP2 (Group 1) antibodies could neutralize BTV-1, -2, -4, -9, -10, -16, -21 and 23. High neutralizing antibody titers of 3 were produced for BTV- 4, -9, -10 and -16, followed by 2.5 for BTV-21, 1.9 for BTV-2 and 1.6 for BTV-1 and BTV-23. No neutralization was observed with BTV-5, -12 and -24. Anti-VP5 (Group 2) antibodies produced high neutralization titers of 3 for BTV-4, -9, -10, -16,- 21 and -23, followed by 2.5 for BTV-2, -1.3 for BTV-1, and no neutralization of BTV-5,-12 and -24. Anti NS1 (Group 3) antibodies neutralization assay showed neutralizing antibody titers of 3 for BTV - 4, -16, -21,- 23, followed by 1.6 for BTV-9 and -10. Absence of neutralization was seen for BTV-1, -2, -5, -12 and -24. Sera from Group 4 mice produced high neutralizing titers of 3 for BTV-4, -16, followed by 2.5 for BTV-9, -21 and -23, 1.9 for BTV-2, and 1.3 for BTV-1. No neutralization was observed for BTV-5, -12 and -24. Serum antibody from animals vaccinated with commercial vaccine (Group 5) was found to neutralize BTV-21 and 23 with titres of 2.8, 2.5 for BTV-1 and 2, 2.2 for BTV- 10, -12 and -16, and 1.9 for BTV-4 and -9. No neutralization was observed for BTV-5, -21 and -24. Inhibition of virus induced CPE was not seen by the sera of the control animals in all the groups (Table 17, and Fig. 41).

Table 16. SNT titres in neutralization against BTV serotypes

	HIS	BTV-1	BTV-2	BTV-4	BTV-5	BTV-9	BTV-10	BTV-12	BTV-16	BTV-21	BTV-23	BTV-24
Group 1	1/10	✓	✓	✓	x	✓	✓	x	✓	✓	✓	X
	1/20	✓	✓	✓	x	✓	✓	x	✓	✓	✓	X
	1/40	✓	✓	✓	x	✓	✓	x	✓	✓	✓	X
	1/80	x	✓	✓	x	✓	✓	x	✓	✓	x	X
	1/160	x	x	✓	x	✓	✓	x	✓	✓	x	x
	1/320	x	x	✓	x	✓	✓	x	✓	✓	x	x
	1/640	x	x	✓	x	✓	✓	x	✓	x	x	x
	1/1280	x	x	✓	x	✓	✓	x	✓	x	x	x
Group 2	1/10	✓	✓	✓	x	✓	✓	x	✓	✓	✓	x
	1/20	✓	✓	✓	x	✓	✓	x	✓	✓	✓	x
	1/40	x	✓	✓	x	✓	✓	x	✓	✓	✓	x
	1/80	x	✓	✓	x	✓	✓	x	✓	✓	✓	x
	1/160	x	✓	✓	x	✓	✓	x	✓	✓	✓	x
	1/320	x	✓	✓	x	✓	✓	x	✓	✓	✓	x
	1/640	x	x	✓	x	✓	✓	x	✓	✓	✓	x
	1/1280	x	x	✓	x	✓	✓	x	✓	✓	✓	x
GROUP 3	1/10	x	x	✓	x	✓	✓	x	✓	✓	✓	x
	1/20	x	x	✓	x	✓	✓	x	✓	✓	✓	x
	1/40	x	x	✓	x	✓	✓	x	✓	✓	✓	x
	1/80	x	x	✓	x	x	x	x	✓	✓	✓	x
	1/160	x	x	✓	x	x	x	x	✓	✓	✓	x
	1/320	x	x	✓	x	x	x	x	✓	✓	✓	x
	1/640	x	x	✓	x	x	x	x	✓	✓	✓	x
	1/1280	x	x	✓	x	x	x	x	✓	✓	✓	x
GROUP 4	1/10	✓	✓	✓	x	✓	✓	x	✓	✓	✓	x
	1/20	✓	✓	✓	x	✓	✓	x	✓	✓	✓	x
	1/40	x	✓	✓	x	✓	✓	x	✓	✓	✓	x
	1/80	x	✓	✓	x	✓	✓	x	✓	✓	✓	x
	1/160	x	x	✓	x	✓	✓	x	✓	✓	✓	x
	1/320	x	x	✓	x	✓	✓	x	✓	✓	✓	x
	1/640	x	x	✓	x	x	✓	x	✓	x	x	x
	1/1280	x	x	✓	x	x	✓	x	✓	x	x	x
GROUP 5	1/10	✓	✓	c	x	✓	✓	✓	✓	x	✓	x
	1/20	✓	✓	✓	x	✓	✓	✓	✓	x	✓	x
	1/40	✓	✓	✓	x	✓	✓	✓	✓	x	✓	x
	1/80	✓	✓	✓	x	✓	✓	✓	✓	x	✓	x
	1/160	✓	✓	x	x	x	✓	✓	✓	x	✓	x
	1/320	✓	✓	x	x	x	x	x	x	x	✓	x
	1/640	x	x	x	x	x	x	x	x	x	✓	x
	1/1280	x	x	x	x	x	x	x	x	x	x	x

Serum(pooled) from all the groups were tested for cross neutralization capacity against BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23 and 24. Neutralization with the particular serotype indicated as (✓), and the absence of neutralization indicated as (**X**).

Table 17. SNT antibody titres expressed as $\log_{10}$ values

	BTV-1	BTV-2	BTV-4	BTV-5	BTV-9	BTV-10	BTV-12	BTV-16	BTV-21	BTV-23	BTV-24
Group 1	1.6	1.9	3.1	nn	3.1	3.1	nn	3.1	2.5	1.6	nn
Group 2	1.3	2.5	3.1	nn	3.1	3.1	nn	3.1	3.1	3.1	nn
Group 3	nn	nn	3.1	nn	1.6	1.6	nn	3.1	3.1	3.1	nn
Group 4	1.3	1.9	3.1	nn	2.5	3.1	nn	3.1	2.5	2.5	nn
Group 5	2.5	2.5	1.9	nn	1.9	2.2	2.2	2.2	nn	2.8	nn
Control	nn	nn	nn	nn	nn	nn	nn	nn	nn	nn	nn

The neutralization titre of each serum was defined as the highest dilution allowing neutralization of the $100TCID_{50}$ and the titres were expressed as $\log_{10}$ reciprocal of the highest positive serum dilution; nn: no neutralization

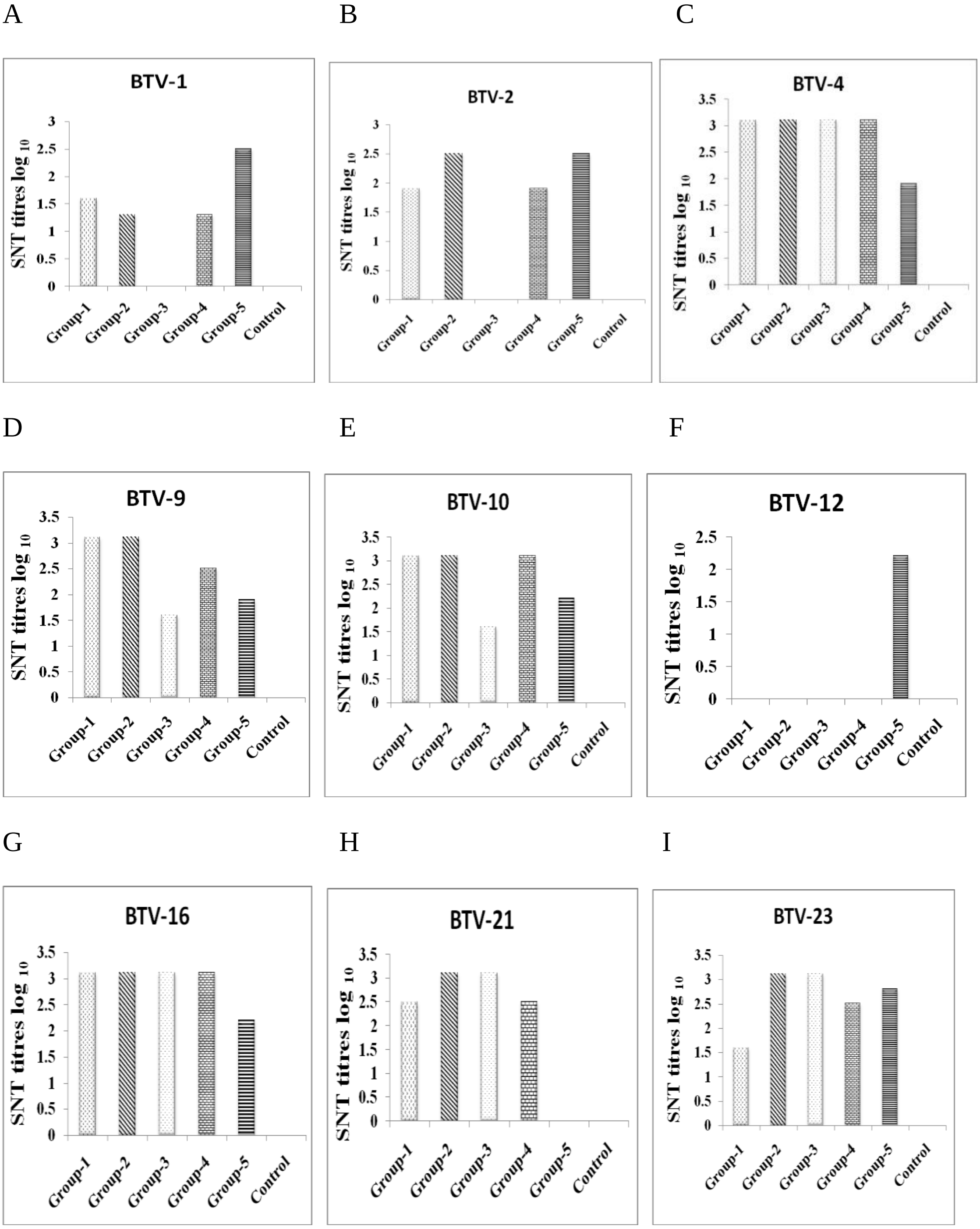


Fig. 41: Graphical representation of neutralizing antibody titres produced after vaccination with the recombination proteins and the pentavalent vaccine in neutralization against BTV serotypes

The neutralizing titres are given in $\log_{10}$ values. BTV-5, 12 and 24 could not be neutralized with sera raised with recombinant proteins. BTV-5, 21, and 24 could not be neutralized with sera raised with commercial inactivated vaccine. BTV 12 that could not be neutralized with recombinant protein sera could be neutralized with sera raised against commercial inactivated vaccine. Absence of neutralization was observed with all the serotypes in the control group.

CHAPTER-V

DISCUSSION

Vaccination is one of the most effective approaches for controlling infectious viral diseases known to date. Extensive knowledge of the basic biology of viruses at the molecular level coupled with recent technology developments have resulted in a number of newly designed vaccines for both human and animal viral diseases. However, the development of effective vaccines for viruses with multiple distinct serotypes remains laborious and highly challenging (Celma *et al.,* 2013). Early attempts at vaccination used serum from infected sheep that had survived the disease (Theiler 1908). Although vaccination has been an effective approach to control BTV spread, currently available vaccines are associated with undesirable side effects. There are two types of vaccines commercially available, namely, conventional live-attenuated and chemically inactivated vaccines. Although both types of vaccines can protect against BTV infection, problems such as incomplete protection, association with teratogenic effects and incomplete attenuation have been reported. Consequently, there are many current efforts to develop new types of vaccines with improved safety and efficacy against a broad range of BTV serotypes. Most of these efforts concentrate on the development of subunit vaccines (Veronesi *et al.,* 2010; Franceschi *et al.,* 2011; Calvo-Pinilla *et al.,* 2012; Matsuo *et al.,* 2011).

Concerns over biosafety regarding the use of conventional vaccines have encouraged the development of new generation vaccines. A number of subunit vaccines have been prepared for the control of BTV to date. Previous studies exploring recombinant BTV structural proteins as subunit vaccine candidates evaluated crude lysates of baculovirus-infected insect cells expressing BTV VP2 and VP5. Immunization of sheep with these proteins induced protection and a significant increase in Nab titres (Anderson *et al.,* 2013; Roy *et al.,* 1990; Stewart *et al.,* 2012).

The current study presented the stages in the development and evaluation of a novel rationally designed recombinant subunit BTV vaccine against multiple serotypes. The central hypothesis of the current study is that administration of recombinant protein conserved epitope of VP2 protein as a subunit vaccine should confer broad-spectrum immunity against most of the available serotypes of BTV, and owing to its subunit nature shall function as differentiating infected from vaccinated (DIVA) vaccine. Since VP5 in combination with VP2 was found to elicit strong immune response and the most highly conserved RNA segment coding of NS1for BTV serotypes from all geographical regions was coding for NS1 which have also been shown to produce specific humoral and cell-mediated immunity, VP5 and NS1 were also included. Advent and latest advances in the fields of genomics and bioinformatics have made a plethora of sequence information of BTV and its different serotypes available, such information was utilized here to identify conserved protein regions of highly immunogenic VP2, VP5 and NS1 of BTV.

5.1 Identification of conserved epitopes in VP2 and VP5 and their homology modelling

The amino acid alignment for segments 2 showed that although VP2 is very variable, several regions are relatively more conserved between serotypes. According to our analysis the most conserved region of VP2 is evident in the 338-383 aa region. Given its proximity to neutralising epitopes and its rather conserved nature, this epitope was targeted as a subunit vaccine. Previous studies reported that a neutralizing epitope was located in VP2 spanning amino acids 321-346 (Gould *et al.,* 1988; De Maula *et al.,* 1993; and De Maula *et al.,* 2000). Despite the overall sequence variability, some features of VP2 appeared to be conserved across serotypes, including the hydrophobicity profile, charge distribution and the position of certain cysteine residues (Maan *et al.,* 2007). Several researchers suggested that developing a multi-serotype BTV vaccine, by including VP2 from different serotypes, and evaluating potential cross-protection against those serotypes, would be an important line of investigation for the future (Anderson *et a*l., 2014). Studies by Jeggo and colleagues showed that cross-neutralisation among BTV serotypes have provided support for speculation that minimum number of BTV serotypes needed in a multivalent vaccine to provide broad protection (Jeggo & Wardley, 1982; Jeggo *et al.,* 1984a, 1984b). This suggests cVP2 to be a strong possible candidate to target for a subunit vaccine moiety.

Since VP5 protein (and cVP2) conserved among BTV serotypes as evident from homology model predictions of the current study, and as several studies suggested that vaccination with recombinant proteins VP2 and VP5 together induced greater protection in sheep than vaccination with either protein alone (Roy *et al.,* 1990; Lobato *et al.,* 1997; Boone *et al.,* 2007; Inumaru and Roy 1987). It was tempting to consider the usage of a combination of cVP2 and VP5 for a broader range protective response among BTV serotypes. VP2 and VP5 are the only BTV proteins shown to induce neutralising antibodies (Lobato *et al.,* 1997 & Roy *et al.,* 1990) with VP2 being the major protein involved in serotype specificity (Huismans *et al.,* 1981 & Mertens *et al.,* 1989).

5.2 Codon optimization of VP5 and NS1

It is important to stress here that the VP5 and the NS1 amplified from BTV-16 failed to produce expression in the bacterial expression systems as a possible consequence of codon bias in the sequences of VP5 and NS1. Hence, the codon optimized sequences were employed for cloning and expression studies. A common cause of low expression levels is the variation in codon usage frequency between different species. This can cause the translation of heterologous expressed genes to stall due to tRNA scarcity, leading to lower protein levels and increased rates of improper protein folding. The degeneracy of the genetic code makes it possible to change the DNA sequence in a way that does not alter the final amino acid sequence, but does have significant effects on the efficiency of transcription, mRNA stability, translation, and protein folding (Burgess-Brown *et al.,* 2008). Hence, the VP5 and NS1 were codon optimized and further used for cloning and expression.

5.3 Cloning and transformation

Amplicons after analyzing with 1% agarose gel electrophoresis were subjected to restriction enzyme digestion before cloning to respective vectors. The cVP2 insert to pRSET-B, VP5 and NS1 into pET-28b (+) were successfully cloned. The transformed clones on amplification with gene-specific primers and having amplicon sizes of 265 bp, 1660 bp, 1671 bp for cVP2, VP5 and NS1, respectively, were considered as positive clones. Further confirmation of the recombinant clones was by restriction digestion and release of 265 bp fragment from pRSET-B, 1660 bp and 1671 bp fragments from pET-28b (+) vector, and the absence of such fragment in pRSET-B and pET-28b (+) when restricted with the same enzymes. The plasmid of each cVP2, VP5 and NS1 were then transformed into BL21 (DE3). Transformed clones on selective antibiotic LB plates were checked for the presence of the insert by colony PCR using gene specific primers. The clones that had amplicon sizes of 265 bp, 1660 bp, 1671 bp were considered as positive and were then proceeded for expression studies.

The vector systems pRSET-B, pET-28b (+) and the bacterial expression system BL 21 (DE3) used in the study have successfully enabled recombinant protein production and are of practical utility for vaccine application because of their easy availability, affordability and potential capability to produce large quantities of functional protein. Bacterial expression systems could present an alternate to inactivated vaccines, particularly if viruses prove to be difficult to produce in cell cultures like BTV-25 (Hofmann *et al.,* 2008). However, difficulties in protein expression are often encountered with the *E. coli* expression systems, particularly for the larger BTV proteins, such as VP2 or VP3, that require production in the native and soluble form for subunit vaccine production (Mohd Jaffar *et al.,* 2014). The downside to bacterial expression systems include lack of post translational modifications, potential misfolding of proteins and risk of lipopolysaccharide contamination. Other expression systems like baculoviruses were also utilized previously for the recombinant protein production (Inumaru *et al.,* 1987; Jones *et al.,* 1997; Mecham and Wilson 2004; Mohd Jaffar *et al.,* 2014).

The present study succeeded in making use of the *E. coli* expression system in cloning and expression, suggesting that the BTV proteins can be expressed in simpler bacterial expression systems without the use of the complex eukaryotic expression systems, and the use of bacterial expressed antigens, could provide a safe and scalable alternative to live-attenuated BT vaccines. Though the proteins expressed were in insoluble form we could further make it soluble by optimizing the conditions and thereby making it useful in immunization studies.

5.4 Recombinant protein expression

In the present study, IPTG was employed for the expression of cVP2 protein and autoinduction was done for VP5 and NS1. Isopropyl β-D-1-thiogalactopyranoside (IPTG) is a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the *lac* operon, and it is therefore used to induce protein expression where the gene is under the control of the lac operator. IPTG is an effective inducer of protein expression in the concentration range of 100 µM to 3.0 mM (Marbach and Bettenbrock, 2012). It is important to stress that the expression trials for VP5 and NS1 were of many and hence autoinduction was tried as an alternative. Autoinduction is an alternative to IPTG induction, depends on glucose catabolite repression and lactose (substrate) induction to tightly control protein expression (Studier 2005). Because of easy processing (e.g., no OD_{600}-dependent induction window) and easy culturing for scaling it is a attractive method for high protein expression. In addition, autoinduction results in several-fold higher yields of the target protein compared with conventional IPTG induction (Studier 2005). Hence, to facilitate and improve the expression, VP5 and NS1 were expressed using autoinduction in *E. coli.*

5.5 Purification of the recombinant proteins

The addition of polyhistidine tag to each recombinant protein enabled Ni-NTA affinity purification using convenient commercial methods. By adjusting the lysis buffer, purification method and elution buffer, we were able to purify cVP2, VP5 and NS1. Since all the three recombinants cVP2, VP5 and NS1 were found to form inclusion bodies, due to misfolding of the proteins, the systems could successfully pyrify proteins according to manufacturers catalog.

5.6 Solubilizing of inclusion bodies and refolding of proteins

Using the said protocol successfully obtained the proteins then were solubilized using appropriate dialyzing solutions thereby obtaining soluble recombinant cVP2, VP5and NS1 for use in immunogenicity studies. Confirmation of these expressed BTV proteins was done by SDS-PAGE analysis with predicted molecular masses of 9.35 KDa (cVP2), 59.8 KDa (VP5) and 61.2 KDa (NS1).

A variety of methods have been published describing refolding of insoluble proteins (Burgess, 1996; Frankel and Leinwand, 1996; Mukhopadhyay, 1997; Kurucz *et al.,* 1995; Rudolph and Lilie, 1996; Marston and Hartley, 1990). Most protocols described the isolation of insoluble inclusion bodies by centrifugation followed by solubilization under denaturing conditions. The proteins are dialyzed or diluted into a non-denaturing buffer where refolding occurrs. Because every protein possesses unique folding properties, the optimal refolding protocol for any given protein must be empirically determined. Optimal refolding conditions can be rapidly determined on a small scale by a matrix approach, in which variables such as protein concentration, reducing agent, redox treatment, divalent cations, etc., are tested. Once the optimal concentrations are found, they can be applied to a larger scale solubilization with refolding of the target protein.

5.7 Immunogenicity studies

Adaptive immune response is initiated by internalizing antigens through major histocompatibility complex (MHC) class I to $CD8^+$ T lymphocytes and MHC II to $CD4^+$ T lymphocytes. While $CD8^+$ T cells are cytotoxic T lymphocytes (CTLs) which secrete molecules that directly target and kill the pathogen, $CD4^+$ T lymphocytes release lymphokines that cause the inflammation to destroy the antigenic material. Also, the T helper cells identify B-cells and cause their differentiation into plasma cells that secrete antibodies. Therefore, identifying epitopes having ability to produce both the B cell and T-cell facilitated immune response could be highly beneficial in developing subunit vaccine candidates.

5.7.1 Subunit vaccine formulation

It has been reported that in BT the humoral response is the main component of the immune response against the virus. The cellular component is also important but, so far, how it works and how important its contribution is to the whole immune response are not well understood. Of the three proteins included in the subunit vaccine formulation, VP2 is the only protein associated with the induction of virus-neutralizing antibodies (Huismans & Erasmus, 1981; Kahlon *et al.,* 1983; Roy *et al.,* 1990). Previous studies have demonstrated that VP5 may also play a role by supporting the tertiary conformation of VP2 (Cowley & Gorman, 1989; Mertens *et al.,* 1989; Roy *et al.,* 1990; DeMaula *et al.,* 2000). Therefore, it has been suggested that the inclusion of VP5 with VP2 would benefit any vaccine (Schwartz-Cornil *et al.,* 2008). NS1 is the most synthesized viral protein in BTV-infected cells and is highly conserved among different serotypes (Owens *et al.,* 2004; Van *et al.,* 2010; Urakawa *et al.,* 1989; Schwartz-cornil *et al.,* 2008). This protein contains epitopes associated with both T cell and humoral responses, and antibody responses against NS1 protein may be important contributors to immune protection (Owens *et al.,* 2004; Anderson *et al.,* 2014; Rojas *et al.,* 2014). The initial inclusion of the NS1 protein in the design of subunit vaccine was based on: A) to find its role in humoral immune response, and B) to determine its potential to induce cross-serotype immune responses, which could contribute to protection against several BTV serotypes, as has recently been shown for BTV (Calvo-Pinilla *et al.,* 2012) and African horse sickness virus (De la Poza *et al.,* 2013)in interferon negative mice (IFNAR-/-). Additionally, the induction of cell-mediated immune responses by NS1 and NS2, combined with neutralizing antibodies induced by VP2, may contribute to broader vaccine efficacy as well as and potentially longer duration of protection, by stimulating diverse immune responses. Recent studies on swine influenza vaccine development have shown that DNA vaccines composed of variable antibody and conserved CTL epitopes provide greater protection against heterologous challenge than those composed of antibody epitopes alone (Wang *et al.,* 2012), which is crucial for viruses such as influenza or BTV in which reassortments can occur (Shaw *et al.,* 2013).

5.7.2 Mice experimentation

To better understand the immunogenicity of the recombinant proteins (cVP2, VP5 and NS1), the BALB/c mouse model was used in the current study to evaluate the immune response elicited by these recombinant proteins. In addition, the commercially available pentavalent vaccine consisting of BTV-1, 2, 10, 16 and 23 was also included in the immunization studies to detect the immunization titres, its ability to cross-neutralize other serotypes, if any, and comparison of the produced neutralizing antibody titres with that against the recombinant proteins.

The immunogenicity studies were carried in BALB/c mice with a total of 36 mice, divided into 6 groups (G1-cVP2, G2-VP5, G3-NS1, G4-cVP2+VP5+NS1, G5-inactivated vaccine, G6-control) of 6 mice each inoculated with 25 µg was administered by subcutaneous injection at the back of the neck on day 0. The mice were boosted on day 14 and day 28 with the same doses using appropriate antigens. Final bleeds were obtained via cardiac puncture at day 41, serum obtained from the blood was stored at -80^{o}C.

Subunit vaccines require adjuvant to induce high immune response following vaccination. Adjuvants provide the advantage of directing the immune response, towards a cellular immune response (Vogel 2000). Selecting the adjuvant is one of the key success of any vaccine in the field. Selecting a flexible adjuvant that will fit with several vaccines dedicated to one or more animal species as a source of economical efficiency. Frequently, the safety or efficacy obtained with one model is different from another: there are few adjuvants with the expectation of more than one animal species. Montanide™ Gel, an innovative polymeric adjuvant has been tested in several animals. Additionally, the Montanide^{TM} ISA series of water-in-oil emulsion adjuvant has shown superior efficacy in conjunction with a variety of human and animal vaccines (Cox *et al.,* 2003), leading to high production of antigen-specific antibody. Based on the data obtained, recombinant proteins with Monatnide^{TM} ISA 20 VG adjuvant was used during the study.

5.7.3 Indirect-ELISA for detection of protection specific antibodies

I-ELISA showed that the three proteins (cVP2, VP5 & NS1) developed specific serum IgG antibodies in immunized mice. The results explained that the three scaled recombinant proteins produced protein-specific antibodies suggesting that the proteins were immunogenic, compared to the control mice that were negative in antibody production. The data indicated that 25 µg of the recombinant proteins with Montanide adjuvant was sufficient to provoke immune response in mice. Since NS1-specific antibodies were detected in I-ELISA, as NS1 is highly conserved among all the serotypes, it can be used to detect BTV-specific antibodies in BTV positive samples collected from field outbreaks of BT disease.

5.7.4 Serum neutralization studies

Neutralizing antibodies have shown to be an essential component of the protective immune response against BTV (Huismans *et al.,* 1987; Roy *et al.,* 1990). So, for confirmation of the protein-specific immune response by I-ELISA these recombinant proteins were evaluated for their neutralization either with BTV-16 alone (since the recombinant protein developed was from BTV-16) or other serotypes. Serum neutralization test was carried with the available serotypes (BTV-1, 2, 4, 5, 9, 10, 12, 16, 21, 23, and 24). These serotypes represent 8 (A-E, G-I) of the 10 (A-J) BTV nucleotype groups (Erasmus 1990) (Fig. 5).

5.7.5 Comparison and evaluation of the neutralization titres produced against BTV serotypes

5.7.5.1 BTV-1

Though only conserved region (cVP2) was used in the subunit vaccine designing, the antibody titre (log1.6) produced was found to be higher than the whole VP5 (log 1.3), and neutralization was not detected with NS1. The higher titre of antibodies produced by inactivated vaccine (log 2.5) could be that commercial inactivated vaccine used has BTV-1 as a part of 5 BTV serotypes (BTV-1, 2, 10, 16, and 23). However, similar pattern was not observed with BTV-2, 10, 16, and 23 in the current study. No significant enhancement of the neutralizing antibody (Nab) titres (log 1.9) were detected in the group immunized with a cocktail of cVP2, VP5 and NS1.

5.7.5.2 BTV-2

The neutralization titres obtained by the commercial vaccine group (log 2.5) were found to be in equivalence with the serum raised against VP5 (log 2.5). Though VP5 had a high titre, no significant additive effect was seen in group immunized with cocktail of cVP2, VP5 and NS1titre (log 1.9). Nabs were not detected with NS1. The higher log titres against BTV-2 in vaccine group may be attributed to its presence in the vaccine formulation.

5.7.5.3 BTV-4

The important finding with regard to the neutralization titres produced against BTV-4 is that sera from all groups could neutralize BTV-4 with high titres of cVP2, VP5 with log 3.1 as compared to the inactivated vaccine that had titre of log 1.9. It is important to note that though the commercial vaccine lacks BTV-4 in its formulation it could produce antibodies against BTV-4 but to a lesser extent as compared to that of the sub unit Groups 1, 2, 3 and 4.

5.7.5.4 BTV-5

Neutralization was not detected with any of the groups against BTV-5. A plausible explanation for this is its recent emergence (Hemadri *et al.*, 2017). However, this needs further study.

5.7.5.5 BTV-9

Even though high neutralizing titres of log 3.1 were detected with the serum raised against cVP2 and VP5 there was no significant added effect in the immunization titres produced in cocktail group (Nab titre log 2.5). Inactivated vaccine lacking BTV-9 in its composition could elicit neutralizing antibodies against BTV-9 (log 1.9) but were found to be less than the titres obtained with the recombinant groups (except for group injected with NS1).

5.7.5.6 BTV-10

High neutralization titres of log 3.1 were produced by cVP2, VP5 and the cocktail, followed by titre of log 2.5 by inactivated vaccine, and a titre of log 1.6 by serum of NS1. Eventhough, the neutralization titre was found to be lower than serum raised against cVP2, VP5 and group that received all the three recombinant proteins, it was found to be higher than the serum raised against NS1. The high antibody titres produced by the recombinant proteins cVP2 and VP5 over the inactivated vaccine despite having BTV-10 in its formulation indicates the greater immunogenic nature of the recombinant proteins.

5.7.5.7 BTV-12

No neutralization was detected with any of the groups against BTV-12 except in the commercial inactivated vaccine group with a titre of log 2.2. It is important to stress that the commercial vaccine lacks the BTV-12 in its composition. The presence of neutralization antibodies against the BTV-12 indicates cross-protection against BTV-12 with the formulated BTV serotypes BTV-1, 2, 10, 16 and 23.

5.7.5.8 BTV-16

High neutralization titres of log 3.1 were produced by all the recombinant protein groups in contrast to the commercial vaccine group had less antibody titre of log 2.2. The commercial vaccine contains BTV-16 in its formulation but the titres obtained were less than the recombinant proteins, indicating higher ability of the recombinant proteins in antibody production than the whole virion. Eventhough the whole virion should have efficiency of higher antibody production than the subunit vaccine, the tires obtained during the study may be attributed to the fact that the subunit vaccines produced were based on BTV-16 nucleotide composition.

5.7.5.9 BTV-21

Less titre of log 2.5 was seen in cVP2 and the cocktail groups. Additive effect was not seen in the cocktail group despite titres of log 3.1 produced by VP5 and NS1. No neutralization was detected with inactivated vaccine serum against BTV-21. The reason for lack of neutralization with the vaccine may be attributed to the fact the vaccine lacks BTV-21 in its composition.

5.7.5.10 BTV-23

High neutralization antibody titres (log 3.1) were detected with VP5 and NS1, and no significant additive effect was seen in group injected with all the three recombinant proteins (Nab titre log 2.5). The Nab titre against cVP2 was also found to be lower (log1.6) than VP5 and NS1. The high neutralization titres (log 2.8) obtained during the study indicated that despite of the BTV-23 in inactivated vaccine, the titres obtained were higher with the recombinant proteins VP5 and NS1.

5.7.5.11 BTV-24

Neutralization was not seen with any of the sera used against BTV-24. This could be attributed to its recent emergence (Krishnajyothi *et al.,* 2016). However, this needs further investigations.

The neutralizing ability of cVP2 strengthens the hypothesis that the conserved region on the VP2 fragment is immunologically significant corroborating the *in-silico* findings of two MHC-I, two MHC-II and B-cell epitopes in the conserved region, thereby making a way for further investigation in the future. There is no information in the literature pertaining to immunization studies for conserved region in BTV VP2 protein. Hence, our study presents the first report of immunization of conserved VP2 segment. However, there are reports where individual or combination of virus proteins have been evaluated as vaccines for BT. The protein VP2 released from the virion (Huismans *et al.,* 1987) or expressed through baculovirus (Roy *et al.,* 1990), bacteria (Mohd Jaffar *et al.,*

2014) or plants (Thenemann *et al.,* 2013) has been shown to elicit neutralizing antibodies and protect sheep against challenge with homotypic virus. Recent studies of Anderson *et al.* (2013 and 2014) highlighted the importance of VP2 and showed that baculovirus-expressed and purified VP2 induced neutralizing antibodies and is stable at +4^{o}C as well as -80^{o}C for two years. Previous findings suggested that VP2 of BTV evolved through duplication and may therefore exist as two related domains, VP2D1 and VP2D2 (Mohd Jaffar *et al.,* 2014). Sera from BALB/c mice immunized with the soluble recombinant VP2D1 of BTV-4 neutralized the homologous virus, while significantly lower Nab titres were observed with sera of mice immunized with soluble VP2D2. This suggests that the majority of the dominant neutralizing epitopes are present in the amino terminal half of VP2. However, when both domains were mixed together on an equimolar basis, higher titers of neutralizing antibodies were elicited. There is also published evidence that neutralization epitopes are located in the first ~350 amino acids (domain 1) of VP2 of BTV-10 (DeMaula *et al.,* 1993).

Although BTV-VP2 is the primary determinant of serotype, the smaller outercapsid protein VP5, also elicits the neutralization response, possibly through interactions with VP2 in the virus capsid (Mertens *et al.,* 1987a and Cowley & Gorman 1987). Moreover, VP5 is the second most variable BTV protein, with only 41% to 79% aa identity between serotypes (Mann *et al.,* 2011 a, b) and appears to influence the conformation of VP2 (Cowley and Gorman 1989; Mertens *et al.,* 1989). Previous studies exploring recombinant BTV structural proteins as subunit /vaccine candidates have evaluated crude lysates of recombinant-baculovirus-infected insect cells expressing BTV VP2 and VP5 (Roy *et al.,* 1990; Stewart *et al.,* 2012 and Anderson *et al.,* 2013). Immunization of sheep with these proteins elicited significant neutralizing antibody titres (up to log 2.408) with transient or undetectable viraemia after a subsequent homologous BTV challenge (Roy *et al.,* 1990).

NS1, being one of the most conserved BTV proteins was included in the study with the aim of detecting neutralizing antibodies against BTV serotypes and to check its role in eliciting broad spectrum immune response. Moreover, it has also been shown to produce specific humoral and cell mediated immunity as cows immunized with an experimental vaccine containing VP2, NS1and NS2 developed neutralizing antibody responses to BTV-8 and a serotype-specific cross-reactive antibody response to NS2 (Anderson *et al.,* 2013). In general, the NS proteins have predominantly been associated with cross-serotype cellular immune responses although VP's (VP2, VP5, and VP7) also have been shown to induce some level of T cell responses (Andrew *et al.,* 1995; Jones *et al.,* 1996; Janardhana *et al.,* 1999). NS1 has been shown to be a strong inducer of cytotoxic T cells in sheep, and it can induce cross-serotype and serotype-specific T cell responses, including helper T cells (Takamatsu *et al.,* 1990; Andrew *et al.,* 1995; Janardhana *et al.,* 1999; Rojas *et al.,* 2014). Despite being largely associated with the induction of cellular immunity, NS1, NS2, and NS3 have also been shown to induce humoral immune responses following BTV infection; although the role of these antibodies in virus clearance or protection is not clearly known (Richards *et al.,* 1988). This could explain the consistent low Nab titres in group injected with NS1 in the current study.

The major intention in including a group injected with all the three recombinant proteins was to assess additive effect of the recombinant proteins in antibody production. Our findings indicated that either an equivalent or less, but not higher antibody production against any of the serotypes tested. However, it cannot be ignored that the doses of recombinant proteins and the inactivated vaccine are not ideally comparable. Further studies in the natural host may shed more light into the role of CTL responses, additive effect of CTL and humoral immune responses.

Compared to other experimental vaccines, high neutralizing antibody titres observed in this study with the recombinant proteins indicated the added advantage of the designed subunit vaccine potential adaptability over the commercial inactivated vaccine. The subunit vaccine using cVP2, VP5 and NS1 was potential to use as a BTV vaccine which is safe, broad spectrum and DIVA compliant. The potentiality of these recombinant proteins should further be evaluated in natural host along with challenge studies so that it can be used safely in the field conditions.

CHAPTER-V

SUMMARY

The present study was aimed to develop a novel subunit vaccine against BTV that could be safe, adaptable to different serotypes, and DIVA compliant. The rationale behind the formulation of the experimental vaccine was to study the immunological response to the conserved VP2, complete VP5, and complete NS1 of BTV and also include a cocktail of BTV proteins that could induce better immune response.

MEGA analysis of databases for the available 29 serotypes of BTV for Segment 2 that codes for VP2, Segment 6 that codes for VP5 protein, and Segment 5 that codes for NS1 obtained from NCBI GenBank revealed conserved regions among different serotypes for VP2 and VP5, and NS1 is very much conserved throughout the gene sequence. In the Seg-2 alignment, a number of gaps due to insertions and deletions among the different serotypes were observed whereas VP5 nucleotide and protein sequences were rather conserved. The amino acid sequence alignment showed that, though VP2 is highly variable, several regions are relatively more conserved between serotypes. According to the present study analysis, most conserved region of VP2 is evident in the region of 338-383aa. *In silico,* two MHCI, two MHCII binding, and three B cell epitopes were predicted in cVP2 with significant percentile ranks indicating strong immunogenicity of cVP2 thereby making it a strong suitable vaccine candidate. 3D homology modelling of VP2 protein of different serotypes of BTV revealed different structures whereas cVP2 region of those serotypes revealed similar protein structure**.** Being rather conserved, VP5 of different BTV serotypes revealed similar protein structure on homology modelling**.** RNA isolation was done by Trizol extraction from BTV-16 (that was isolated from 2016 field outbreaks of BT in Andhra Pradesh) infected BHK-21 cell culture fluid. The isolated RNA was used to synthesize cDNA followed by PCR with cVP2, VP5 and NS1 gene-specific primers. The amplified cVP2 (265 bp) as above was used to clone in pRSET B vector. However, VP5 and NS1 as amplified above could not be expressed upon cloning in different bacterial vectors. Hence, to enhance the expression of these proteins, these two genes were codon optimized then cloned and expressed in *E. coli* BL21 (DE3) host system. With this the gene sequence was altered for efficient codon usage by *E. coli's* transcription machinery but the protein sequence remained unchanged. Thus codon optimized genes were used to amplify products for cloning using the designed primers.

Protein production was IPTG dependent for cVP2 and autoinduction was employed for VP5 and NS1. Fractionation studies showed that all the three recombinant proteins were in inclusion bodies (insoluble fraction). Optimized conditions were used for the purification, dialysis and solubilization of the recombinant proteins cVP2, VP5 and NS1. The purity of the eluted His-tagged fusion protein was checked by SDS-PAGE and was found to be 90%, 80% and 85% for cVP2, VP5 and NS1, respectively.

Immunization studies were carried out with 36 BALB/c mice. Animals were divided into six groups with six animals in each group. Group 6 containing 6 animals were kept as controls that received plain Montanide. Test animals (Group 1 to Group 4) were injected with 25 μg of the purified protein mixed with equal quantity of Montanide subcutaneously at the back of the neck. Group 5 received 100 μL of BTV commercial pentavalent vaccine (Raksha Blu, Indian Immunologicals). Animals were boosted twice with the same antigen concentration at days 14, 28 and were monitored till day 40. All the animals in the study were examined daily for the presence of local and systemic reactions till day 40. On day 41, mice were anaesthetized with isopropyl alcohol, and around 1 mL of blood was collected from each mouse, serum separated and stored at -80^{o}C till use.

Indirect ELISA was standardized using the respective recombinant proteins, and our results showed that the immunized mice developed antibodies against the given recombinant proteins. In addition, the NS1 recombinant protein was used as detection tool for detection of BTV antibodies in BT suspected field samples. The known tested samples found to be positive for BTV antibodies by commercial VP7 ELISA kit and tested against NS1 coated plate. Recombinant NS1 was capable of detecting the field samples suspected of BT.

Neutralization studies on immunized mice sera were carried out with virus (BTV-1, -2, -4, -5, -9, -10, -12, -16, -21, -23 and -24) concentration of 100 TCID$_{50}$ incubated with equal amount of diluted serum (1/10 to 1/1280). Plates were observed under inverted microscope at 12 h interval for CPE and the final titer was taken on day five by staining. Varying neutralizing titres were observed for different BTV serotypes against different groups. Recombinant proteins elicited higher Nab titres as compared to inactivated vaccine group, except for neutralization of BTV-1 where the vaccine group elicited higher Nab titres. Interestingly, the neutralizing antibody titres generated by BALB/c mice immunized with a cocktail of cVP2, VP5, and NS1 was either similar to or less than individual protein groups. BTV-12 could only be neutralized by serum raised against inactivated vaccine. Moreover, BTV-5 and 24 could not be neutralized by any of the groups tested. There was no neutralization of any of the serotypes with the control group serum.

Conclusion

In conclusion, the study began with cloning and expression of three BTV proteins (cVP2, VP5 & NS1) followed by evaluation of immunogenicity of these proteins with Montanide as adjuvant in BALB/c mice. Since, the developed subunit proteins were shown to induce specific immune response and could neutralize other serotypes, the recombinant proteins cVP2, VP5 and NS1 are of significance in development of broad spectrum BT vaccine. Furthermore, by using this rational vaccine design, the recombinant subunit vaccine may be adapted to additional or multiple serotypes of BTV depending on the local epidemiology. Additional investigations on the determinants of cellular immune response of these proteins and immunogenicity studies in the natural host will shed more light into the immune response generated by these vaccines. To conclude, with the rapidly changing dynamics between the climate, vector ecology, and the risk posed by newly emerging BTV strains, development of a universal BTV vaccine is of major importance.

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APPENDIX

PBS

Sodium chloride (NaCl)	8.00 g
Potassium chloride (KCl)	0.2 g
Di- sodium Hydrogen orthophosphate (Na_2HPO4)	1.44 g
Potassium dihydrogen orthophosphate (KH2PO4)	0.24 g
Double distilled water (make up to)	1.00 Litre.

Sterilized through autoclave at 121°C/15lb/15 min and stored at 4°C. (pH-7.4)

Dulbecco's Modified Eagle Medium (DMEM)

Dulbecco's Modified Eagle Medium (Ref.No.12800-058 1L; Gibco) powder of 13.48 g was dissolved in 1000 mL of autoclaved distilled water in a conical flask gradually with constant mixing. To this 3.7 g of sodium bicarbonate was added. To that 100 IU/ mL of Benzyl Penicillin, 100 µg/ mL of Streptomycin sulphate, 5 µg/ mL of Ciprofloxcin and 2 µg/ mL of Amphotericin B were added. Medium was filtered through 0.22 µm membrane filter using filter assembly. Sterility of medium was checked using Nutrient Agar and Sabouraud Dextrose Agar, for any bacterial or fungal contamination. Prepared medium was properly sealed and stored at 4°C. Before use, medium was brought to room temperature.

Growth Medium

Growth medium was prepared by supplementing DMEM with 10% TBP and 5% FBS (Ref.No.RM1112-500 mL; HiMedia).

Maintenance Medium

Maintenance medium was prepared by supplementing DMEM with 1% FBS. Maintenance medium was used during the maintenance period of the cell layer and no for subculture. During maintenance phase, the cells will survive but the rate of division is slow. Hence only 1% FBS was used.

Trypsin (0.2%)

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

0.5M EDTA (pH-8.0)

18.61 g of EDTA was added to 80 mL of water stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH and volume was made up to 1 litre with double distilled water. The solution was sterilized by autoclaving.

1M NaCl

5.844 g of NaCl dissolved in 75 mL of water after proper mixing the volume was made upto 100 mL

10 X TRIS ACETATE EDTA BUFFER (TAE BUFFER)

48.4 g Tris base was dissolved in 800 mL of distilled water followed by addition of 11.42 mL Glacial acetic acid and 20 mL 0.5 M EDTA. The pH was adjusted to 8.0 with NaOH and volume was made up to 1 litre with double distilled water.

ETHIDIUM BROMIDE

0.5 g of Ethidium bromide was added to 50 mL of water and was stirred on a magnetic stirrer for several hours to allow the dye to dissolve. The container was wrapped in aluminum foil and stored at room temperature.

TE Buffer

10Mm Tris(Ph 8.0) – 1.21g

1Mm EDTA (di-sodium salt)-0.37g

Distilled water-1000 mL.

10%SDS

SDS -10G

DDH2o-100 mL

10% Ammonium persulphate (APS)

Ammoniumpersulphate- 0.10g

Distilled water-1 mL

Staining solution

Coomassie brilliant blue (CBB R-250) - 0.25 g

Methanol-50 mL

Glacial Acetic acid-10 mL

Distilled water-40 mL

Destaining solution

Methanol-45 mL

Glacial acetic acid-10 mL

Distilled water-45 mL

LB (Luria-Bertani broth)

LB broth- 2.5g in 100 mL of distilled water

Autoclaved at 121^0 C for 15 min.

LB agar

LB agar- 2.5g in 100 mL of distilled water

Agar agar-1g

Autoclaved at 121^0 C for 15 min

SOB media

Tryptone-8 g

Yeast-2 g

Sodium Chloride-0.2 g

0.25 M potassium chloride-4 µ mL

Sterile water upto 4 mL

Allowed to cool and added 1/100 volume of sterile 2M mg2+ (Magnesium ions).

SOC

SOB-98 mL

2M mg2+- 1 mL (sterilized by filtrate by 0.2 µL syringe filter)

2M glucose-1 mL (sterilized by filtrate by 0.20.2 µL syringe filter)

180.16 g/1L=1M glucose

Gel loading Dye

Tris-HCL (pH-7.6)-10 mM

Bromophenol blue-0.03%

Xylene cyanl FF-0.03%

Glycerol-60%

EDTA-60 mM

IPTG (Isopropylthio-β-D-galactoside) 0.1M

IPTG-238.3 mg

Distilled water-10 mL

Filtered, through 0.22μ and stored at -20^0 C.

Ampicillin

Ampicillin Powder-500 mg

Sterile distilled water-10 mL

Filtered, through 0.22μ and stored at -20^0 C.

Kanamycin

Kanamycin powder-300 mg

Sterile distilled water-10 mL

Filtered, through 0.22μ and stored at -20^0 C.

1M Calcium Chloride Solution

$CaCl_2$, $6H_2O$-54 g

Distilled water-200 mL

Filtered, through 0.22μ and stored at -20^0 C.

Protein Marker

ProteinMarker (Mediumrange)-2 µL

Water-4 µL

Lamelli Buffer-4 µL

Western blot reagents

Transfer buffer (1000 mL pH 8.30)

Tris base -3.03 g

Glycine -14.4 g

Methanol – 100 mL

Distilled – 900 mL

Phosphate buffer saline tween-20 (PBS-T)

PBS (pH-7.4)-1000 mL

Tween-20 (0.1%)-1 mL

Blocking buffer

PBS-T – 1000 mL

Skim milk powder -50 mg

DAB substrate solution (100 mL)

PBS (pH-7.4)-1000 mL

DAB-50mg

H_2O_2 (30%)- 10 µL

Coating Buffer

3.7g Sodium Bicarbonate ($NaHCo_3$)

0.6g of Sodium Carbonate (Na_2Co_3)