NONSTRUCTURAL GENE BASED MOLECULAR CHARACTERIZATION OF BLUETONGUE VIRUS

T H E S I S

Submitted

in partial fulfilment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY MICROBIOLOGY

BY

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2022

DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled **"NONSTRUCTURAL GENE BASED MOLECULAR CHARACTERIZATION OF BLUETONGUE VIRUS"** or part thereof has not been submitted for any other degree or diploma of any university, nor the data have been derived from any thesis/publication of any university or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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This is to certify that the thesis entitled, **"NONSTRUCTURAL GENE BASED MOLECULAR CHARACTERIZATION OF BLUETONGUE VIRUS"** submitted by **GHARAT DIPALI KESHAORAO** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **Master of Veterinary Science** in the subject of **Veterinary Microbiology** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

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***Place**: Nagpur*
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LIST OF ABBREVIATIONS

AGE	Agarose gel electrophoresis	Amp	Ampicillin
BHK	Baby hamster kidney	BT	Bluetongue
aa	Amino acids	BTV	Bluetongue virus
Blastn	Basic Local Alignment Search Tool	bp	Base pair
CPE	Cytopathic effect	CLPs	Core-like particles
°C	Degree Celsius	°F	Degree fahrenheit
°N	Degree North	°S	Degree South
DNA	Deoxyribonuclease Acid	dsRNA	Double stranded RNA
dNTP	deoxynucleotide tri phosphate	DSS	Dried sample spot
DIVA	Differentiation of infected from vaccinated animals	DISA	Disabled Infectious Single Animal vaccine
ELISA	Enzyme-linked immunosorbent assay	EDTA	Ethylenediamine tetraacetic acid
ETEC	Enterotoxigenic Escherichia coli	Fig.	Figure
e.g.	For example	FTA	Flinders Technology Associates
g/gm	Gram	HBV	Hepatitis B virus
Hrs/hrs	Hours	%	Percentage
IFN	Interferon	IL	Interleukin
IU	International unit	LB	Luria broth
mRNA	Messenger RNA	mAbs	monoclonal antibodies
Min	Minute	µg	Microgram
ml	Milliliter	µl	Microliter
NCBI	National Center For Biotechnology Information	NS	Non-structural Protein
OIE	Office International des Epizooties	pg	Picogram
PAGE	Poly Acrylamide	PCR	Polymerase Chain

	Gel Electrophoresis		Reaction
RPM	Revolution Per Minute	RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium Dodecyl Sulfate	TE	Tris-EDTA buffer
TNF	Tumor necrosis factor	VLPs	Virus-like particles
VP	Viral Protein	Viz.	It is permitted to see

INTRODUCTION

The Livestock sector is an important part of the agricultural production system in India and plays an important role in national economy. According to the 20th Livestock census, the total Livestock population is 536.76 million in the country. Out of that, sheep population is 74.26 million and goat population is 148.88 million in 2019. When compared to previous census, the increase in livestock population by 4.8% is observed, whereas 14.1% and 10.1% increase is observed in sheep and goat population respectively. Maharashtra contributes 3.63% sheep population and 7.12% of goat population. The 20th census counts 2.7 million sheep and 10.6 million goat population in Maharashtra which shows 3.87% and 25.72% increase over the previous census (20th Livestock census report, Government of India, 2019). As the population of sheep and goats is increasing, the threat of infectious diseases also increasing. The infectious viral diseases like sheep pox, goat pox, *peste-des petits ruminants*, Border disease, orf/contagious pustular dermatitis, Bluetongue disease, etc. affect the economy of the Livestock industry (Kardjadj and Ben-Mahdi, 2017).

Bluetongue virus (BTV) is classified as a notifiable disease by the World Organization for Animal Health because of severe economic damage as a result of trade restrictions in outbreak-prone areas (OIE, 2012). Bluetongue has an economic impact on livestock that is both direct (production) and indirect (income loss). Worldwide there are 28 serotypes, however, in India, there are more than 23 serotypes identified either based on serological or virological methods. The estimated global economic loss as a result of BT was US$ 3 billion (Ruston and Lyons, 2015). The maximum yearly average loss due to BT in India was roughly Rs. 299.09 lakh, accounting for 1% of total economic loss owing to all diseases in sheep in the country (Singh and Prasad, 2009). The losses are both direct (death, abortions, weight loss, or reduced milk yield and meat efficiency) and, what is more important, indirect as a result of export restrictions for live animals, their semen, and some products such as fetal bovine serum and costs of preventive and control measures. These measures could have a major influence on the quantity of meat and animal products accessible for the consumer market if bluetongue spreads further; thus,

bluetongue is considered a possible biological weapon (Zendulkova and Pospisil, 2007).

Bluetongue (BT) is a viral disease transmitted by *Culicoides spp.* that infects ruminant and camelid species. Clinical infection is mostly reported in sheep; however, subclinical BT infections are seen in both domestic and wild ruminants (Chand *et al.*, 2015). Cattle and goats are the main hosts of the virus; however, infection is frequently asymptomatic in these animals in spite of high virus levels, allowing the disease to spread without symptoms. In most cases, sheep and deer are the only species that show signs of illness. In general, older animals are more susceptible (Elbers *et al*., 2008). Bluetongue is spread whenever susceptible animal species get imported into areas where virulent *Bluetongue virus* (BTV) strains were circulating, and also when virulent BTV strains widen their distribution to previously unaffected ruminant populations (Zientara *et al.*, 2010).

The incubation period of the disease is 4 to 8 days. The disease is characterized by fever (105-106°F), depression, anorexia, tachypnea, salivation, nasal discharge, hyperemia of the lips and nostrils, edema of the tongue and face, ulcers on the oral mucosa, and conjunctivitis are some of the clinical symptoms seen. There is cyanosis of the tongue in a few cases. Animals with coronitis, laminitis, lameness, and muscle weakness develop after the pyretic phase, and they stand with an arched back. Dermatitis, torticollis, and wool break are all possible side effects (Brewer and MacLachlan, 1994; Elbers *et al.*, 2008).

The disease is caused by the *Bluetongue virus*, which belongs to the *Orbivirus* genus in the *Reoviridae* family. BTV is a non-enveloped virus with icosahedral symmetry, a diameter of 80-90 nm, and a 10-segmented linear dsRNA genome (Verwoerd *et al*., 1972; Mertens and Diprose, 2004; Stewart *et al*., 2015). Seven structural (VP1–VP7) and five nonstructural (NS1, NS2, NS3/NS3A, NS4, and NS5) proteins are encoded by the 10 viral genome segments (Stewart *et al*., 2015).

The NS1 and NS2 proteins are widely produced and two closely related proteins, NS3 and NS3A, are hardly detectable. The sequences of all NS proteins are

highly conserved (96%) across BTV serotypes (Roy *et al*., 1990). In BTV-infected cells, NS1 and NS2 synthesis coincides with tubules and granular viral inclusion body (VIB) synthesis, that characterize the cytoplasm of orbivirus-infected cells (Cromack *et al*., 1971). Tubules are composed of NS1 protein. This protein is highly expressed than any other BTV protein (Huismans and Els, 1979). The NS2 protein is virus-specific phosphoprotein which is identified in BTV-infected cells (Huismans, 1979). It has important role in the virus life cycle. NS3 and NS3A proteins are synthesized in low level in BTV-infected BHK cells (Mertens *et al.,* 1984). Insufficient NS3 and NS3A synthesis in mammalian cells by BTV has prevented the structural analysis, while cDNA analyses and the use of expression vectors have provided some information on their structural and immunological properties. NS3 and NS3A are related, according to peptide mapping and immunological investigations (French *et al*., 1989). NS4 and NS5 involved in viral nuclear localization (Stewart *et al.,* 2015).

In mammals, Seg-10/NS3 proteins affect BTV replication kinetics, but not in insects. NS3 protein turnover may vary in ovine but not in Culicoides cells due to a single amino acid residue that, most likely, leads to rapid and host-dependent protein degradation. The genetically distant BTV Seg-10/NS3 influence BTV biological properties in a host-specific manner and increases understanding of how NS3 proteins contribute to the outbreak of BTV infection (Ftaich *et al.,* 2015).

The clinical signs of Bluetongue are similar to those of other ruminant diseases. Thus, BT is easily confused with other ruminant diseases like foot-and-mouth disease, vesicular stomatitis, peste-des-petits ruminants, photosensitization, contagious ecthyma, sheep pox, oestrus ovis infestation, malignant catarrhal fever, pododermatitis, infectious bovine rhinotracheitis, bovine viral diarrhoea, etc. (Mehlhorn *et al.,* 2009; Williamson *et al.,* 2008; Savini *et al.,* 2011). BTV can infect animals with several other viruses that have similar symptoms, causing diagnosis difficult. Antigen, antibody, and nucleic acid-based assays were used to identify the mixed infection of BTV and *Peste des petits ruminants virus* (PPRV) in goats (Mondal *et al.,* 2009). Hence, apart from clinical symptoms of the disease, several

sensitive molecular biological methods should be used to confirm the diagnosis of Bluetongue in ruminants.

Early detection and response to such diseases are still delayed by infrastructure limitations. Many epidemics go unreported or result in diagnosis failure due to a lack of facilities and professional technicians to collect samples, transport them to laboratories in good condition, and so on. As a result, certain simple, practical, and user-friendly sample collection procedures to minimal infrastructural requirements and independent of cold-chain conveyance to the laboratory are urgently required.

The most sensitive approach for detecting the *Bluetongue virus* is reverse transcriptase polymerase chain reaction (RT-PCR) (Prasad *et al.,* 1999). The Dried Sample Spot (DSS) or Dried Blood Spot (DBS) has been mentioned as the most suitable method for biomaterial sampling due to specific inherent advantages, such as the small volumes of biomaterials required, the lack of a need for specific conditions for sample storage and transportation, improved analyte stability, and reduced risk of infection from contaminated samples (Malsagova *et al.,* 2020).

Many of the major replication and assembly activities within the infected target cell are controlled by NS proteins, which could make them good targets for antiviral intervention techniques. Understanding the evolution and epidemiology of the bluetongue virus requires sequence analysis and phylogenetic research based on the epidemiologically important genes of BTV. NS3 was selected for phylogenetic analysis because it is useful for topotype analysis and it has been suggested that NS3 may exert an important role in determining the vector status of individual species of Culicoides insects for *Bluetongue virus* (Balasuriya *et al.*, 2008).

Further information on the use of nonstructural genes for *Bluetongue virus* characterization is obscured, topotyping based on nonstructural genes is limited, diagnosis need and difficulty in sample collection and dispatch remains critical in identification and confirmation, which leads to failure in planning the control of

disease. Looking into these issues, the present study was planned with the following objectives.

1. To amplify the nonstructural gene of *Bluetongue virus* by RT-PCR.
2. To standardize the Dried Sample Spot-RT-PCR for detection of *Bluetongue virus*.
3. To sequence the nonstructural gene of *Bluetongue virus* and analyze the phylogenetic relationship of *Bluetongue virus* based on the nonstructural gene.

REVIEW OF LITERATURE

Bluetongue (BT) is a notifiable disease (World Organization for Animal Health, OIE). The disease cause significant economic damages due to decreased trade and increased mortality and morbidity. *Bluetobgue virus* (BTV) reacts with a wide range of antigenically similar viruses, including those that cause African Horse Sickness and Deer Epizootic Haemorrhagic Disease (Rojas *et al.,* 2019).

Along with regions of Southeast Asia, India is most likely one of the major BTV source populations in Asia. Bluetongue outbreaks in India are linked to meteorological conditions in various sections of the country and the density and breeds of sheep. Most sheep are affected by the disease and die as a result of it, primarily in the southern areas of the country (Rao *et al.,* 2016).

Since the initial report of Bluetongue from India (Sapre, 1964), several serotypes of BTV have been found, either by anti-BTV antibody detection or virus isolation. Until the year 2000, most virus isolation and serotyping were done at one of two World Reference Laboratories for Bluetongue (Prasad *et al.,* 2009). All-India Network Programme on Bluetongue, and other research groups, have been isolating and typing BTV since 2001. *Bluetongue virus* (BTV), the *Orbivirus* genus in the family *Reoviridae*, causes a non-contagious, infectious, arthropod-borne viral disease of ruminants (Pandrangi, 2012).

2.1 History of Bluetongue

Bluetongue was discovered initially in South Africa at the end of the 18th century, following the import of fine wool sheep from Europe (Spreull, 1905). Hutcheon reported the clinical aspect of the disease in his annual report in 1880. However, it was not until 1902 that the disease "Malarial catarrhal fever" or "Epizootic catarrh" was first reported in the scientific literature (Hutcheon, 1902). The word "bluetongue" is derived from the African "bloutong," which was coined by South African farmers after observing cyanotic tongue in clinically infected animals (MacLachlan *et al.,* 2009). Although the disease has certainly occurred there since at least 1924, the first well-documented epizootic of BT outside of

Africa occurred among sheep in Cyprus in 1943 (Worwa, 2009). Later, BT was recognized in the United States, the Iberian Peninsula, the Middle East, Asia, and southern Europe. The increased recognition of BT in widely separated regions of the world in the middle of the 20th century was interpreted at the time to reflect the emergence of BT from its presumed ancestral origin in Africa (Verwoerd and Erasmus, 2004).

BTV has recently spread across North America and China, with cases reported up to 50°N. Since 1998, the BT disease range has changed significantly, with the disease also affecting countries in northern Europe and Scandinavia (Saegerman *et al.,* 2008). It spreads northward from the Caribbean Basin, invading the southeastern United States and northern Australia, where it had not previously been recorded (Johnson *et al.,* 2006). The rapid appearance and spread of BTV serotype 8 began in northern Europe from 2006-2007 in Germany (Elbers *et al.,* 2008).

2.2 Etiology

Bluetongue disease is caused by *Bluetongue virus* belongs to the genus *Orbivirus* within the family *Reoviridae*. *Bluetongue virus* with closely related species African Horse Sickness Virus (AHSV) and Epizootic Hemorrhagic Disease Virus (EHDV) belongs to the genus *Orbivirus* in the family *Reoviridae* (Eschbaumer *et al.,* 2009; Maan *et al.,* 2012a).

2.3 Structure of *Bluetongue virus*

The virus is non-enveloped and has 10 linear double-stranded RNA segments enclosed within a triple-layered icosahedral protein capsid (90 nm in diameter) (Maan *et al.,* 2012a). It has seven structural (VP1–VP7) and five non-structural (NS1, NS2, NS3/NS3A, NS4, and NS5) proteins encoded by the 10 viral genome segments. Except for Seg-9 and -10, each genomic segment encodes a single protein. Seg-9 encodes VP6 and NS4 proteins. NS3 and NS3A proteins are encoded by Seg-10 (Ratinier *et al.,* 2011; Stewart *et al.,* 2015).

BTV's whole genome is about 19.2 kbp in length (Roy *et al.,* 1990). To decrease molecular weight, genomic segments range in size from 3954 to 822 bp (Seg-1 to Seg-10). The non-coding region of BTV varies in length from 8 to 34 bp at the 5' end and 24-116 bp at the 3' end (Mertens and Diprose, 2004). BTV's dsRNA is made up of 57% AU (adenine and uracil) and 43% GC (guanine and cytosine), with conserved hexanucleotides (GUUAAA at the 5' end and ACUUAC at the 3' end of the positive strand) at the non-coding end of both the 5' and 3' terminal sequences (Mertens and Diprose, 2004; Stewart *et al.,* 2015).

BTV virion has three concentric capsid layers. Outer layer composed of VP2 and VP5 structural proteins. VP2 is responsible for receptor binding, haemagglutination, and triggering serotype-specific neutralizing antibodies, and has a strong affinity for BTV binding to erythrocytes (Dahiya *et al.,* 2004; Schwartz-Cornil *et al.,* 2008), and is the major determinant of BTV serotype and also revealed variations between strains of the same serotype (Dahiya *et al.,* 2004; Maan *et al.,* 2012a). VP5 is much more conserved shows slight variations that reflect the geographical origin (Singh, 2009). It has a membrane penetration protein that allows the release of viral particles from endosomal compartments into the cytoplasm (Forzan *et al.,* 2008).

The middle layer comprises VP7 and VP3 proteins that are hydrophobic and form a major core protein. They play a significant role in the structural integrity of the virus core (Anthony *et al.,* 2007). In the absence of VP2 or VP5, VP7 can mediate insect cell adhesion and penetration. The VP3/VP7 complex prevents the viral dsRNA genome from intracellular damage, preventing type I interferon (IFN) production from activation (Schwartz-Cornil *et al.,* 2008).

The inner shell is made of VP3 with minor amounts of three enzymatic proteins associated with transcription and replication, namely the RNA-dependent RNA polymerase VP1, the RNA capping enzyme VP4, and the dsRNA helicase VP6, all of which are situated along the particle's five-fold symmetry axis (Boyce, 2012). In both insect and human cells, VP1 has permits efficient replication. VP6 is an ATP-binding protein with RNA-dependent ATPase and helicase activities.

BTV's non-structural (NS) proteins are absent in mature BTV virions and are only found in infected cells (Van Dijk and Huismans, 1988; Ratinier *et al.*, 2011). The NS proteins are involved in viral replication, maturation, and virus release from infected cells. The proteins NS1 and NS2 have highly expressed proteins in infected cells. The most abundant NS1 protein creates tubules that allow translocation of progeny virus particles to the cell membrane and release from infected host cells. The NS1 protein also aids BTV's cytopathogenesis. The NS2 protein is an ssRNA-binding protein with nucleotidyl phosphatase activity that produces inclusion bodies in infected cells' cytoplasm. NS2 is involved in early morphogenesis, stops spindle development, and prevents host cell division. The NS3 protein is highly conserved and has two forms, full-length NS3 and short truncated NS3A (lacks 13 amino acids at N-terminal end of methionine codon) (Van Dijk and Huismans, 1988). The NS3 protein is the smallest non-structural membrane glycoprotein, and it produces viroporin, which causes cytoplasmic membrane permeabilization and permits virion particle release from infected cells through the budding process (Hyatt *et al.*, 1993). An open reading frame (ORF) in Seg-9 encodes the NS4 protein, overlapping the ORF encoding VP6. NS4 is found in the cytoplasm and nucleoli of BTV-infected cells during the early stages of infection (Belhouchet *et al.*, 2011; Ratinier *et al.*, 2011). The NS4 protein is involved in the virus-host interaction and counteracts the host's antiviral response. It inhibits cellular transcription, which regulates the host's IFN responses from overlapping ORF of Seg-10, the NS5 protein was identified (Stewart *et al.*, 2015). The NS5 is believed to play a similar role to BTV NS4 in viral nuclear localization (Stewart *et al.*, 2015).

2.4 Transmission

BTV is transmitted by various hematophagous biting midges from the genus *Culicoides*, order Diptera, and family *Ceratopogonidae*. From India, 63 species of *Culicoides* were identified (Maheshwari, 2012*). Culicoides actoni, Culicoides brevitarsis, Culicoides dumdum, Culicoides fulvus, Culicoides imicola (Culicoides minutus), Culicoides oxystoma,* and *Culicoides peregrines* were the

most common vectors for BTV transmission in India. (Jain *et al.,* 1986; Ilango *et al.,* 2006; Maheswari, 2012; Archana, *et al.*,2016).

BTV has been isolated from various arthropods, including sheep ked *(Melophagus ovinus*), ticks (Bouwknegt *et al.,* 2010), and mosquitoes. These play a minor role in disease epidemiology. Bull semen can also transmit the virus, but only if the bull has viremia and the semen contains red or white blood cells with which the virus is associated (Wilson *et al.,* 2008). Another route of transmission for BTV is through the placenta. It has been found in cattle, sheep, and dogs (Sternberg *et al.,* 2010; Santman-Berends *et al.,* 2010).

2.5 Pathogenesis of Bluetongue disease

The virus is transferred by the host dendritic cells from the epidermis to the local lymph nodes, the sites of early virus replication, after being introduced through the bite of an infected midge (Hemati *et al.,* 2009). It then travels to the bloodstream, causing a primary viremia that seeds secondary organs such as lymph nodes, spleen, and lungs (Sanchez-Cordon *et al.,* 2010). The virus replicates in vascular endothelial cells, macrophages, and lymphocytes in the early viraemic stage (Drew *et al.,* 2010), the virus associates with all blood constituents, but later in the disease, it only associates with erythrocytes (MacLachlan, 2009).

BTV infection causes necrosis and apoptosis in cells. It also stimulates the synthesis of TNF, IL-1, IL-8, IL-6, IFN-I, and cyclooxygenase-2 and increases the plasma concentration of prostacyclin and thromboxane, resulting in the increased inflammatory response and consequent damage to the infected animal cells and tissues (Chiang *et al.,* 2006; Schwartz-Cornil *et al.,* 2008).

Pathogenesis of Bluetongue is characterized by injury to small blood vessels in a target tissue, which leads to vascular occlusion and tissue infarction. Virus-induced vasoactive mediators secreted by thrombocytes, macrophages, dendritic cells, and BTV-infected endothelial cells cause endothelial damage,

impair its function, and increase vascular permeability, resulting in edema and effusions (MacLachlan *et al.,* 2009; Drew *et al.,* 2010).

2.6 Host range

The vertebrate hosts for BTV include both domestic and wild ruminants, such as sheep, goats, cattle, buffaloes, deer, most species of African antelope and other Artiodactyla such as camels (OIE, 2012).

2.7 Epidemiology of Bluetongue disease:

Bluetongue was first discovered in Africa in the late eighteenth century (Spreull, 1905). From then on, the disease has been documented in a variety of continents and countries, along with South and North American continents, Europe, Australia, Asia, and the Indian subcontinent (Wilson and Mellor, 2009; Sperlova and Zendulkova, 2011; Ranjan *et al.,* 2015; Rao *et al.,* 2016; Sun *et al.,* 2016; Bumbarav *et al.,* 2020). Except for Antarctica, the disease is now seen in all continents (Gould and Pritchard, 1990). Bluetongue was endemic across Africa, Europe, West Asia, and the countries of the Indian Subcontinent. In South Africa, BTV is endemic, and 22 of the 28 known serotypes were identified (Coetzee *et al.,* 2012). BTV-10 sporadic outbreaks were documented in Europe until 1998 (Wilson and Mellor, 2009). BTV-6 was observed in the Netherlands and Germany in2008, and BTV-11 was reported in Belgium (Wilson and Mellor, 2009). In Australia, twelve different BTV serotypes were identified (Firth *et al.,* 2017).

Bluetongue is an endemic disease in India, with most BTV outbreaks occurring in crossbreds and exotic sheep breeds. However, the majority of outbreaks in south India were observed in native sheep breeds. Because of its large animal population, India is one of Asia's largest sources of BTV. Since the initial report of BT from India in 1964, 23 serotypes of BTV were identified from India using serological assays and virus isolation (excluding22, 25-28). Virus isolation has detected fifteen serotypes (BTV-1-6, 9, 10, 12, 16-18, 21, 23, and 24), whereas serological testing has detected 22 serotypes (BTV-1-20, 23, and 24). The majority of the serotypes were found in southern Indian states (Mehrotra

et al., 1996; Prasad *et al.,* 1992; Sreenivasulu *et al.,* 2004; Maan *et al.,* 2012a; Susmitha *et al.,* 2012; Chauhan *et al.,* 2014; Ranjan *et al.,* 2015; Krishnajyothi *et al.,* 2016; Rao *et al.,* 2016; Hemadri *et al.,* 2017).

2.8 Non-structural genes of *Bluetongue virus*:

Bluetongue virus and recombinant vaccinia virus (expressing NS3) infected cells have been found to have NS3 and NS3A in their plasma membranes. The presence of NS3 and NS3A and BTV release had a strong association. The NS3 protein is linked with membrane fragments, and it is unable to detect the extracellular aspect of whole cells showing that it was not exposed extracellularly. The findings suggest that NS3 and NS3a are involved in the final stages of BTV morphogenesis, such as BTV release from infected cells (Hyatt *et al.,* 1991).

The association of virus-like particles (VLPs) of *Bluetongue virus* (VP2, VP3, VPS, and VP7) and cell cytoskeleton, and also the release of such particles from infected cells in the presence of BTV NS3/NS3A, but not when co-expressed with BTV NS1 protein were demonstrated using recombinant baculoviruses and immunoelectron microscopy. The expression of the non-structural NS1 or NS3/NS3A proteins in combination with core-like particle (CLPs) did not result in particle interaction with the cytoskeleton or release from cells. CLPs produced with VP2 or VP5 failed to connect to the cytoskeleton, indicating that both outer coat proteins are necessary for a stable virus-cytoskeleton interface. The results showed that the presence of NS3/NS3A is essential for the budding and subsequent release of VLPs from infected cells (Hyatt *et al.,* 1993).

Bluetongue virus produces large numbers of tubules during infection. The tubules are formed from a 552-amino-acid, 64-kDa NS1 protein encoded by the viral double-stranded RNA segment S-6. A series of deletion and extension mutants of *Bluetongue virus* serotype 10 NS1 has been generated and expressed in insect cells in order to identify the carboxy-terminal components of the protein which are important for tubule formation. Extension mutants including foreign

antigenic sequences involving up to 16 amino acids added to the C terminus of NS1 were shown to form tubules, although an extension of 19 amino acids inhibited tubule formation. Analysis of monoclonal antibodies has established that an NS1 antigenic site is located near the carboxy terminus of the protein. It appears to be exposed on the surface of tubules (Monastyrska *et al.,* 1995).

The 229-amino-acid NS3 and the 216 NS3A are non-structural proteins encoded by segment 10 of the *Bluetongue virus*. Infected cells contain both glycosylated and nonglycosylated proteins. Two hydrophobic domains (aa 118–141 and 162–182) and two asparagine-linked glycosylation sites (aa 63 and 150) are found in the NS3/NS3A proteins. They created several S10 gene mutants and expressed them using the vaccinia virus T7 polymerase transient-expression system to determine if these properties were utilized in the mature protein forms. The findings showed that both hydrophobic domains of NS3 cross the cell membrane, but only the location at aa 150 is responsible for NS3 protein N-linked glycosylation (Bansal *et al.*, 1998).

In BTV field strains, individual gene segments differ; hence, the study hypothesized that essential viral genes undergo genetic drift during the alternating passage of BTV in its ruminant and insect hosts. To test this hypothesis, variation in the consensus sequence and quasispecies heterogeneity of the VP2 and NS3/NS3A genes of a plaque-purified strain of BTV serotype 10 was determined. Individual BTV gene segments evolve independently of one another by genetic drift in a host-specific manner, generating quasispecies populations in both ruminant and insect hosts. The study concludes that genetic drift and the founder effect play a role in the diversity of individual gene segments in BTV field strains (Bonneau *et al.*, 2001).

Bluetongue virus infects and replicates in both insect and mammalian cells. BTV infected mammalian cells have a considerable CPE and BTV infected insect cells have very little CPE. Expression analysis showed that one protein, the non-structural (NS) protein NS3, is variably expressed in each infected cell type, indicating that it may play a role in virus egress. The study showed the interaction of the NS3 region with p11 to 13-residue peptide at the N terminus of the protein

that efficiently competes with p36 (annexin II heavy chain) for p11 ligand binding. The study also reported that the C-terminal domain of NS3 interacts with VP2, the fully assembled virus particle's outermost protein, indicating that NS3 works as a bridging molecule (Baeton *et al.,* 2002).

The S2 gene of *Bluetongue virus*, serotype 17, has been cloned, and the nonstructural protein NS2 has been expressed. A prokaryotic expression system was used to generate a series of deletion mutants with the ssRNA-binding domains of NS2 removed. These truncated proteins were expressed on a large scale and purified to near homogeneity. The affinity of each truncated protein towards ssRNA was assayed by electrophoretic mobility shift assays. The result showed that the three ssRNA-binding domains of BTV nonstructural protein NS2 have been conclusively localized, and removal of domains completely abrogates the ability of NS2 to bind to ssRNA (Fillmore *et al.,* 2002).

The study showed that NS3 has properties associated with viroporins. Results indicate that NS3 localize in the plasma membrane and Golgi apparatus in transfected cells, NS3 can homo-oligomerize in transfected cells, targeting of NS3 to the plasma membrane and Golgi apparatus related with enhanced permeability of cells to the translation inhibitor hygromycin B (hyg-B), amino acids 118–148 comprising transmembrane region 1 (TM1) of NS3 are critical for Golgi targeting and hyg-B permeability, and deletion of amino acids 156–181 comprising transmembrane region 2 (TM2) of NS3 has little to no effect on Golgi targeting and hyg-B permeability. These viroporin-like properties can contribute to the role of NS3 in virus release and have significant implications for *Bluetongue virus* pathogenicity (Han and Harty, 2004).

The study examined, through RNA binding assays using highly purified NS2, the specificity of interaction with different single-stranded RNA (ssRNA) species in the presence of appropriate competitors. The data showed that NS2 has a preference for BTV ssRNA over nonspecific RNA species and that NS2 recognizes a specific region within the BTV10 segment S10. The secondary structure of this region was determined and found to be a hairpin-loop with substructures within the loop. Modification-inhibition experiments highlighted

two regions within this structure that were protected from ribonuclease cleavage in the presence of NS2. These data imply that a function of NS2 may be to recruit virus messenger RNAs selectively from other RNA species within the infected cytosol of the cell during virus replication (Lymperopolus *et al.,* 2003).

Bluetongue virus released from infected host cells by cell lysis and budding. Two non-structural proteins, NS3 and NS3A, have been implicated in this process. The study showed that both proteins bind to human Tsg101 and its ortholog from *Drosophila melanogaster* with similar strengths. This interaction is mediated by a conserved PSAP motif in NS3 and appears to play a role in virus release. The depletion of Tsg101 with small interfering RNA inhibits the release of BTV and African horse sickness virus from HeLa cells up to fivefold and threefold. NS3 also harbors PPXY late-domain motif that allows NS3 to bind NEDD4-like ubiquitin ligases in vitro. The late-domain motifs in NS3 do not effectively facilitate the release of mini Gag virus-like particles from 293T cells as the late domains from human immunodeficiency virus type 1, human T-cell leukemia virus, and Ebola virus. A study showed that the arginine residue in PPRY motif is responsible for the low activity of NS3 late-domain motifs and suggest that the BTV late-domain motifs either recruit an antagonist that interferes with budding or fails to recruit an agonist, different from NEDD4 (Wirblich *et al.,* 2006).

The *Bluetongue virus* is released from infected cells by cell lysis and budding. NS3 is related to the budding process because it interacts with the VP2 and is a component of the cellular ESCRT pathway. The study used the newly developed T7 transcript-based reverse genetics system on BTV to induce mutations in the NS3 sequence into the viral genome and analyze the effect of mutations in a replicating virus. While specific NS3 mutations significantly affected newly produced viral release, others had a lesser effect. Mutations in two residues in the Tsg101 binding motif presumed L domain of NS3 disrupted regular virus egress patterns and left nascent particles attached to the cellular membrane and trapped in the budding process. No budding particles were visible in cells treated with a mutant virus that could not form an NS3-VP2 connection.

This shows that NS3 functions similarly to enveloped viruses' membrane protein, regulating intracellular trafficking and virus particle budding. Thus, during virus egress, NS3 act as a bridge between mature virion particles and cellular proteins (Celma and Roy, 2009).

The *orbivirus* genome, composed of 10 segments of dsRNA, encodes 7 structural proteins (VP1–VP7) and 3 non-structural proteins (NS1–NS3). An open reading frame of genome Seg-9 encodes VP6. Bioinformatic analysis of the *orbivirus* genome identified an overlapping ORF (ORFX) in Seg-9. The study showed that ORFX encodes a new non-structural protein, identified as NS4. Western blotting and confocal fluorescence microscopy, using antibodies raised against recombinant NS4 from *Bluetongue virus* demonstrate that these proteins are synthesised in BTV infected mammalian cells and culicoides insect cells. Bioinformatic analysis indicate that NS4 contains coiled-coils which are related to proteins that bind nucleic acids. NS4 was found to associate with lipid droplets in cells infected with BTV or transfected with a plasmid expressing NS4 (Belhouchet *et al.,* 2011).

BTV mRNAs are synthesised by the viral RNA-dependent RNA polymerase as exact plus sense copies of the genome segments. NS1-mediated upregulation of expression is restricted to mRNAs which lack the cellular 30 poly (A) sequence identifying the 30 end as a necessary determinant in specifically increasing the translation of viral mRNA in the presence of cellular mRNA. NS1 is identified as a positive regulator of viral protein synthesis (Boyce *et al.,* 2012).

The heptad regions and oligomer formation were investigated in the NS3 protein sequences of *Bluetongue virus* serotypes. At least three coiled-coil motifs (CCMs) were found in the NS3 sequences of all 26 BTV serotypes. According to bioinformatics research at 14–26 aa (CCM-I), 185–198 aa (CCM-II), and 94–116 aa (CCM-III), a conserved a-helical heptad sequence were identified (CCM-III). CCM-I, which is found near the N-terminus of NS3, is involved in oligomerization. In the prokaryotic expression system, the NS3 (1M-R117 aa) N-terminus was also overexpressed as a recombinant fusion protein. The biochemical characterization of recombinant NS3Nt protein shows that it

produces SDS-resistant dimers and high-order oligomers (hexamer and octamer). Coiled-coil motifs are thought to be important for NS protein oligomerization and may have a role in developing the viroporin ring/pore, which contains six or eight subunits, and it characterize CCMs in NS3 of *Bluetongue virus* (Chacko *et al.*, 2015).

The NS3 protein encoded by Seg-10 of the *Bluetongue virus* (BTV), plays an essential role in BTV infection. The study evaluated the impact of Seg-10 and NS3 proteins on *Bluetongue virus* infection and host interactions. Results showed that NS3 protein turnover differs in ovine but not in Culicoides cells caused by a single amino acid residue, leading to rapid and host-dependent protein degradation (Ftaich *et al.*, 2015)

The role of NS3/NS3a in the replication and dissemination of BTV1, was investigated. Virus strains were generated using reverse genetics, and their growth was examined *in vitro*. A laboratory colony of *Culicoides sonorensis*, a known competent BTV vector, was fed or injected with BTV with or without expressing NS3/NS3a, and replication in the midge was examined using RT PCR. Results showed that although the parental NS3/NS3a expressing strain could not replicate and disseminate within *C. sonorensis* after oral feeding, this virus could replicate efficiently when the midgut infection barrier was bypassed by intrathoracic injection. In contrast, the NS3/NS3a knockout mutant was unable to replicate. This demonstrates that NS3/NS3a is required for viral replication within Culicoides. The lack of viremia and the inability to replicate within the vector demonstrates the inability of NS3/NS3a knockout disabled infectious single animal (DISA) vaccine strains to be transmitted by midges (Feenstra *et al.*, 2015).

The study developed five BTV NS3-reactive monoclonal antibodies (mAbs), labeled 3D8, 2G9, 1B5, 4H8, and 2B12. To determine linear peptide epitopes identified by each mAb, a panel of overlapping NS3-derived peptides representing the entire BTV15 NS3 protein was screened. A series of progressively shortened peptides was created to find the smallest linear peptide sequence needed to maintain mAb binding. This identified linear epitopes, could

be effective reagents for studying the function of the NS3 protein and developing BTV group-specific diagnostics (Qin *et al.,* 2015).

In BTV, the ORF (S10-ORF2) encodes a putative protein 50–59 residues in length and appears to be under strong positive selection. S10-ORF2 inhibited gene expression, but not RNA translation, in transient transfection reporter assays. In both mammalian and insect cells, BTV S10-ORF2 deletion mutants (BTV8DS10-ORF2) displayed similar replication kinetics to virus. The results showed that S10-ORF2 is a fifth non-structural protein of BTV (NS5), given its localization in the nucleolus of cells transfected with expression plasmids encoding tagged versions of this protein (Stewart *et al.,* 2015).

The study found that the BTV interacts with serine/threonine-protein kinase B-Raf (BRAF), a critical component of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, through its non-structural protein NS3 (BTV-NS3). The study discovered that BTV-NS3 enhances the MAPK/ERK pathway, which is BRAF dependent activation. The pharmacologic inhibitor U0126 decreases viral multiplication in the MAPK/ERK pathway, demonstrating that BTV manipulates a pathway for its benefit (Kundlacz *et al.,* 2019).

The BTV NS3 is linked with host membranes in infected cells and traffics from the endoplasmic reticulum to the plasma membrane. The study discovered two polybasic motifs (PMB1/PMB2) consistent with membrane binding. The Mutant PBM2 motif reduces NS3 export to the cell surface and virus production. Although, both mutant viruses produced mostly inner core particles which remained close to their assembly site. The data showed that proper NS3 protein trafficking is required for viral maturation and release (Labadie *et al.,* 2020).

In this study, the applicability of the fusion construct was examined and optimized for field application. Compared to a pre-established test panel, the fusion construct utilized in an ELISA platform showed a relative diagnostic sensitivity and specificity of 98.1% and 95.5%, respectively. The rNS1-NS3

ELISA was shown to have a high level of agreement with a commercial BTV antibody detection kit (Mohanty *et al.,* 2021).

2.9 Diagnosis of Bluetongue disease

The diagnosis of BTV infection is based on either pathogen identification or immune response detection. Real-time RT-PCR, RT-PCR (Reverse transcriptase-polymerase chain reaction), and classical virus isolation are the methods for pathogen identification (Lakshmi *et al.,* 2018; Rojas *et al.,* 2019). For the detection of the immune response in the host, c-ELISA (Competitive enzyme-linked immunosorbent assay) (serogroup specific), virus neutralization test (VNT, serotype-specific), agar gel immunodiffusion test (AGID), and complement fixation test (CFT) are available (Rojas *et al.,* 2019). In earlier times, CFT was used to detect BTV antibodies, and now it is replaced by AGID in many parts of the world. The AGID test is easy and simple to perform, but the major disadvantage is that it cannot differentiate between antibodies to the BT and epizootic hemorrhagic disease (EHD) serogroups, and hence less specific. VNT is used to identify BT serotype-specific neutralizing antibodies (OIE, 2014). The OIE recommended serodiagnostic tests are complement fixation, agar gel immunodiffusion (AGID), competitive enzyme-linked immunosorbent assay (ELISA), and indirect ELISA. Indirect ELISA based on detecting BTV NS3 antibodies can be used for Differentiation of Infected from Vaccinated Animals (DIVA) (Barros *et al.,* 2009). Though there are many techniques developed for BTV diagnosis, time consuming nature and efficacy, and high cost of this techniques are major constraints in its wide usage. Most of the seroepidemiological studies in India are conducted by detecting BTV group-specific antibodies with ELISA and AGID assay (Rao *et al.,* 2016). After the inception of the AINP-BT, the prevalence and distribution of anti-BTV antibodies were carried out as per the OIE recommended monoclonal antibody-based competitive-ELISA (c-ELISA) (OIE, 2014).

2.10 Amplification of *Bluetongue virus* by RT-PCR:

The BTV reverse transcriptase-PCR (RT-PCR) is a 1-tube reaction and involves chemical denaturation of the double-stranded viral RNA target, a complementary DNA (cDNA) synthesis step, and PCR amplification of the cDNA. A 251-base pair (bp) product is obtained from BTV RT-PCR using primers chosen from highly conserved genome segment 10. BTV RNA from all USA prototype serotypes 2, 10, 11, 13, and 17; USA BTV field isolates including serotypes 10, 11, 13, and 17; Israeli field isolates including serotypes 2, 4, 6, 10, and 16 were detected by BTV RT-PCR. The BTV RT-PCR using primers derived from genome segment 10 can detect the USA and Israeli BTV serotypes and has the potential for detection of infection by the BTV serogroup (Akita *et al.,* 1992).

The use of nonradioactive chemiluminescent hybridization enhanced the sensitivity of the BTV serogroup PCR. BTV PCR with nonradioactive chemiluminescent hybridization resulted in a level of sensitivity comparable to virus isolation (VI) and likely more sensitive than VI on Vero cells for blood. This BTV PCR shows a great potentiality for detecting active BTV infection in a variety of clinical samples in a quick, sensitive, and specific manner (Akita *et al.,* 1993).

Bluetongue virus was detected in cell culture and tissue samples using a nested polymerase chain reaction. The nested PCR was performed in two phases with two sets of oligonucleotide primers (BTV-1 and BTV-4 and BTV-2 and BTV-3) selected from the non-structural protein 1 (NSl) gene of BTV-17. The 826-bp product was amplified using an outer primer pair BTV-l and BTV-4. The second amplification produced a 517-bp PCR product using the nested primer pair BTV-2 and BTV-3. The BTV-PCR assay's sensitivity was improved by using nested primers BTV-2 and BTV-3. The BTV-PCR-based assay is a useful tool for studying BTV infection epidemiology in susceptible wild ruminants and domestic livestock (Aradaib *et al.,* 1998).

The highly expressed non-structural protein gene was used as the target gene in RT-PCR to detect bluetongue viral RNA, with specific primers targeted to

274 bp of the 5' end of the NS1 gene. According to the findings, RT-PCR can be used to detect BTV in Indian conditions with a sensitivity limit of 10 infectious virus particles (Prasad *et al.,* 1999).

The RT-PCR was standardized to amplify the VP-7 gene sequences of an Indian isolate of bluetongue virus serotype 23 (BTV-23). Using two different sets of primers, a sequence of 1156 bp comprising the complete coding sequence of the VP-7 gene and its 770 bp internal sequence were amplified. The sensitivity of RT-PCR, using these two sets of primers individually was 40 picogram and 4 picogram, with the external and internal primers, respectively, whereas the nested PCR was 100-fold more sensitive than the single PCR with the external primers (Tiwari *et al.,* 2000).

Various RT-PCR protocols and strategies, which target genome segment 7, were evaluated for their ability to detect all members of the BTV species (serogroup), with the aim of developing a fully validated reverse transcriptase-polymerase chain reaction (RT-PCR) based diagnostic assay. A nested PCR strategy, using near terminal and internal segment 7 primers, detected all 24 BTV serotypes, but also cross-reacted with some other related Orbivirus species. In an attempt to circumvent these problems, conventional PCR and touch-down PCR methods, using similar primers were also investigated. Both methods were able to amplify cDNA from only 21 of the 24 BTV types (Anthony *et al.,* 2007).

The diagnostic potential of RT-PCR for detecting BTV sequence in cell culture and tissue samples from infected ruminants from the United States, Sudan, South Africa, and Senegal was assessed. For PCR amplification, the non-structural protein 1 (NS1) gene of North American BTV serotype 11 was chosen. The findings suggested that the RT-PCR technique could be used in the United States and Africa to detect BTV in cell culture and clinical samples from susceptible ruminants during a disease outbreak (Aradaib *et al.,* 2005).

NS3 specific RT-PCR for detection of bluetongue virus (BTV) in clinical samples has been standardized using NS3 primers. Out of 32 field blood samples collected from bluetongue suspected outbreaks of Andhra Pradesh during the

months of August and September, 2005, ten were found positive for the presence of BTV RNA by NS3 RT-PCR. BTV was isolated from PCR positive blood samples by intravenous inoculation into embryonated chicken eggs and subsequent passages on to Baby Hamster Kidney-21 (BHK-21) cell line and identified as BTV by nucleic acid migration pattern in 1% agarose gel electrophoresis. This confirmed the utility of NS3 RT-PCR as a test for rapid and confirmatory diagnosis of bluetongue infection in sheep (Devi *et al.,* 2006).

Bluetongue virus was detected in blood samples using real-time RT-PCR. The primers and Taqman probes were designed to target a conserved region of BTV RNA segment 5, which codes for the non-structural protein NS1. It can detect BTV serotypes 2, 4, 9, and 16, as well as their vaccination strains that have been discovered in Italy. From 104 blood samples collected in containers with EDTA, its accuracy was tested, and the results were compared to those obtained with the conventional RT-PCR employed in routine diagnosis. Both tests gave negative results on 40 blood samples from bluetongue-free farms. Real time PCR detected BTV RNA in 64 sentinel cows (Polci *et al.,* 2007).

An RT-PCR-based typing assay has been developed for BTV-1 to 26 types by using the Seg-2 database. Multiple primer pairs were tested and identified serotypes by amplification of a cDNA product of the expected size. Sequencing of the cDNA amplicons confirmed the serotype. For all 26 BTV serotypes and field isolates tested, the RT-PCR and sequencing results were in perfect agreement with VNT. The primers and RT–PCR assays developed in this study provide a rapid, sensitive, and reliable approach for identifying and differentiating the twenty-six BTV serotypes (Maan *et al.,* 2012a)

Using BTV-specific real-time RT-PCR, a small but considerable proportion of animals were detected weakly positive during surveillance programs in Western Europe in 2010–2011. A study was conducted to investigate the risks of detecting bluetongue vaccine-associated RNA using real-time RT-PCR in the blood and spleen of cattle. The findings showed that vaccine viral RNA can enter the bloodstream in large quantities to be identified by real-time RT-PCR in cattle (De Leeuw *et al.,* 2015).

In this study, for detecting BTV from a cell-adapted strain of BTV-23 and blood samples, a nested polymerase chain reaction (PCR) based on the BTV NS1 gene (genome segment 5) was optimized. The nested PCR revealed that 19 out of 70 of cattle blood samples and 9 out of 30 of sheep blood samples were positive for BTV RNA (Ayanur *et al.,* 2016).

In this study, real-time polymerase chain reaction (PCR) was standardized to detect the *Bluetongue virus* in blood samples collected from sheep during bluetongue epidemics in the Indian states of Andhra Pradesh and Telangana in 2014. Compared to RT-PCR, real-time PCR was proven to be a very sensitive and reliable approach for detecting BTV in various materials, including blood samples taken from BTV-infected sheep (Lakshmi *et al.,* 2018).

2. 11 Use of Dried sample spot method for detection of viruses:

The stability of measles virus, another member of the morbillivirus genus, was also demonstrated on Whatman 3 MM filter paper (Swart *et al.,* 2001; Katz *et al.,* 2002). These researchers have shown the utility of filter paper to transport and recover measles virus RNA from blood samples.

EDTA blood samples collected from 100 Human Immuno Deficiency Virus (HIV) seronegative and 109 HIV seropositive individuals, were tested on dried blood spots; Whatman, Schleicher and Schuell (S and S) No. 903 and S and S IsoCode filter paper. Extracted nucleic acid was used as a template for HIV-1 proviral DNA detection by multiplex PCR which showed 94% sensitivity and 100% specificity (Uttayamakul *et al.,* 2005).

Infectious Bursal Disease Virus (IBDV) causes immune suppression and lesions in the Fabricius bursa in poultry. For IBDV tissue collection and transportation, phenol inactivation has been the standard. Bursas from diseased birds were printed on Flinders Technology associates (FTA)® cards and then immersed in phenol. The virus's nucleic acid was found in 85% of FTA® card inactivated samples versus 71% of phenol inactivated samples. For FTA® sampling, no changes were found; however, for tissues in phenol, nucleic acid

was only detectable in the tissues stored at 4°C before the sample for up to 2 hours post-mortem. These data suggest that using the FTA® card as an alternate collection method for molecular identification and characterization of IBDV is both efficient and reliable (Purvis *et al.,* 2006).

The study reported use of filter papers for rapid sample collection and the molecular detection and genotyping of viruses when stored over long periods at elevated temperatures. Infected blood was collected on filter papers, dried, and stored at different temperatures (22, 32, and 37 °C) for various periods. *African swine fever virus* and *Peste des Petits Ruminants virus* were used in the study. PCR products obtained from the filter papers were sequenced, and phylogenetic analysis was carried out (Michaud *et al.,* 2007).

The study describes the use of Classic Cards for the collection, storage, shipment, and identification of the *Foot and mouth disease virus* (FMDV) genome by RT-PCR and real-time RT-PCR and showed the stability of the viral RNA, the absence of infectivity, and ease of processing the sample for molecular methods make the FTA cards a useful option for the transport of FMDV genome for identification and serotyping (Muthukrishnan *et al.,* 2008).

FTA cards preserve RNA on a dry storage basis and render pathogens inactive upon contact. The study showed that FTA cards can be used to detect *Avian influenza virus* (AIV) RNA in reverse-transcription PCR and that the resulting cDNA could be sequenced and virus genes are determined (Kraus *et al.,* 2011).

Dried blood spot (DBS) was tested as a possible substitute to blood for detecting Hepatitis C virus (HCV) RNA in this study. The method endpoint detection limit, as well as its inter-assay and intra-assay variability, were determined. The DBS approach was compared to a routine assay using paired DBS and blood samples. Various storage temperatures and times on HCV RNA stability in DBS were also investigated. According to the findings, the method's sensitivity and specificity were determined to be 100% and 95.8%, respectively. Over one year at various temperatures, there was no substantial fluctuation in the

stability of HCV RNA in DBS. A sensitive and stable technique for detecting HCV RNA in DBS was developed (Bennet *et al.,* 2012).

According to a study conducted to monitor the viral load of Human Immuno Deficiency (HIV) virus-infected individuals in India, dried blood spot (DBS) samples have a high positive correlation with standard plasma-based assays. DBS samples have the utility, feasibility, and cost advantage of measuring the viral load. DBS might be a suitable choice in resource-constrained situations, particularly in rural and isolated areas of the country (Neogi *et al.,* 2012).

In the Study of Porcine reproductive and respiratory syndrome viruses, FTA cards carrying dried sample spot found a safe, simple, and sensitive alternate method to transport serum and tissues of infected animals for RT-PCR diagnosis (Linhares *et al.,* 2012).

In this study, the capillary blood samples blotted on filter papers can be used to diagnose Chikungunya virus (CHIKV) infection. During the CHIKV outbreak in Madagascar (2010), use of venous blood and dried blood blotted on filter paper (DBFP) as routine surveillance of dengue-like disease. Real-time RT-PCR was used to examine samples, and results were compared in between serum and DBFP samples. The sensitivity and specificity of DBFP tests using venous samples were 93.1% and 94.4%, respectively. This work demonstrated that DBFP specimens can be utilized as a low-cost alternative sample approach for CHIKV surveillance and emergence in underdeveloped countries, as well as other arboviruses (Andriamandimby *et al.,* 2013).

Dried blood spots (DBS) have been recommended for newborn screening for *Congenital cytomegalovirus* (CCMV). The single-target PCR assays have a sensitivity of 69 percent in both asymptomatic and symptomatic infants with laboratory-proven CCMV. The single-tube nested assay demonstrated an enhanced sensitivity of 81% and 100% specificity. The test detected CMV from a DBS equivalent to a 500 IU/ml original blood sample. This single tube nested approach allows for simultaneous amplification and detection of CMV DNA in

1.5 hours, eliminating the contamination risk associated with a two-step nested PCR (Atkinson *et al.,* 2014).

The study demonstrated that the performances of filter papers for archiving RNA from PPRV field isolates for further molecular detection and genotyping of PPRV, at -70°C combined with ambient temperature, for periods up to 16 months. PPR-suspected live animals were sampled, and their blood and nasal swabs were applied on filter papers then air-dried. RT-PCR appeared positive for both blood and nasal swabs when animals were in the febrile stage, and only nasal swabs were detected positive in a non-febrile stage. Those tested positive were monitored by RT-PCR up to 10 months by storage at -70°C. At 16 months, using real-time RT-PCR adapted to amplify the N gene from filter paper, high viral loads could still be detected from nasal samples (Bhuiyan *et al.,* 2014).

The filter paper–dried sera samples were used to detect dengue virus (DENV) RNA during an outbreak in American Samoa, and real-time reverse RT-PCR tested RNA. Of 18 RT-PCR–positive liquid specimens, 14 matched filter paper–dried specimens were positive for a sensitivity of 78%. Of 82 RT-PCR–negative liquid specimens, all filter paper–dried specimens were negative for the specificity of 100% (Curren *et al.,* 2020).

The researchers devised methods to evaluate antigen-specific antibody responses in cholera, enterotoxic *E. coli* (ETEC), and typhoid fever patients and oral cholera vaccination (OCV) recipients, using dried blood spots (DBS). They prepared DBS and plasma specimens from patients with cholera, ETEC, typhoid, and OCV recipients, using heparinized blood. To assess vibriocidal antibody response in DBS eluates, they optimized the conventional vibriocidal method. Using the Bland-Altman method, they reported a significant agreement of lipopolysaccharide (LPS) and cholera toxin B (CTB)-specific antibody responses between DBS eluates and plasma in cholera patients (ICC = 0.9) and OCV recipients (ICC = 0.8). DBS eluates and plasma samples from ETEC and typhoid patients showed an agreement of heat-labile toxin B (LTB) and membrane protein (MP)-specific antibody responses. The findings showed that dried blood specimens can be utilized as an alternative approach for preserving samples for

measuring antibody responses in enteric infections and vaccination trials, as well as for assessing reactions in humanitarian crises and other challenging field environments (Bhuiyan *et al.,* 2019)

The study evaluated the performance and acceptability of dried blood spot (DBS) sampling with filter paper to collect blood for viral load (VL) quantification in everyday situations. They evaluate the performance of DBS VL quantification using the Biocentric technique with plasma VL quantification using Roche and Biocentric as reference methods. DBS samples were collected using a phlebotomist's finger prick (DBS-1), a phlebotomist's pipetting of whole venous blood (DBS-2), and a laboratory technologist's pipetting of whole venous blood (DBS-3). The results revealed that test characteristics were equivalent between DBS-sampling methods regardless of the reference method. Estimates of sensitivity ranged from 85.3 % to 89.2%, while specificity ranged from 94.5% to 98.6% (Kerschberger *et al.,* 2019).

Biomedical research and healthcare rely heavily on blood cell analysis. The dried blood spot from a fingerstick permits biological molecules to be preserved for months. Leucocyte elution from dried blood spots is optimized here. The recovered cells are analyzed using flow cytometry and mRNA expression profiling. After shaking the support with buffer and drying the leucocytes on solid polyester support, 50–70% of the leucocytes are recovered through elution. The bulk of leucocytes is determined to be intact. The permeabilization of leucocytes allows for simple staining of all cellular compartments. The mRNAs and immunophenotyping both retained. The capability of a novel biomarker (CD169) to distinguish between patients with and without Severe Acute Respiratory Syndrome (SARS-CoV-2) infections has also been preserved. Blood leucocytes can be dried, transferred, and stored for at least one month before being recovered for various assays, possibly easing biomedical applications around the world (Ait *et al.,* 2021).

Dried blood spot (DBS) samples are a valuable resource for obtaining viral DNA and help increase Hepatitis B virus (HBV) diagnosis accessibility. In this study DBS samples to test the accuracy of four DNA extraction methods for

detecting HBV in both qualitative and quantitative terms was used. HBV DNA was spotted on filter paper in a panel of serially diluted HBV DNA in whole blood (Whatman 903 paper and Whatman FTA cards). The four methods QIAamp® DNA Blood Mini Kit (Qiagen); High Pure Viral Nucleic Acid Kit (Roche); Invisorb Spin Blood Midi Kit (Invitek); and DBS Genomic DNA Isolation Kit (Norgen Biotek) used to extract DNA. All extraction and PCR procedures were effective in detecting HBV DNA. For the surface/polymerase HBV area, the lowest detection limit was discovered using Whatman 903 paper, Roche extraction, and qualitative PCR (20 copies of HBV DNA per ml). These findings imply that in detecting HBV DNA in DBS, both the extraction procedure and the PCR protocol are essential. The study showed that the usefulness of DBS samples in HBV molecular diagnosis and their feasibility in low-resource settings where cold storage and transportation may be challenging (Bezerra *et al.,* 2021).

A unique approach for completely automated dried blood spot (DBS) sample handling and extraction for serological testing of human IgG antibodies against SARS-CoV-2 utilizing a commercial enzyme-linked immunosorbent assay (ELISA) testing kit is shown in the study. This proof-of-principle pilot investigation effectively showed the recovery of antibodies in its intact form from DBS employing automated, direct sample elution within 100 µl of extraction buffer. Minimally invasive DBS sampling can replace existing analytical procedures like venipuncture or nasal swabs. No third party is required for DBS collection, allowing for an at-home sample during quarantine (Gaugler *et al.,* 2021).

In this study, the suitability of filter cards were tested for long-term storage of fecal samples from animals and humans that were positive for the diarrhea-causing protozoan parasites *Giardia duodenalis* and *Cryptosporidium hominis*. The FTA® Classic Card, the FTA® Elute Micro Card, and the 903 Protein Saver Card were assessed. Human fecal samples positive for *G. duodenalis* and *C. hominis* were used to impregnate the selected cards over a while (1, 3, and 6 months) and at different temperatures (20°C, 4°C, and room temperature). PCR-based approaches were used to detect parasite DNA. Under

the tested conditions, all three Whatman® cards were helpful for detecting and molecular characterizing *G. duodenalis* and *C. hominis* (Koster *et al.*, 2021).

2.12 Sequencing and Phylogenetic analysis NS3 gene of *Bluetongue virus*

This study used the Clamp-R method to create full-length cDNA copies of segment 10 genes from *Bluetongue virus* serotypes 2, 11, 13, and 17, which were then inserted into plasmid pUC19. The nucleotide sequences of four cognate genes were sequenced, and the smallest of the 10 genes in the bluetongue virion was identified as 822 nucleotides long. BTV-10 and BTV-11 are relatively closely related, while BTV-2 is the most distantly related serotype among the five US *Bluetongue virus* serotypes, according to the phylogenetic analysis (Guang-Yuh *et al.*, 1992).

In this study, the sequence of the S10 gene segment of the American Type Culture Collection (ATCC) prototype strains of BTV serotypes 10, 11, 13, and 17, the commercial modified live virus vaccine strains of BTV serotypes 10, 11, and 17, and 20 field isolates of BTV serotypes 10, 11, 13, and 17 was determined. The S10 genes across field isolates of BTV have nucleotide variations ranging from zero (100% identity) to 142 (81.8% identity). The S10 gene segments from the US prototype ATCC BTV 10 and 11 strains differed from previously published sequences of putative US prototype viruses of the same serotypes. When comparing the NS3/NS3A proteins encoded by the S10 gene, there was little difference between the viruses (93% to 100% identity). The apparent conservation of NS3/NS3A across BTV strains and serotypes is most likely due to functional constraints on the protein that allows little change. The S10 sequence data show that reassortment of the S10 gene segment likely occurs in nature (Pierce *et al.*, 1998).

In this study, for 19 field isolates of *Bluetongue virus* (BTV) serotypes BTV-1, BTV-4, BTV-9, and BTV-16, the sequence of the genome segment 10 (Seg-10) encoding NS3/NS3A was identified. Regardless of the year of isolation, geographical location, host species, or tissue origin, most BTV-4 isolates had similar NS3/NS3A sequences. However, the clustering of the NS3/NS3A

sequences was independent of the viral serotype, showing that genome segment reassortment occurred during virus evolution (Nikolakaki *et al.*, 2005).

In this study, for 10 strains of *Bluetongue virus* serotype 4, the nucleotide sequences of genome segments 2, 7, 8, 9, and 10, which code for viral proteins (VP) and non-structural proteins (NS)-VP2, VP7, NS2, VP6, and NS3/NS3A, respectively, were determined and compared. Results showed that the nucleotide and deduced amino acid sequences of the BTV 4 strains within each lineage were found to be identical, regardless of isolation year or geographical location. An alignment of the NS3 sequences from the attenuated BTV 4 vaccine strain and the field strains revealed 13 amino acids substitutions which may be responsible for attenuation and hence defining the virus's pathogenicity, either individually or in combination (Breard *et al.*, 2007).

The deduced amino acid sequences of the genes (S10) encoding the NS3 protein of 137 BTV strains from Africa, the Americas, Asia, Australia, and the Mediterranean Basin revealed only minor variations. Potential glycosylation sites at amino acid residues 63 and 150 and a cysteine at residue 137 were found in all NS3 sequences, while a cysteine at position 181 was not. Phylogenetic analysis of these sequences revealed two main clades that classified the viruses by serotype and isolation year (1900-2003). Positive selection on the S10 gene was not detected using phylogenetic reconstruction of ancestral codon states; instead, the functional constraints of the NS3 protein are expressed through significant negative (purifying) selection (Balasuriya *et al.*, 2008).

In this study, the nucleotide sequences of Taiwan strain 10 genome segments and their coding regions were determined and evaluated. Except for the VP2 genes, the two strains BTV2/KM/2003 and BTV12/PT/2003 shared >96.8% nucleotide and >97.9% deduced amino acid identities. Except for the NS1 and VP2 genes, genome sequences clustered in the Asian lineage, and were closely related to strains from China, India, Indonesia, and Japan. The distribution of and amounts of changes in nucleotide identities were evaluated to see which genes were more suited for topotyping BTVs, finding that VP3, NS2, and NS3 genes were more suitable for topotyping BTVs (Lee *et al.*, 2011).

In this study, segment (Seg)-2, Seg-3, Seg-6, Seg-7, and Seg-10 sequence comparisons and phylogenetic analysis of the first Argentinian field isolates of BTV were described. The Seg-2 and Seg-6 sequences analysis yielded a single cluster of Argentinian serotype-4 sequences. The Argentinian isolates were grouped into the western topotype, indicating that the circulating virus had an African/European origin, according to Seg-3, Seg-7, and Seg-10 analyses. The phylogenetic analysis found that the Argentinian sequences have a South American genetic identity, indicating that they evolved independently (Legisa *et al.,* 2013).

The study aimed at characterizing the NS3 gene sequence of Indian BTV serotype-2. Compared to different BTV serotypes, the NS3 gene of BTV-2 revealed moderate levels of diversity, with nucleotide sequence identities varying from 81% to 98%. With multiple BTV serotypes, the site revealed a high sequence homology of 93-99% at the amino acid level. BTV isolates are divided into four topotypes, according to phylogenetic studies, and Indian BTV-2 in subclade IA is closely related to Asian and Australian strains. The NS3 gene analysis revealed that the Indian BTV-2 isolate is closely related to isolates from Asia and Australia, indicating a common infection origin (Pudupakam *et al.,* 2017).

Bluetongue virus NS3/3A and putative NS5 are encoded by the shortest polycistronic dsRNA segment-10 (S10). The S10 sequence data of 46 Indian BTV field isolates were determined and compared to cognate sequences of worldwide BTV strains between 1985 and 2011. Due to an alternative translation initiation site, the biggest ORF on S10 produces NS3 (229 amino acids), an amino-terminal truncated version of the protein (NS3A), and a putative NS5 (50-59 amino acids). At the next and deduced amino acids sequences, the overall mean distance of the global NS3 was 0.1106 and 0.0269, respectively. The global BTV strains were divided into four clusters. The viruses found from Australia and China were closely connected to the primary cluster of Indian BTV strains. A small sub-cluster of Indian BTV strains were closely linked to USA strains, while a few Indian strains were similar to South African reference and vaccine strains. The

worldwide trait correlation of phylogenetic structure suggests that global BTV S10 evolution was not homogeneous, but instead displays a moderate amount of geographical heterogeneity. There was different selection pressure on the alternate coding sequences of the S10, with NS3/3A evolving under strong purifying (negative) selection and NS5 evolving under positive selection (Biswas *et al.,* 2021).

2.13 Recombinant proteins of *Bluetongue virus*.

Segment 10 (S10) codes for two similar proteins, NS3 and NS3A. Instead of the 5' coding region of the AcNPV polyhedrin gene, a cDNA copy of BTV-10 (BTV-10) S10 RNA was inserted into Autographa californica nuclear polyhedrosis baculovirus (AcNPV). By Western blot (immunoblot) and peptide map analysis, *Spodoptera frugiperda* cells infected with the recombinant baculovirus produced two polypeptides identified as NS3 and NS3A. Antibodies produced by immunizing mice with expressed NS3 identified both NS3 and NS3A in BTV-10-infected BHK cells but not in pure BTV-10 viral particles. NS3 was the primary product both in the baculovirus expression system and *in vivo* in BTV-infected cells, in contrast to *in vitro* translation of BTV S10 RNA when NS3 and NS3A are produced in equimolar quantities. The expressed NS3, and NS3A proteins responded significantly with sera from sheep infected with homologous and heterologous BTV serotypes, indicating that the S10 gene products are group-specific antigens that are highly conserved (French *et al.,* 1989).

BT outbreaks with six different serotypes have occurred in the Mediterranean basin since 1998. Only serotype-specific BTV vaccinations are currently available. In this study, sheep (n=1) and goats (n=4) were inoculated with bluetongue recombinant capripoxvirus (BTV-Cpox), which expressed four distinct genes encoding two capsid proteins (VP2 and VP7) and two non-structural proteins (NS1, NS3) of *Bluetongue virus* serotype 2 (BTV-2). In both groups of animals, seroconversion to NS3, VP7, and VP2 was seen. Partial protection in sheep exposed with a virulent strain of BTV-2 correlates with a particular BTV antigens lymphoproliferation seen in goats (Perrin *et al.,* 2007).

The study reported the purification of *Bluetongue virus* (BTV) group-specific VP7 protein expressed in a prokaryotic system as a histidine-tagged fusion protein. The primary antigenic region of the BTV-23 VP7 gene was amplified and cloned from extracted RNA using a reverse transcription-polymerase chain reaction. The polymerase chain reaction and sequencing analysis discovered the recombinant expression construct (pET-VP7). Anti-His antibody was used to assess the expression of histidine-tagged fusion truncated VP7 protein having a molecular mass of 36 kDa. The expressed VP7 was purified to near homogeneity by chromatography on a nickel-agarose column, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In Western blot analysis, the purified VP7 protein was identified by an antibody to BTV. In the enzyme-linked immunosorbent test, the capacity of the recombinant VP7 protein to distinguish hyperimmune rabbit serum to BTV from normal rabbit serum was demonstrated (ELISA). The purified VP7 responded satisfactorily to bluetongue with 24 BTV serotype-specific sera supplied from the OIE Reference laboratory. The results suggest that the produced VP7 protein might be used as an antigen in developing an antibody-capture ELISA for detecting antibodies specific to the BTV group. This recombinant protein can also be used as an antigen (Pathak *et al.,* 2008).

Due to challenges in distinguishing infected from vaccinated animals (DIVA), vaccination programs for managing bluetongue (BT) in ruminants have limitations. A DIVA test was created to address this issue that looks at a differential immune response to the non-structural protein 3 (NS3) of the *Bluetongue virus* (BTV). The BTV4/ 22045/PT04 NS3 encoding gene was introduced into the expression vector pET-28a and expressed in Escherichia coli strain JM109. In an indirect ELISA (NS3- ELISA), recombinant NS3 protein was utilized as an antigen to evaluate the serologic response to NS3 protein in cattle and sheep. A total of 562 serum samples from uninfected, BTV-infected, and vaccinated animals were analyzed for NS3 antibodies to determine the usability of the NS3-ELISA in a field environment. The findings showed that NS3 antibodies are generated at the highest levels in animals infected with BTV compared to those inoculated with inactivated BTV vaccines, demonstrating that antibody

response to NS3 permits infected and vaccinated animals to be distinguished (Barros *et al.,* 2009).

To develop a novel subunit vaccine targeting BTV-8 that allows differentiation of infected from vaccinated animals, five His-tagged recombinant proteins, VP2 and VP5 of BTV-8 and NS1, NS2, and NS3 of BTV-2, were expressed in baculovirus or *Escherichia coli* expression systems for further study. Optimized purification protocols were determined for VP2, NS1, NS2, and NS3, which remained stable for detection for at least 560 to 610 days of storage at 4°C or 80°C, and Western blotting using sera from vaccinated or experimentally infected cattle indicated that BTV-specific antibodies recognized VP2 and NS2. Mice were subcutaneously immunized twice at a 4-week interval with one of three protein combinations with immunostimulating complex ISCOM-Matrix adjuvant or with ISCOM-Matrix alone to characterize murine immune responses to the four proteins (n 6 per group). In immunized mice, serum IgG antibody titers specific for VP2 and NS2 were significantly greater than in controls. When immunized mice's spleen cells were restimulated, VP2, NS1, and NS2, but not NS3, caused distinct lymphocyte proliferative responses. According to the findings, these recombinant purified proteins, VP2, NS1, and NS2, could be a vital component of a unique BTV-8 vaccine formulation (Anderson *et al.,* 2014).

Non-structural proteins (NS1-NS4) of the BT virus are presumed candidate antigens in the development of DIVA diagnostics. NS3 fusion gene encoding for NS3 protein containing the N- and C-termini with a deletion of two hydrophobic domains (118A to S141 amino acids and 162S to A182 amino acids) and an intervening central variable domain (142D to K161 amino acids) of *Bluetongue virus*-23 was constructed, cloned and over-expressed using prokaryotic expression system. The recombinant NS3DHD fusion protein (~38 kDa), including a hexahistidine tag on both its termini, was non-cytotoxic to recombinant *Escherichia coli* cells. The purified rNS3DHD fusion protein detect BTV-NS3 specific antibodies in indirect-ELISA format with diagnostic sensitivity (DSn ¼ 94.4%) and specificity (DSp ¼ 93.9%). The study indicated the potential utility of rNS3DHD fusion protein as a candidate diagnostic reagent

in developing an indirect-ELISA for sero-surveillance of animals for BTV antibodies under DIVA strategy, wherever monovalent/polyvalent killed BT vaccine formulations devoid of NS proteins are being utilised for immunization (Mohanty *et al.,* 2016).

A partial NS2 gene encoding for two non-structural protein-2 fragments, N-terminus (1M-A177 amino acids) and C-terminus (178P-V354 amino acids) of BTV-23, were cloned separately, expressed, and purified as recombinant NS2Nt and NS2Ct fusion proteins (˜39 kDa each) from prokaryotic expression system (*Escherichia coli*). After affinity chromatographic purification under non-denaturing conditions, both rNS2Nt and rNS2Ct fusion proteins were obtained in sufficient quantity and quality. Both antigens were found to possess good reactivity in detecting NS2 specific BTV antibodies irrespective of serotypes in ruminant serum samples by indirect ELISA. Of two fragments, rNS2Nt was more efficient as a diagnostic candidate (Chacko *et al.,* 2019).

This study has targeted proteins of the *Bluetongue virus* (BTV). They developed a novel NS1 and NS3 fusion gene (1302bp) encoding for NS1 N-terminus (1M to G252 amino acids) and NS3 protein containing the N- and C-termini with a deletion of two hydrophobic domains, as well as an intervening variable central domain (118A to A182 amino acids) of *Bluetongue virus*-23 to avoid the difficulties associated with production/purification. Single-step affinity chromatography was used to clone, over-express, and efficiently purify this construct under unique denaturing/renaturing conditions. In an indirect ELISA, the purified fusion protein was suitable for detecting antibodies against BTV (Mohanty *et al.,* 2019).

Bluetongue is a viral disease of small ruminants spread by insects (Culicoides). *Bluetongue virus non*-structural proteins (NSPs) have always been a favorite target for distinguishing infected from immunized animals. The applicability of the fusion construct was examined and optimized in this work. Compared to a pre-established test panel, the fusion construct utilized in an ELISA platform projected a relative diagnostic sensitivity and specificity of 98.1% and 95.5% respectively. The rNS1-NS3 ELISA was shown to have a high

level of agreement with a commercial BTV antibody detection kit (Mohanty *et al.,* 2021).

MATERIALS AND METHODS

3.1 Materials

3.1.1 Place and Facilities

The research work was performed with the facilities available at the Department of Microbiology and Animal Biotechnology, Teaching and Research cell, Nagpur Veterinary College, Nagpur.

3.1.2 Clinical Samples:

Blood samples available at the department leftover from numerous samples received from the BTV suspected outbreaks in sheep population from the Dhule and Nandurbar District of Maharashtra were used. Approximately 100µl blood was spotted on the FTA card, ordinary filter paper, newspaper avoiding printed area in and around 1.5 cm circle (Appendix 2). The samples were allowed to dry at room temperature and kept at room temperature until further use (Approx. 24 hrs).

3.1.3 Chemical, Reagents, Glasswares, Plastic wares and Kits:

Chemicals and reagents used in the present study were of molecular biology and analytical grade procured from SIGMA (USA), Invitrogen (USA), Qiagen (Germany) etc. All plastic wares were procured from Axygen (USA). Glassware were procured from Borosil (India). The formulation of reagents and solutions used in the present study are given in appendix.

3.1.4 Oligonucleotide primers and clones:

The oligonucleotide primers used for RT-PCR to amplify Nonstructural gene of bluetongue virus are given in Table No. 1. Primers were synthesized commercially from Eurofins Genomics India Pvt. Ltd., Bengaluru, India.

The pET-32a-NS3 recombinant clone in BL-21(DE3) cells present in Virology laboratory of Department of Vererinary Microbioloy and Animal Biotechnology was revieved on LBAmp agar plate and use for further study.

Table 3.1: List of primers used for RT-PCR:

Primers	Primer sequence	Amplicon size	Reference
BTV-NS1-F2-724	ATG ATT TCG CGA AGC ATT TT	967bp	Designed primers
BTV-NS1-R2-1691	CAT CCA CAT CTG AGA CAT GC		
BTV-NS2-F-19	CAT GGA GCA AAA GCA ACG TA	1087bp	Designed primers
BTV-NS2-R-1106	CTA ACC ACG CGG TCA CAA G		
BTV-NS3-7	AAG TGT CGC TGC CAT GCT AT	725bp	Designed primer
BTV- NS3- 732	GGG CGC AAC TCT ACC TAC TG		
BTV- F-217	TGC TAC GCA AAC ACA CAA GG	248bp	Designed primer
BTV-R-465	AGC TTA AAT GCC ACG CTC AT		
BTV-VP2-F	GAG AGG CGG TAC TTG ATG AA	507bp	Designed primer
BTV-VP2-R	AAC GTG CGA ACT CTC TGT CA		
BTV-VP7-Trm-F	GTT AAA AAT CTA TAG AG	1154bp	Wade-Evans *et al.*, (1990)
BTV-VP7-Trm-R	GTA AGT GTA ATC TAA GAG A		
BTV-VP7-Nst-F	AAG CTT GGA TCC CGC GGA TCC CCA TAT GGT TTC TTT CTT	542bp	Designed primer
BTV-VP7-Nst-R	CTC GAC GAA TTC CCC AAG CTT ATA TGC TAT CTC GGT CG		
Cycl-AF1-Forward	GGA TTT ATG TGC CAG GGT GGT GA	119bp	Jarczak *et al.*, (2014)
Cycl-AF0-Reverse	CAA GAT GCC AGG ACC TGT ATG		

3.1.5 Scientific Equipments:

- Autoclave (M. C. Dalal, Chennai, India)
- Hot air oven (M. C. Dalal, Chennai, India)
- Refrigerator 4°C (LG, India)

- Deep freezer -20°C (Blue Star, India)
- Deep freezer -80°C (Eppendorf, UK)
- Laminar Flow (Microfilt, India)
- Micropipette (Eppendorf, Germany and Fisherbrand®, USA)
- Table top centrifuge (Tab spin^{Tm}, Himedia)
- Nanodrop 1000 (Thermo Scientific, USA)
- Refrigerated Centrifuge Machine (Sorvall biofuge primerR, Thermo Scientific, Germany)
- Thermocycler (Prima-Trio^{TM} Thermal Cycler, Himedia)
- Electrophoresis System (Hoefer, Germany)
- Mini Gel apparatus (Hoefer, Germany)
- Gel documentation System (BioZen Laboratories)

3.2 Methodology

3.2.1. Sample processing

Approximately, 100 µl blood sample spotted on the FTA card, ordinary filter paper and newspaper (within around 1.5 cm circle) avoiding printed area (Appendix 2) were processed for the RNA extraction followed by subsequent reaction as detailed below.

3.2.2. Extraction of Total RNA from samples

Total RNA from blood sample, blood spotted on newspaper, FTA cards, ordinary filter paper were extracted using TRI reagent (Cat#T9424, SIGMA, USA) by the method described by Pundlik, (2014). Protocol used for extraction of RNA from different samples is described below.

3.2.2.1. Extraction of total RNA from blood

1) Whole blood (200 µl) was taken in a labeled microcentrifuge tube and to it 20 µl of 5N acetic acid was added. Then mixing was done by tapping and it was further processed for RNA extraction with TRI reagent.
2) The DNAse/ RNAase free microcentrifuge tubes were pre-labeled according to the sample numbers.
3) As postive control 250 µl virus suspension was taken in labeled 1.5 ml microcentrifuge tube.
4) The TRI reagent, 750 µl was added and mixed with pipette and vortexed several times and incubated at room temperature for 5 min.
5) Around 200 µl of chloroform was added vortexed vigorously for 15 seconds and incubated at room temperature for 10 min.
6) It was then centrifuged at 13000 rpm for 15 min at 4°C.
7) The aqueous phase was transferred in fresh labeled tube.
8) To this 500 µl Iso-propanol was added and incubated at room temperature for 15 min.
9) The centrifugation was carried out at 13000 rpm for 10 min at 4°C.
10) The pellet was visible and supernatant was carefully discarded to avoid dislodging of pellet.
11) To the pellet, 1 ml of 70% chilled ethanol was added and mixed by tapping. Incubated at room temperature for 3-4 min.
12) The microcentrifuge tube was centrifuged at 10,000 rpm for 8 min at 4°C.
13) The ethanol was carefully removed with proper care to avoid dislodging of pellet.
14) The tubes was placed upside down on paper towel for 1-2 min to drain the residual ethanol then invert and kept upside up for air drying for 15-30 min with utmost care to avoid the over drying of the pellet.

15) To the pellet, 20 µl nuclease free water (NFW) was added and incubated at room temperature for 10 min.

16) The RNA quality & quantity was chacked using Nanodrop 1000 instrument and stored at -70°C until further use.

3.2.2.2 Extraction of total RNA from Filter papers

1) Filter paper approximately 1.2 cm square area having blood spot was cut into smaller pieces taking care to avoid unspotted portion from coming into reaction.

2) The cut pieces of the same samples were taken into labeled microcentrifuge tube and 300 µl of RNA elution buffer was added and incubated on ice for 30 min.

3) The elute, approximately 250 µl was collected in separate labelled microcentrifuge tube.

4) To this elute, 1000 µl of TRI reagent was added.

5) Then routine RNA extraction procedure was followed as mentioned in section 3.2.2.1 with additional one wash with 70% chilled ethanol.

3.2.2.3 Extraction of total RNA from FTA Cards

1) Approximately 1 cm square area of FTA cards was cut into smaller pieces. It was ensured that the cut portion covers the dried blood spot for RNA extraction.

2) The cut pieces from the same sample as above were pooled together in labeled microcentrifuge tube.

3) To this labeled microcentrifuge tube, 500 µl of RNA elution buffer was added and incubated on ice for 2 hours.

4) The elute was collected in new labeled microcentrifuge tube.

5) To the leftover pieces of FTA sample card, 1ml TRI reagent was directly added.

6) The mixture was incubated for 5-10 min on ice.

7) The TRI reagent was pipetted out and added into respective previously eluted extract obtained in step 4.

8) Mixture of TRI reagent and eluted extract was mixed properly by vortexing the microcentrifuge tubes for 2-5 min and incubated for 5 min at room temperature.

9) To above mixture, 200 µl chloroform (per 1 ml of TRI reagent used) was added and mixed by vortexing and incubated for 10 min at room temperature.

10) The tubes were centrifuged at the rate 13,000 rpm for 15 min at 4°C.

11) After centrifuging the microcentrifuge tubes three separate phases were obtained, out of which upper aqueous phase was transferred to the new labeled microcentrifuge tube.

12) To the aqueous phase, 500 µl isopropanol was added (per ml of TRI reagent used).

13) The mixture was incubated at room temperature for 15 min followed by incubation at -20°C for 2 hours.

14) The mixture was centrifuged at the rate of 13000 rpm for 15 min at 4°C.

15) The supernatant was carefully discarded with care to avoid the dislodging of the pellet.

16) The RNA pellet was washed twice with 75% chilled ethanol. Briefly, 1 ml chilled 75% ethanol was added and incubated at room temperature for 5 min and then the microcentrifuge tubes were centrifuged at the rate 12000 rpm for 10 min at 4°C. Ethanol was carefully discarded with proper care to avoid dislodging of the pellet.

17) Pellet was air dried for 5-10 min (complete drying was avoided).

18) To this pellet 15 µl 1X TE buffer was added.

19) Microcentrifuge tube containing pellet was incubated at room temperature for 5 min and then at 55°C for 10 min with intermittent vortexing and brief spin to draw the content at the bottom. RNA was stored at -70°C until further use.

3.2.2.4: Extraction of total RNA from Newspaper

1) The blood spotted on newspaper were chopped into pieces and were taken into labeled microcentrifuge tubes.

2) The cut pieces of the same samples were taken into labeled microcentrifuge tube and 300 µl of RNA elution buffer was added and incubated on ice for 30 min.

3) The elute, approximately 250 µl was collected in separate labelled microcentrifuge tube.

4) To these labeled microcentrifuge tubes 1 ml TRI reagent was added. The routine RNA extraction procedure was followed as mentioned in section 3.2.2.1

3.2.3: cDNA synthesis of the total RNA

The cDNA synthesis was carried out by Superscript^{TM}IV First -Strand Synthesis System (Invitrogen Cat. No. 18091050) as per manufacturer's instructions in 20 µl volume containing the following reagents in labeled microcentrifuge tubes.

The Reaction was set up as follows:

Name of Reagent	Quantity
Template RNA	11 µl
Random hexamer	1 µl
10mM dNTP mix (10mM each)	1 µl
Total	13 µl

This mixture was incubated in Thermal cycler at 65°C for 5min and snap chilled on ice for 2 min, thereafter following reagents were added to each tube (7 µl per tube).

The RT reaction mixture was prepared as follows:

Name of Reagent	Quantity
5X SSIV Buffer	4 µl
100mM DTT	1 µl
Ribonuclease inhibitor	1 µl
Superscript^{TM} IV Reverse transcriptase(200U/µl)	1 µl
Total reaction	7 µl

Capped the tube, tap and mixed, and briefly spun the content. RT reaction mixture was added to the annealed RNA.

The cDNA synthesis reaction was carried out in thermal cyclic conditions as follows:

Step I	23°C	10 min
Step II	23°C	30 min
Step III	80°C	10 min
Step IV	4°C	∞

The obtained cDNA was stored at -20°C until use.

3.2.4: Standardization of Polymerase chain reaction for Amplification of *Bluetongue virus* (BTV) genes and Housekeeping genes.

The BTV gene amplification was carried out using the primers NS1, NS2, NS3, VP2 and VP7. Similarly, the Cyclophilin A gene (housekeeping gene) amplification was carried out to confirm cDNA synthesis using published primer (Table No. 3.1).

The reaction was set up as follows.

Name of Reagents	Quantity
cDNA	3 µl
10X PCR Buffer	5 µl
$MgCl_2$ (50mM)	3 µl
dNTP (10mM)	1 µl
forward primer (10 pmol/µl)	2 µl
Reverse primer (10 pmol/µl)	2 µl
Taq DNA polymerase (5U/µl)	0.3 µl
Nuclease Free Water	33.7 µl
Total Volume	50 µl

The reaction was carried out using following cyclic conditions:

For BTV NS3 gene amplification

Step no.	Step	Temperature	Time	
1.	Initial denaturation	95°C	2 min	
2.	i) Denaturation	95°C	20 sec	3 step cycling for 35 cycles
	ii) Annealing	#52°C *56°C	#45sec *1 min	
	iii) Extension	72°C	45sec	
3.	Final extension	72°C	10 min	
4.	End of the PCR cycles	4°C	∞	

for BTV NS3 gene amplification

* for cyclophilin gene amplification

For BTV NS1 and NS2 gene amplification

Step no.	Step	Temperature	Time	
1.	Initial denaturation	94°C	2 min	
2.	i) Denaturation	94°C	20 sec	Repeated 10 cycles each
	ii) Annealing	45°C	1 min	
	iii) Extension	72°C	1:30 min	
3.	i) Denaturation	94°C	20 sec	Repeated 30 cycles each
	ii) Annealing	50°C	1 min	
	iii) Extension	72°C	1:30 min	
4.	Final Extension	72°C	10 min	
5.	End of the PCR cycles	4°C	∞	

*****1°C increment/cycle upto 10 cycle

For BTV VP7-Trm gene amplification

Step no.	Step	Temperature	Time	
1.	Initial denaturation	94°C	2 min	
2.	i) Denaturation	94°C	20 sec	3 step cycling for 35 cycles
	ii) Annealing	39°C	1:30 min	
	iii) Extension	72°C	1:30 min	
3.	Final extension	72°C	10 min	
4.	End of the PCR cycles	4°C	∞	

For BTV VP2 and VP7-Nst gene amplification

Step no.	Step	Temperature	Time	
1.	Initial denaturation	94°C	2 min	
2.	i) Denaturation	94°C	20 sec	3 step cycling for 38 cycles
	ii) Annealing	55°C	1 min	
	iii) Extension	72°C	1 min	
3.	Final extension	72°C	10 min	
4.	End of the PCR cycles	4°C	∞	

The PCR products were removed from the Thermal cycler and analyzed by gel electrophoresis.

3.2.5: Confirmation of Nonstructural NS1, NS2, NS3 gene and structural VP7-Trm gene and cyclophilin A gene amplicons by agarose gel electrophoresis.

The PCR products were resolved by agarose gel electrophoresis (AGE) using 1% agarose containing 0.5 ug/ml of ethidium bromide (SIGMA) in 0.5X Tris borate-EDTA buffer (TBE). 10 µl of PCR product was mixed with 2 µl of 6X loading dye and loaded into the wells of gel. The DNA ladder (100bp Cat # SM0321, Thermo, USA) was loaded along with the PCR products. Electrophoresis was carried out at 12V/cm of gel in 0.5X TBE running buffer in submarine electrophoresis apparatus (HE33.3 Hoefer, Germany/ Bangalore Genei submarine gel electrophoresis unit) and power supply (Cat # EV265 Hoefer, Germany) till the bromophenol blue (6X loading dye) reached 2/3rd of the gel. The gel was visualized for amplificons/PCR products using the U.V. transilluminator and photographed by using gel documentation system (BioZen Laboratories, India).

3.2.5 Sequence and phylogenetic analysis

3.2.5.1 Nucleotide Sequencing

The NS3 PCR amplicon was subjected for sequencing by both orientations (Forward and Reverse) from commercial sequencing services from Eurofins Pvt. Ltd., Bangalore, India.

3.2.5.2 Sequence analysis using Bioinformatics tools

The nucleotide sequences obtained were analyzed using online bioinformatics tools for phylogeny with global BTV sequences.

The raw sequences of BTV-NS3 obtained after commercial sequencing, were first analyzed for the base call and trimmed using the Chromas software. The cleaned sequences of the forward and reverse sequences of single NS3 amplicons were analysed for the homology search using online BLASTn interface of GenBank (http://blast.ncbi.nlm.nih.gov/). The further mismatched base pairs were reanalyzed with Chromas for base call. The reverse oriented sequences were reverse complemented using the Chromas. The contig were prepared by using the online contig software and consensus sequences were saved for afterward analysis using the BLASTn program. The consensus sequences were further analysed for the BLASTn. The consensus nucleotide sequence of NS3 was subjected to ORF search using online ORFinder programme. The 49 global BTV NS3 sequences of 27 serotypes were retrieved from the GenBank, their nucleotide and amino acid accession numbers are depicted in table no. 3.2. The sequences were analyzed, using the Clustal X standalone programme, and the output file was further analysed using MEGA 11 software. The nucleotide sequence based evolutionary history was inferred using the Maximum Likelihood method and kimura-2 parameter. The bootstrap consensus tree inferred from the 1000 replicates. The nodes values below 50% were collapsed and the tree for the heuristic search were obtained using Neighbour-Joining and BioNJ algorithm to matrix of pairwise distances estimated using Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the

number of substitutions per site. All positions containing gaps and missing data were eliminated.

For the amino acid sequences analysis, the best fit model was first analysed with lowest BIC number and LG model was selected. The evolutionary analysis was inferred using the Maximum Likelihood method and Le_Gascuel_2008 model. The bootstrap consensus tree was inferred from the 500 replicates. The divergence was analyzed. The initial tree(s) for the heuristic search were obtained automatically by applying the NJ and BioNJ algorithm to a matrix of pairwise distances estimated using JTT model and selecting the topology with superior log likelihood value. The evolutionary analysis was conducted in MEGA 11 software.

3.3. Expression and confirmation of recombinant NS3 protein

3.3.1. Expression of NS3 protein in BL-21(DE3) (*E. coli*) cells

The pET-32a-BTV-NS3 recombinant clones from glycerol stocks of the transformed bacteria were freshly streaked on the LB agar with Ampicillin (50µg/ml) plate. A single colony of transformed colony harboring recombinant pET-32a-BTV-NS3, recombinant clone was inoculated into 5 ml of LBAmp broth and incubated in orbital shaker incubator with 220 rpm at 37°C for overnight. From this, 100µl culture was transferred to 10 ml LBAmp broth in 100 ml conical flask and incubated in orbital shaker incubator with 220 rpm at 37°C till OD_{600} reached to 0.60. The uninduced 1ml bacteria was removed and pelleted by centrifuging at 5000 rpm for 5 min. The supernatant was discarded and pellet was saved in the deep freeze. The rest of the bacterial culture was inoculated with IPTG to final concentration of 1mM for three hours at 37°C and 200 rpm.

3.3.2: Harvesting of rNS3 protein

After post induction, from the culture, 1 ml bacterial suspension was centrifuged at 13000 rpm for five min. The supernatant was discarded and to the pellet was saved. To the uninduced (previously collected as in above) and induced bacterial pellet 100 µl of 1X Laemmli buffer was added. The samples

were boiled for five min and centrifuged briefly and loaded into sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for further analysis.

3.3.3: Confirmation of rNS3 protein by SDS-PAGE

The molecular weights of expressed rNS3 recombinant protein was confirmed by SDS-PAGE as per the method described by Moore and Burke (1974).

The SDS-PAGE gel was prepared using Mini Gel apparatus (Hoefer, Germany). The gel slabs (two) were prepared between glass plates. The spacers (2 mm thick) were placed in position. The assembly was fixed in gel casting tray. The resolving gel (12.5%) was poured between the plates avoiding air bubble. The resolving gel was gently overlayered with n-butanol and kept at room temperature for polymerization. After polymerization, the n-butanol layer was removed by tilting the apparatus and using blotting paper. The gel surface was rinsed twice with Tris-glycine buffer. The comb was placed in position and 5% stacking gel was poured. After polymerisation about 20-30 min, the gel stack was removed from casting tray and put in electrophoresis apparatus. The electrode buffer was poured in upper and lower tanks. The comb was removed and the wells were gently washed with Tris-glycine buffer using the gel loading tips and micropipette.

The bacterial lysate and supernatant from section 3.3.2 were subjected for SDS-PAGE: Un-induced recombinant BL-21(DE3)NS3, cells and untransformed BL-21(DE3) cells were processed in parallel for SDS-PAGE analysis. The respective wells were loaded with 15 µl of each sample. A protein marker (cat # BM003-0500, BR BIOCHEM, India) was also loaded. The samples were electrophoresed at constant voltes of 90V for 1.5-2 hours at room temperature until the dye reaches to the bottom of gel.

3.3.4: Coomassie brilliant blue staining

The gel was stained with Coomassie brilliant blue staining solution for four hours at room temperature and destained with destaining solution till the background became clear. The molecular weights of separated proteins were

calculated based upon their relative migration (Rf) corresponding to the Protein Ladder.

3.4: Optimization, production, purification and quantification of rNS3 protein production

The small scale optimization of recombinant protein expression was carried out as per Pathak et al., (2008), with brief modifications. The truncated rNS3 protein production conditions were optimized for producing specific and volumetric yield. For this, independent parameters viz., incubation intervals and IPTG concentration were considered.

3.4.1: Inducer concentration optimization

The mid exponential phase of *E. coli* BL-21(DE3) harboring pET-32a-BTV-NS3 plasmid was obtained in 20 ml volume in 250 ml conical flask (OD 0.45 0.65). The culture was dispensed in 20 ml test tubes containing 2 ml each. The 2 ml culture from a single tube was saved as uninduced culture. The culture of other tubes were induced with varying concentration of IPTG viz. 0.50, 1.00 and 1.50 mM and incubated in orbital shaker incubator at 37°C and 220 rpm for three hrs. The cultures (2 ml) were centrifuged. The uninduced pellets and induced pellet were dissolved in 200 µl of 1X Laemmli buffer and analyzed by SDS-PAGE.

3.4.2: Incubation optimization

The mid exponential phase of *E. coli* BL-21(DE3) harboring pET-32a-NS3 recombinant clone was grown in 10 ml volume in 250 ml conical flask till OD value reached to 0.45-0.65. The culture was induced with 1mM concentration of IPTG and incubated in orbital shaker incubator at 37°C and 220 rpm for 3 and 5 hours. The un-induced (1.5 ml) and induced cultures (1 ml) were centrifuged. The pellets were dissolved in 100 µl of 1X Laemmli buffer and analyzed by SDS-PAGE.

3.4.3: Large scale production of BTV-NS3 protein

The overnight grown culture (100µl) of recombinant *E. coli* BL-21(DE3) was inoculated in 10ml LBAmp broth and incubated in orbital shaker incubator at 37°C and 150 rpm upto OD reached to 0.45-0.65 (at mid exponential phase). The culture was induced with final concentration of 1mM IPTG and incubated in orbital shaker incubator at 37°C and 150 rpm for 6 hrs. The culture was harvested, centrifuged and pellet was stored at -80°C. The protein expression was confirmed by SDS-PAGE analysis.

3.4.4 Purification and quantification of rNS3 protein by Ni-NTA batch chromatography

3.4.4.1: Dissociation of expressed protein by ultrasonication

The bacterial pellet was resuspended in 900 µl of binding buffer, freeze thawed six times and subjected to three cycles of sonication at 5 µm amplitude for 10 seconds pulse each in ice bath with 30 seconds gap between each cycle. The slurry was centrifuged at 8000 g for 15 min at 4°C. The supernatant was collected carefully and the pellet was also saved. This supernatant was then subjected for further purification using Ni-NTA chromatography.

3.4.4.2: Purification of BTV-NS3 protein Ni-NTA chromatography

For purification 250µl of Ni-NTA slurry was taken into 1.5 ml microcentrifuge tube. To this 500µl of equilibration buffer was added and mixed property. It was centrifuged at the rate 700g for 2min and supernatant was discarded carefully. The cell supernatant was added to Ni-NTA pellet and was mixed well by slowly vortering (by hand) and placed it horizontally to hybridization oven for 45 min and further centrifuged at 700g for 2 min The supernatant was collected as flow through (F1) and stored at -20°C. For washing, 500µl washing buffer was added to resin pellet. It was mixed and centrifuged at 700g for 2 min and supernatant was collected and stored at -20°C. To pellet again 500µl wash buffer was added and mixed gently and centrifuged at 700g for 2 min and the supernatant was collected (wash 2) and stored at -20°C. Again the pellet was washed with 500µl of

wash buffer and mixed gently and centrifuged at 700g for 2 min. and supernatant was collected (wash 3) and store at -20°C. To this one bed of resin volume (approx.100-125µl) elution buffer was added and mixed properly and incubated horizontally in hybridization oven for 10 min. The tube was centrifuged at 700g for 2 min. and the eluted protein (E1) was collected in labelled microcentrifuge tube and stored at -80°C again re-elution was carried out using same volume and stored -80°C.

The Ni-NTA resin was regenerated by washing with 1000µl regeneration buffer and it was centrifuged at 700g for 2 min and supernatant was discarded. To the pellet 1000µl regeneration buffer was added again and re-centrifuged at 700g for 2 min and supernatant was discarded. To the pellet 1ml MilliQ water was added and centrifuged at 700g for 2 min and supernatant was discarded. To the resultant pellet 200 µl of 20% ethanol was added and mixed well and the resuspended resin was stored at 4°C for further use. The pellets, crude extract, flow through, wash and eluted fractions saved at appropriate time points were analyzed by SDS-PAGE.

TABLE 3. 2. Accession numbers of BTV NS3 gene sequences retrived from GeneBank (http://blast.ncbi.nlm.nih.gov/).

	Origin/ Country	Serotype	Species	Accession NO.	
				Nucleotide	Amino acid
1.	INDIA	BTV/NS3/DHULE	Sheep		
2.	INDIA	BTV1	Sheep	KF664132	AGW27490.1
3.	INDIA	BTV	Sheep	JQ681265	AFH57190.1
4.	INDIA	BTV16	Sheep	KF664112	AGW27466.1
5.	INDIA	BTV23	Sheep	EU131027	ABX10965.1
6.	INDIA	BTV23	Sheep	EU131022	ABX10960.1
7.	INDIA	BTV18	Sheep	EU131026	ABX10964.1
8.	INDIA	BTV18	Sheep	EU131024	ABX10962.1
9.	INDIA	BTV10	Sheep	JQ740780	AFI73134.1
10.	INDIA	BTV2	Sheep	KF460444	AHA46536.1
11.	INDIA	BTV2	Sheep	MK516729	QED41481.1
12.	INDIA	BTV12	Sheep	MK516724	QED41476.1
13.	INDIA	BTV9	Sheep	JQ424789	AFI45079.1
14.	INDIA	BTV	Sheep	JQ424790	AFI45080.1
15.	INDIA	BTV12	Sheep	MF615246	ASV51743.1
16.	USA	BTV17	Sheep	MT952980	QOP57873.1
17.	INDIA	BTV9	Sheep	JX003696	AFN68311.1
18.	SOUTH AFRICA	BTV2	Sheep	MG255698	AYA21890.1
19.	SOUTH AFRICA	BTV24	Sheep	MG255598	AYA21880.1
20.	SOUTH AFRICA	BTV16	Sheep	MG255538	AYA21874.1
21.	SOUTH AFRICA	BTV1	Sheep	MG255528	AYA21873.1
22.	SOUTH AFRICA	BTV3	Sheep	MG255518	AYA21872.1
23.	SOUTH AFRICA	BTV17	Sheep	MG255488	AYA21869.1
24.	SOUTH AFRICA	BTV13	Sheep	MG255478	AYA21868.1
25.	SOUTH AFRICA	BTV5	Sheep	MT070948	QIQ51110.1
26.	SOUTH AFRICA	BTV 4	Sheep	MT070928	QIQ51080.1
27.	SOUTH AFRICA	BTV6	Sheep	MT070918	QIQ51070.1
28.	SOUTH AFRICA	BTV12	Sheep	MT070908	QIQ51060.1
29.	SOUTH AFRICA	BTV14	Sheep	MT070898	QIQ51050.1

30.	SOUTH AFRICA	BTV19	Sheep	MT070878	ADI49550.1
31.	USA	BTV22	Sheep	KX164148	AOX24029.1
32.	INDIA	BTV18		EU131025	ABX10961.1
33.	INDIA	BTV1		EU131023	AFY12611.1
34.	INDIA	BTV2		JQ904064	ADI49512.1
35.	INDIA	BTV12		MF615246	AKM21158.1
36.	SAF	BTV11	Sheep	KT885074	ALX36914.1
37.	SAF	BTV9	Sheep	KT885064	ALU65954.1
38.	SAF	BTV10	Sheep	KT317704	ALU65944.1
39.	SAF	BTV8	Sheep	KT317694	ALU65934.1
40.	ZAMBIA	BTV7	Cattle	LC570004	BCI59324.1
41.	ZAMBIA	BTV15	Cattle	LC569998	BCI59318.1
42.	CHINA	BTV21	Goat	MK250965	QDK54963.1
43.	GERMANY	BTV25	Goat	LR993309	CAD9197767.1
44.	FRANCE	BTV27	Goat	KU761006	AMQ36836.1
45.	USA	BTV 20		FJ713331	ACX69797.1
46.	INDIA	BTV5	Sheep	MK516719	QED41471.1
47.	KUWAIT	BTV26	Sheep	JN255162	AER60539.1
48.	INDIA	BTV16	Cattle	JX007931	AGC08405.1
49.	CHINA	EHDV7	Cattle	MK656462	QES86655.1
50.	SOUTH AFRICA	AHSV5	Horse	MK656462	AJU57441.1

RESULTS AND DISCUSSION

Bluetongue (BT) is an arthropod-borne infectious disease that affects domestic and wild ruminants, especially prominent in sheep, spread by Culicoides biting midges. The causative agent is the bluetongue virus, having 28 serogroups/serotypes belonging to genus *Orbivirus* in the *Reoviridae* family (Mellor *et al.,* 2009). Bluetongue primarily affects native sheep populations causes severe morbidity, mortality, abortion, fetal death, and malformations, as well as milk, meat, and fleece losses. Native sheep are mostly reared by farmers, it causes enormous economic losses to the less-affluent farming community. Bluetongue infection can have a negative impact on the trade of such animals due to the strict restrictions on the transfer of animals and animal products from BT-endemic nations to BT-free countries (Prasad *et al.,* 2016).

In India, bluetongue is endemic, owing to favorable climatic conditions and the density of the natural host population that are essential for the survival of the Culicoides vector and the virus. In India, 23 serotypes have been reported as of now; out of 28 serotypes of *Bluetongue virus* (BTV) that exist globally owing to its reassortment ability due to the segmented genome. However, data on the temporal or spatial distribution of particular serotypes of BTV are not available in India. South Indian states are frequently and more severely affected when compared to north Indian states. So there is a need for continuous sero-surveillance programs to monitor the endemicity, emerging, and reemerging status of BTV serotypes in India to identify the most commonly circulating serotypes in endemic areas to plan the development of multivalent vaccines for control and eradication of BT. The diagnosis of BTV is done by serological tests, virus isolation in the chicken embryo or cell culture, and detection of viral nucleic acid. The serological tests used for the diagnosis of BTV include agar gel immunodiffusion, cELISA, and indirect ELISA, among which cELISA is the most preferred and reliable (OIE, 2014). RT-PCR is the most sensitive method for detecting the *Bluetongue virus* (Prasad *et al.,* 2016, Chand *et al.,* 2015). The RT-PCR can detect BTV in various biological samples for ‘serogrouping’ and ‘serotyping’ of BTV (Ranjan, 2015). The efficient diagnostic system is one of the

prerequisites for sensitive detection and the declaration/confirmation of the virus-free status of non-endemic regions of the world. BTV is detected and isolated routinely, by direct inoculation onto cultured mammalian or insect cells, through the intravenous route into 10-12 days embryonated chicken eggs, followed by one passage in insect cell culture and up to three passages in mammalian cell cultures (Wechsler *et al.,* 1998; Anthony *et al.,* 2007, Wilson *et al.,* 2009). However, both the cell culture-based methods and serological methods are labor-intensive, cost-ineffective, and time taking. With interesting and desirable properties such as speed, high specificity, sensitivity, cost-effectiveness, and reduced contamination risk, polymerase chain reaction (PCR) has evolved as a very convenient alternative to the conventional methods of BTV detection. By targeting any one of the several conserved BTV genome segments, such as those encoding VP1, VP3, VP7, NS1, NS2, and NS3, reverse transcription-PCR and real-time RT PCR assays are carried out for detecting BTV (Wilson *et al.,* 2009; Anthony *et al.,* 2007; Shaw *et al.,* 2007; Toussaint *et al.,* 2007; Maan *et al.,* 2015; Orru *et al.,* 2006).

The sample in proper conditions signifies the outcome of any test. In India, due to different climatic zones and rearing of sheep and goats by marginal farmers in infrastructure lacking regions make it difficult to receive the samples in a pristine state to any laboratory. So avoiding cold chain maintenance and viral transport media for transportation would be of immense help in such conditions. Therefore, the need for easy-go sample collection methods which can solve the purpose in a limited resource scenario could be of more importance. The Dried sample spot is one of the easiest ways, but sample card cost is a limiting factor; hence the present study also plans to address this issue, trying to use the sample card and other cellulose-based material viz. filter paper and ink-free area of newspaper which can be readily available to any locations. Molecular epidemiology is one of prime importance in the endemic/enzootic area to ascertain the disease prevalence and its emergence or re-emergence owing to the introduction of different serotypes in the newer regions; therefore, the BTV molecular epidemiology has also been addressed in the current study. Further molecular characterization of the BTV using the RT-PCR, sequencing, and

attempting the protein analysis using the recombinant NS3 clone have been employed.

Detection of *Bluetongue virus* from blood samples by Conventional Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

A total of 10 BTV suspected sheep blood samples were processed for RNA extraction using TRI reagents as per the manufacturer's instruction. Total RNA was used as a template for cDNA synthesis, and BTV gene-specific primers were used in RT-PCR to amplify target BTV genes and cyclophilin A (housekeeping gene) gene of host using cyclophillin A specific primers on the same cDNA to confirm the cDNA synthesis to avoid any false-negative results. This technique was utilized to avoid PCR-based BTV disease diagnosis failing due to errors in RNA extraction, cDNA synthesis, or PCR. In molecular biology, the use of a two-step RT-PCR technique is common.

Using the designed diagnostic primers targeted to amplify BTV-NS3 gene, the PCR was carried out on the cDNA synthesized from the blood samples. Out of the 10 samples of blood, only five blood samples showed the expected product of 248bp (Fig. No. 4.1). A cyclophilin A gene (housekeeping gene) was also amplified from blood samples, and samples show a specific product of 119 bp (Fig. No. 4.2 and 4.2a). Similarly, it was observed that other researchers have also used the NS3 primers based RT-PCR for the detection of BTV. Akita *et al.* (1992; 1993) detected BTV using primers derived from seg 10 by RT-PCR and found that BTV PCR is rapid, sensitive, and detect active BTV infection in clinical samples. Billinis *et al.* (2001) developed a duplex RT-PCR assay to detect BTV in clinical samples, which detects the highly conserved S10 region of BTV, and found that the duplex RT-PCR could be a useful method for monitoring *Bluetongue virus* infection in the field. Devi *et al.* (2006) used the NS3 specific RT-PCR for detection of *Bluetongue virus;* out of 32 field blood samples, 10 samples were found positive for *Bluetongue virus.* Subhadra *et al.* (2014) also used conventional NS3 RT-PCR for the detection of *Bluetongue virus;* out of 32 blood samples, 17 were found positive for *Bluetongue virus*.

In the present study to amplify the complete CDS of NS3 was attempted, where only one sample has shown the 725bp amplicon as confirmed by agarose gel electrophoresis (Fig. No. 4.3) The other BTV primers targeting NS1, NS2, VP2, and VP7 were also used on BTV positive sample no. 2. The agarose gel electrophoresis revealed the expected size of 967bp, 1087bp, and 1156bp for NS1, NS2, and VP7, respectively, but unable to amplify VP2 and VP7nst amplicons (Fig. no.4.4). To further investigate the geographical origin of BTV in the present study for a better understanding of its incursion in the Dhule district of Maharashtra, the NS3 amplicon of 725bp was sequenced and genetically and phylogenetically analyzed.

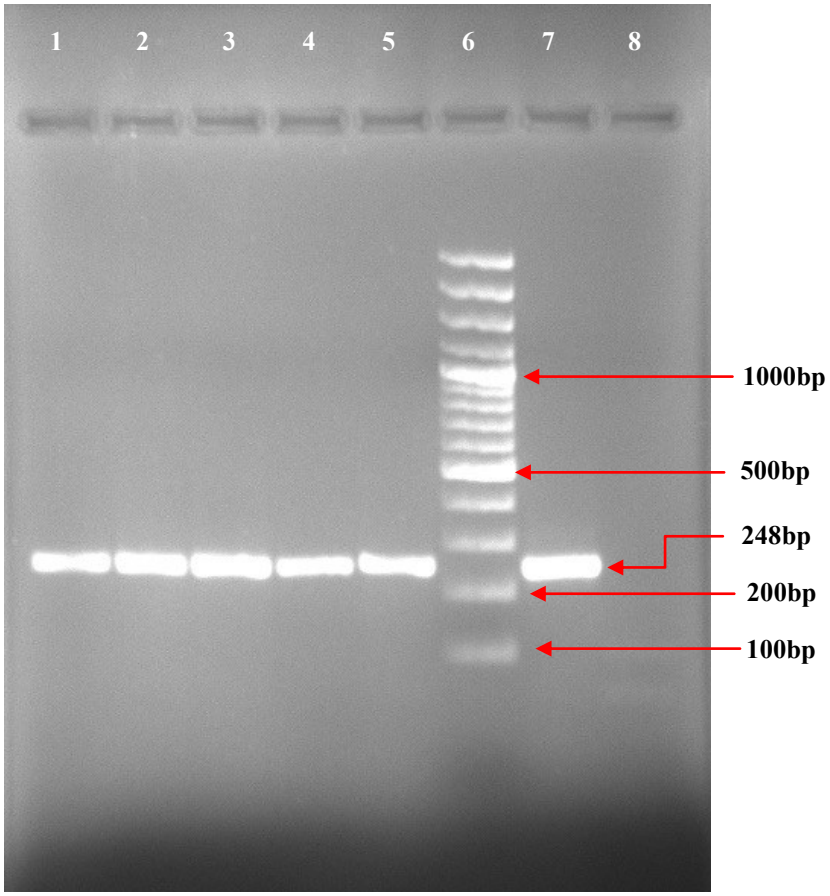
Detection of *Bluetongue virus* from Dried sample spots by DSS-RT-PCR

Sample collection and transportation issues frequently obstruct the diagnosis and surveillance of tropical infectious diseases. To overcome the sample collection and transportation in the cold chain for diagnosis, the other methods have been evaluated. Cellulose-based material viz. FTA, Filter paper provides a medium to overcome such problems.

Based on the previously analyzed blood samples by RT-PCR, five positive blood samples were considered for the alternative samples for checking to omit the cold chain maintenance and easy transportation in resource-limited conditions.

The alternative samples viz. dried blood spot on FTA, ordinary filter paper, and newspaper were processed for RNA extraction using TRI regents as per manufacturer's instruction with modification for extraction of RNA from FTA, Filter paper, and newspapers. While extracting RNA from filter paper and newspaper, 300µl RNA elution buffer was used, and incubation with elution buffer was given on ice for 30 minutes to release more amount of sample material from the spot. While extracting RNA from FTA cards, 500µl RNA elution buffer was used, and incubation on ice was increased up to 2 hours owing to the thickness of the FTA card. Further, two washings with 75% chilled ethanol were given to RNA pellet isolated from FTA cards to minimize the effect of DTT

Figure 4.1 Agarose gel electrophoresis showing NS3 gene specific product (248bp) by RT-PCR from blood samples



Lane 1- Blood sample no. 1

Lane 2- Blood sample no. 2

Lane 3- Blood sample no. 3

Lane 4- Blood sample no. 4

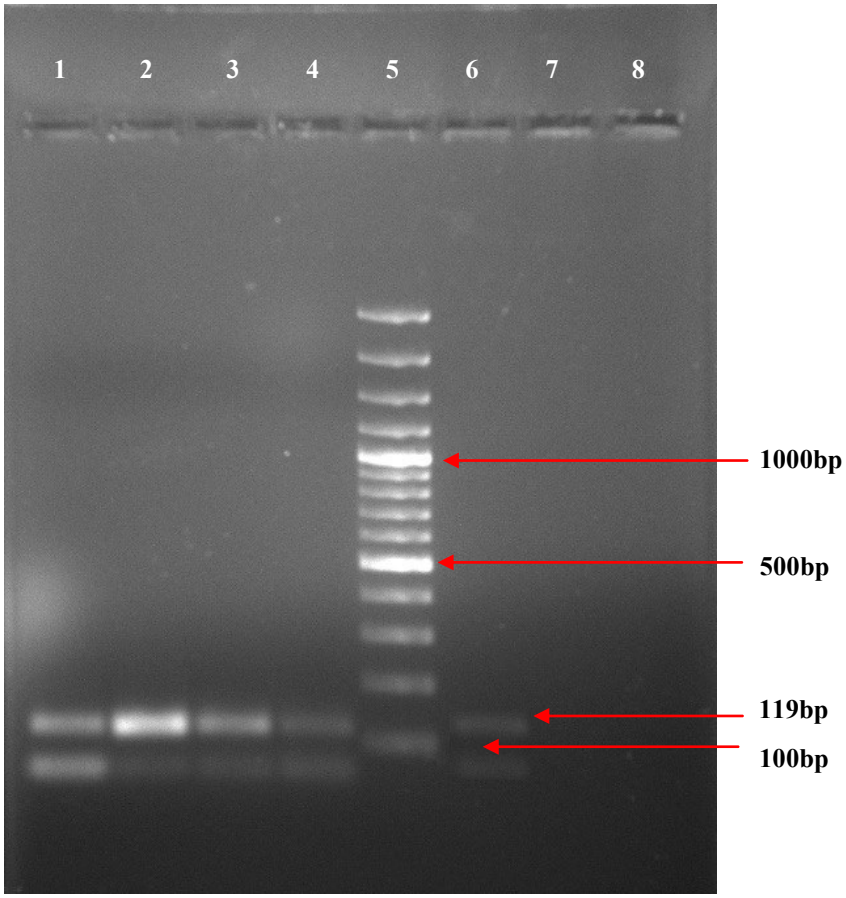
Lane 5- Blood sample no. 5

Lane 6- DNA Ladder (100bp Cat # SM0321, Thermo, USA)

Lane 7- Positive control (cell culture adopted BTV-2 suspension)

Lane 8- Negative control (No template)

Figure 4.2 Agarose gel electrophoresis showing Cyclophilin A (Housekeeping gene) amplification(119bp) by RT-PCR from blood samples



Lane 1- Blood sample no. 1

Lane 2- Blood sample no. 2

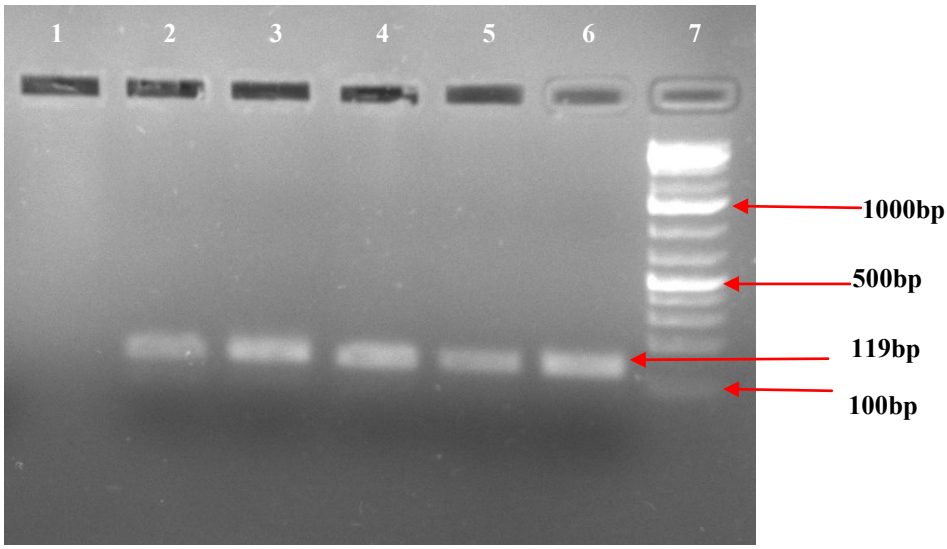
Lane 3- Blood sample no. 3

Lane 4- Blood sample no. 4

Lane 5- DNA Ladder (100bp Cat # SM0321, Thermo, USA)

Lane 6- Blood sample no. 5

Figure 4.2a. Agarose gel electrophoresis showing Cyclophilin A (Housekeeping gene) amplification (119bp) by RT-PCR from blood samples



Lane 1- Negative control

Lane 2- Blood sample no. 6

Lane 3- Blood sample no. 7

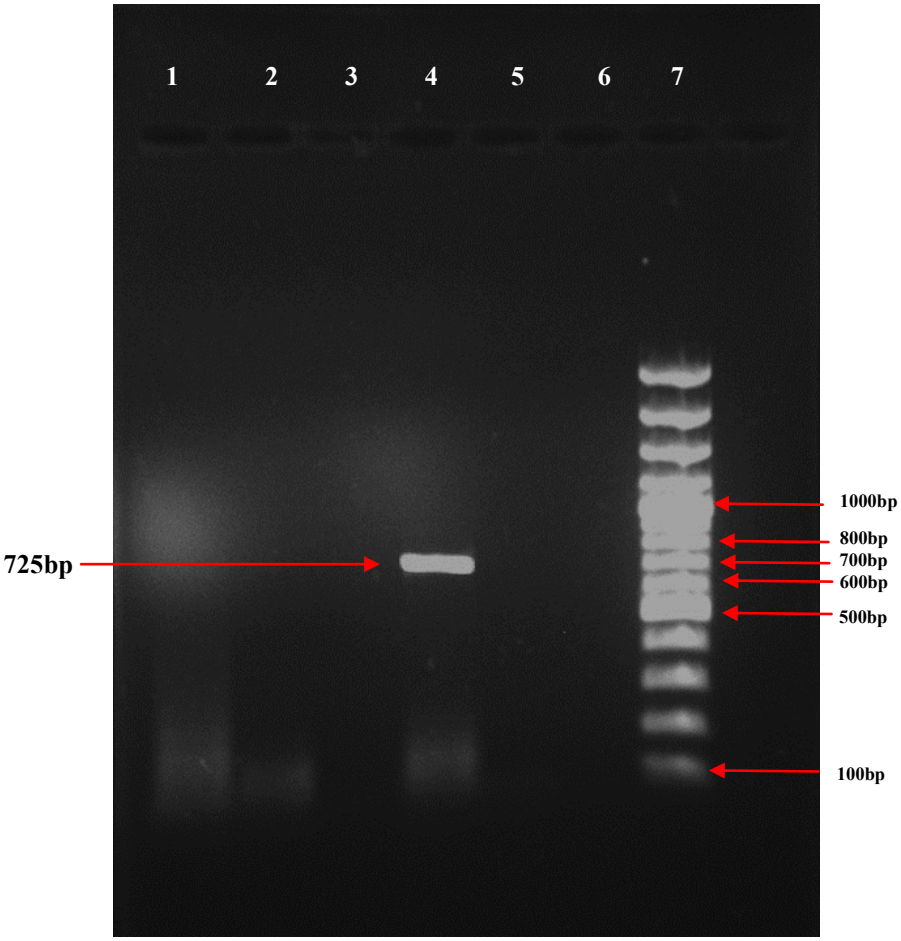
Lane 4- Blood sample no. 8

Lane 5- Blood sample no. 9

Lane 6- Blood sample no. 10

Lane 7- DNA Ladder(100bp Cat # SM0321, Thermo, USA)

Figure 4.3 Agarose gel electrophoresis showing BTV NS3 gene specific product (725bp) by RT-PCR from blood samples



Lane 1- Negative for NS3 sample no. 5

Lane 2-. Negative for NS3 sample no. 4

Lane 3- Negative for NS3 sample no. 3

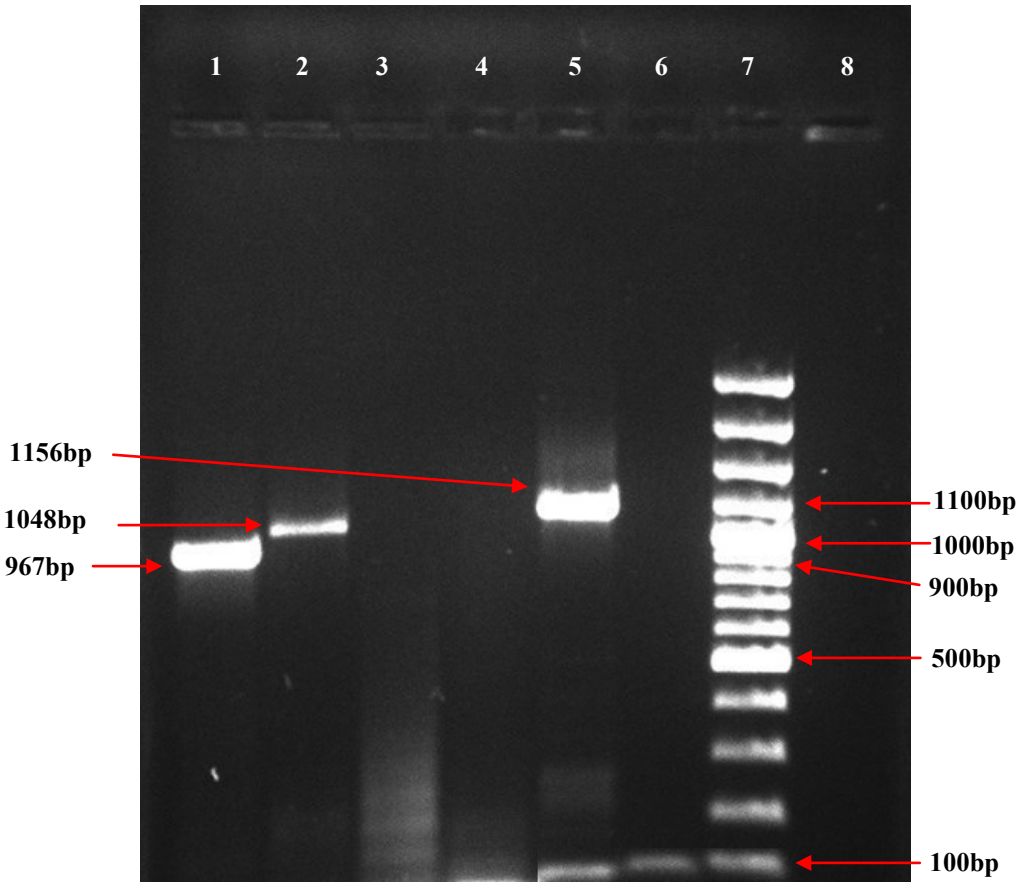
Lane 4- Positive for NS3 sample no. 2

Lane 5- Negative for NS3 sample no. 1

Lane 6- Negative control

Lane 7- DNA Ladder(100bp Cat # SM0321, Thermo, USA)

Figure 4.4 Agarose gel electrophoresis showing BTV genes specific product by RT-PCR from blood sample no. 2



Lane 1– sample no. 2 showing NS1 gene amplicon (967bp)

Lane 2- sample no. 2 showing NS2 amplicon (1087bp)

Lane 3- sample no. 2 showing Negative for VP2 gene

Lane 4- Negative control

Lane 5- sample no. 2 showing VP7ter amplicon (1156bp)

Lane 6- sample no. 2 showing Negative for VP7nst gene

Lane 7- DNA Ladder (100bp Cat # SM0321, Thermo, USA)

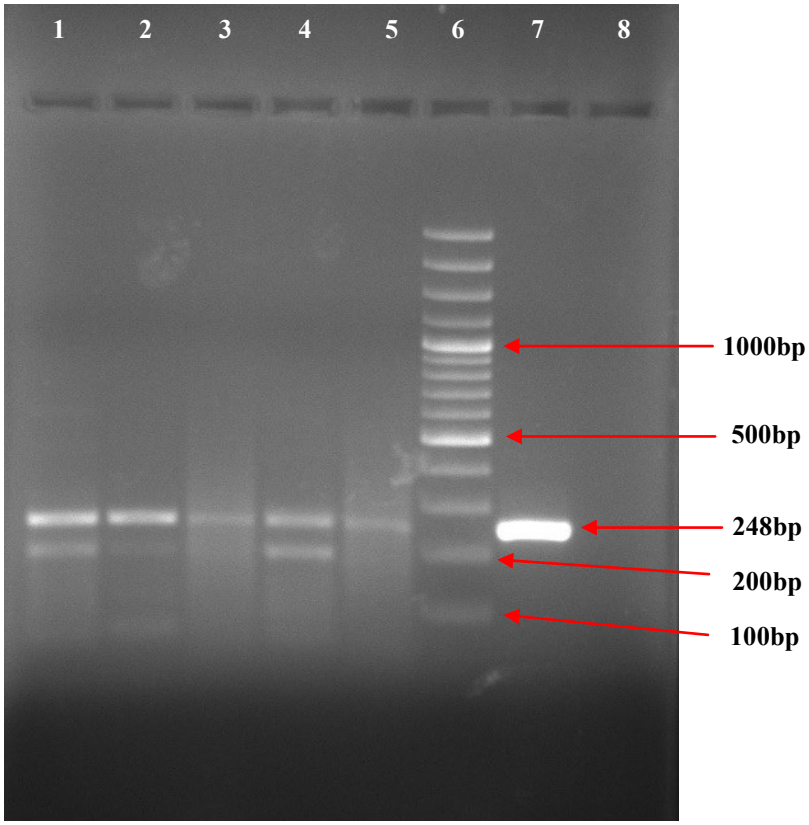
during downstream processing of RNA extraction, cDNA synthesis and PCR. These dried samples were processed by extraction of RNA, cDNA synthesis, and subsequent RT-PCR.

The NS3 primers targeted to amplify 248bp amplicons were used for the RT-PCR employed for alternative samples for BTV detection. The blood sample on FTA card when analyzed by the NS3-RT-PCR showed the amplification of 248bp in all the five samples (Fig. no. 4.5), as observed in the conventional RT-PCR on blood, suggesting the blood can be easily spotted on the FTA card with 100% positivity and such DSS-RT-PCR on FTA are suitable for the BTV detection. There was several documentation indicating the employment of cellulose material for the sample transportation. In 1815, Jons Berzelius, a Swedish chemist, was the first to use filter paper as a scientific tool. Robert Guthrie is widely recognized for being the first to use dried blood on filter paper (Guthrie cards) to test phenylalanine in infants for phenylketonuria detection. For blood sample collection some researchers used commercially available Gutherine cards (S and S 903, Schleicher and Schull UK) and FTA sample cards. Researchers also proved that FTA technology would be used for dried blood spot (DBS) and other discharges. The filter paper disk (no. 903; Schleicher and Schuell) was used for measles virus from oral fluid (Chibo *et al.,* 2005), for NDV (Perozo *et al.,* 2006). Bursas from experimentally infected birds was imprinted on FTA® cards, placed in phenol and used for molecular detection of IBDV (Purvis *et al.,* 2006). The Whatman 3MM filter paper (VWR, Fontenay-sous-Bois, France) and Whatman FTA cards was used for nucleic acid detection of ASFV and PPRV (Michuad *et al.,* 2007). The FTA® Classic Cards was used for identification of FMDV (Muthukrishnan *et al.,* 2008). The FTA cards viz. Whatman 903® Protein Saver untreated cards and Whatman FTA® Elute Micro treated cards were used for spotting blood in prodrug discovery study (D' Arienzo *et al.,* 2010). The FTA card was used for sampling pharayngeal swab and cloacal material for detection of Avian influenza in wild birds (Keeler *et al.,* 2012), the Porcine Reporductive and Respiratory virus (PRRSV) from blood, tissue and oral fluid was detected on FTA (Linhares *et al.,* 2012). Protein Saver™ 903® Cards were used for Hepatitis C virus (Bennett *et al.,* 2012). The FTA classic card for

detection of PPR virus (Pundlik, 2014), Whatman 903™ DBS cards for congenital cytomegalovirus (CMV), Whatmann 903 cards, FTA DMPK type A, B, C and FTA elute cards for therapeutic drug monitoring (TDM) and diagnosis of diseases in humans from blood (Ayre *et al.,* 2018). The Whatman 1001 filter paper was used for human African trypanosomiasis (HAT) (Compaore *et al.,* 2020). Similarly other researchers also used varied type of cards viz. HBV DNA in whole blood was spotted onto Whatman 903 paper and Whatman FTA cards (Bezerra *et al.,* 2021), TFN filter paper cards for SARS-CoV-2 antibodies (Gaugler *et al.,* 2021), FTA® Classic Card, the FTA® Elute Micro Card, and the 903 Protein Saver Card for storage of faecal sample for protozoan *G. duodenalis* and *C. hominis*. In humans, samples other than blood like serum, plasma, sputum, saliva, CSF, cervical smears, urine, milk, and fecal samples have been used for investigating different bacteriological, virological, and parasitological specimens by dried sample spot (Chibo *et al.,* 2005; Mharakurwa *et al.,* 2006; Desbois *et al.,* 2009, Gustavsson *et al.,* 2009, Neogi *et al.,* 2012, Olagunju *et al.,* 2015, Zheng *et al.,* 2016, Curren *et al.,* 2020).

The NS3-RT-PCR performed on BTV suspected sheep blood spotted on ordinary filter paper, instead of the suggested expensive filter papers (Whatman 903 and Perkin Elmer (Beaconsfield, UK) 226 filter paper) showed amplicon of 248bp in all the five blood samples, showing the 100% positivity as resembles in blood on FTA cards and in conventional blood samples (Fig. No. 4.6). The other researchers also used commercially available filter paper for storing the DNA/RNA from the samples. Vilcek *et al.* (2001) employed whole blood and serum samples on Whatman paper No. 1 to store Bovine viral diarrhea virus for diagnosis and RNA extraction, proving that it is safe to store and transport BVDV-infected blood for up to 6 months. Katz *et al.* (2002) investigated the detection of measles virus using dried blood spots stored on filter paper and discovered that measles virus RNA could be stored for two months at ambient temperature (25°C) and one month at 37°C. Andriamandimby *et al.* (2013) used venous blood, and dried blood blotted on filter paper (DBFP) for molecular diagnosis of *Chikungunya virus* (CHIKV) and found that DBFP specimens can be used as a cost-effective alternative sampling method for the surveillance and

Figure 4.5 Agarose gel electrophoresis showing NS3 gene specific product (248bp) by RT-PCR from dried blood spot on FTA



Lane 1- Blood sample no. 1 spotted on FTA

Lane 2- Blood sample no. 2 spotted on FTA

Lane 3- Blood sample no. 3 spotted on FTA

Lane 4- Blood sample no. 4 spotted on FTA

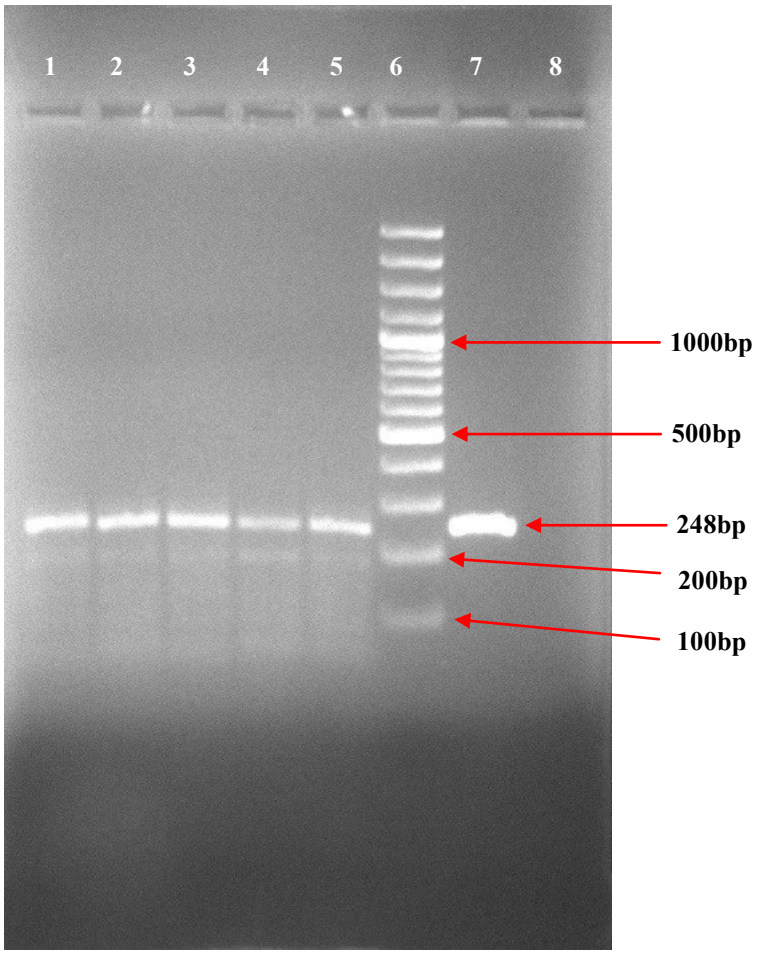
Lane 5- Blood sample no. 5 spotted on FTA

Lane 6- DNA Ladder(100bp Cat # SM0321, Thermo, USA)

Lane 7- Positive control (cell culture adopted BTV virus spotted on FTA)

Lane 8- Negative control (No template)

Figure 4.6 Agarose gel electrophoresis showing NS3 gene specific product (248bp) by RT-PCR from dried blood spot on Filter paper



Lane 1- Blood sample no. 1 spotted on Filter paper

Lane 2- Blood sample no. 2 spotted on Filter paper

Lane 3- Blood sample no. 3 spotted on Filter paper

Lane 4- Blood sample no. 4 spotted on Filter paper

Lane 5- Blood sample no. 5 spotted on Filter paper

Lane 6- DNA Ladder(100bp Cat # SM0321, Thermo, USA)

Lane 7- Positive control (cell culture adopted BTV virus spotted on Filter paper)

Lane 8- Negative control (No template)

monitoring of CHIKV. Bhuiyan *et al.* (2014) were used Whatman® 3MM filter paper (GE Healthcare, France) for molecular detection and genotyping of PPRV, at -70°C combined with ambient temperature, for periods up to 16 months and noticed excellent capacity of filter papers to store genetic material that can be sampled using a non-invasive approach. Pundlik, (2014) used ordinary filter paper by DSS-PCR for the detection of the PPR virus and found that it is economical for sample collection. Kerschberger *et al*. (2019) used filter paper to collect capillary or venous whole blood for dried blood spot (DBS) HIV Viral Load quantification. Curren *et al.* (2020) used a filter paper-dried sera sample for detection of dengue virus (DENV) RNA by real-time reverse RT-PCR. The use of filter paper in the diagnosis of tropical diseases indicated that almost all clinical samples might be stored on filter paper for subsequent analysis (Smit *et al.*, 2014).

This is the first report of work using regular filter paper for archiving the Bluetongue virus by spotting the blood and detecting BTV by RT-PCR.

For the manufacturing of numerous filter papers, including FTA, Whatman no. 903, no. 1, 3mm Paper, etc., the basic matrix material used is cellulose. Newspaper is one of the cheapest cellulose materials available. Newspapers are the cheapest and cost-free material available even in a remote area and can be easily carried anywhere. Therefore, the inclusion of newspapers in the present study had been done to use readily available material as an alternative approach to sample collection and dispatch. Blood spotting on the newspaper can also be done by the farmer or untrained individuals. Hence, in the present study, we used the ink-free portion of the newspaper, i.e., the margin of the newspaper and side of the newspaper, to spot the blood. Previously mentioned 5 positive blood samples in the study were spotted on the newspaper and analyzed by extracting the RNA, synthesizing cDNA, and RT-PCR as per the given protocol. The NS3-RT-PCR showed the 248bp amplicons in all five samples (Fig. No. 4.7), indicating 100% positivity with conventional blood samples, DSS on FTA, and Filter paper. So agreement of NS3-RT-PCR on dried sample spot on newspaper with 100% positivity rate is a breakthrough in sample collection and transportation in the resource-limited scenario. Similar work has been previously

reported by Pundlik, (2014), where he spotted 6 positive nasal secretion samples in the newspaper and found all positive for the detection of *Peste des petits ruminants virus* by conventional RT-PCR.

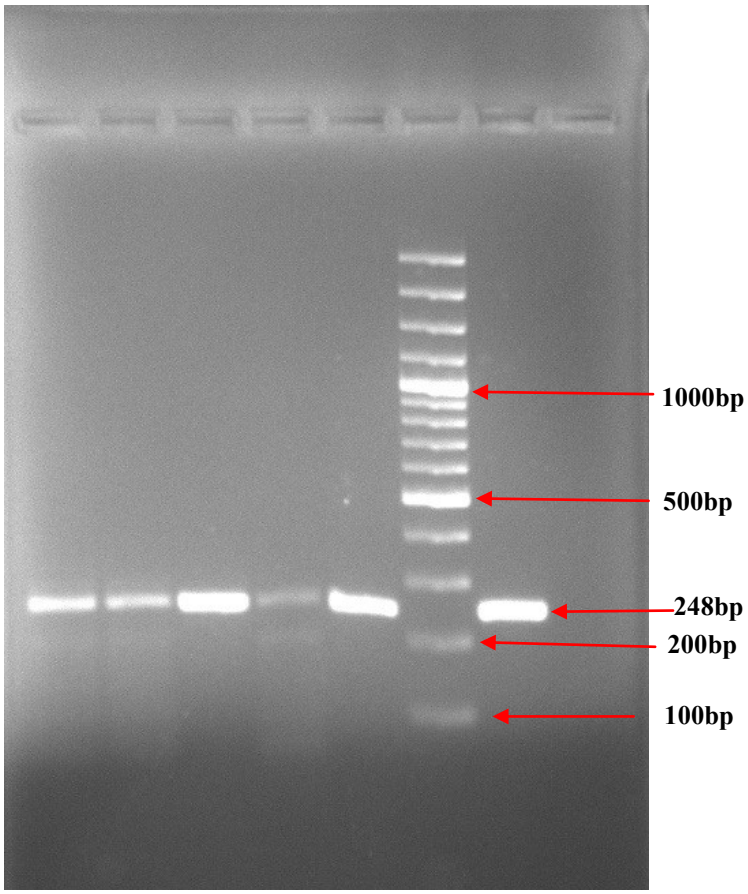
As previously stated, all of the blood samples spotted on the newspaper were able to archive BTV RNA and had 100% agreement with a conventional sample (unclotted blood) to detect BTV by PCR. This is the first report of work using newspaper for archiving the Bluetongue virus by spotting the blood and detection of BTV by RT-PCR.

As a result, the present study highlighted successful use of alternative cheapest and cost-effective sample collection and dispatch methods, such as blood spots on filter papers and ink-free portions of newspapers, which will change the way we look for BTV diagnosis in developing countries where resources are limited. BTV is endemic, and disease diagnosis is critical in the event of an eradication program.

Molecular epidemiology of BTV based on Segment 10 (NS3) gene sequence and phylogenetic analysis:

In the current study, the topographical investigation of bluetongue virus causing the disease in Dhule district of Maharashtra was carried out by sequencing the amplicon of NS3 coding DNA strand of 725bp size using the commercial sequencing facility and its analysis. The raw sequence data of the NS3 gene obtained by forward and reverse sequencing were analyzed for the base call and quality using the Chromas software. The primer location was identified and any excessive data beyond was removed. The identification of BTV was carried out using BLASTn analysis, which revealed the 99.40% to 97.52% identity with forward sequence and 97.94% to 96.90% identity with the reverse sequence as depicted in the appendix (Appendix 3 and 4). The contigue was generated using the curated forward and reverse sequence. The consensus sequence was obtained and designated as BTV/NS3/Dhule/MH/India/sheep/2019. It was reanalyzed by using BLASTn, revealed 99.72% to 97.97% identity with global sequences of BTV available in the GenBank with 1000 blast hits, thus

Figure 4.7 Agarose gel electrophoresis showing NS3 gene specific product (248bp) by RT-PCR from dried blood spot on Newspaper



Lane 1- Blood sample no. 1 spotted on Newspaper

Lane 2- Blood sample no. 2 spotted on Newspaper

Lane 3- Blood sample no. 3 spotted on Newspaper

Lane 4- Blood sample no. 4 spotted on Newspaper

Lane 5- Blood sample no. 5 spotted on Newspaper

Lane 6- DNA Ladder(100bp Cat # SM0321, Thermo, USA)

Lane 7- Positive control (cell culture adopted BTV virus spotted on Newspaper)

Lane 8- Negative control (No template)

indicating the obtained sequence is of BTV and the confirmation of outbreak as BT (Appendix 5). The sequence data showed that the amplified and sequenced NS3 is 725bp in size, with 55.94% A+T and 44.05% C+G, indicating obtained partially sequenced is AT-rich. The ORF finder revealed that there were two longest open reading frames, the largest first ORF started from 14bp to 703bp having a CDS of 690bp, encoding 229 amino acid protein as translated, indicating NS3 protein of expected size of 25524 daltons (25.52 kDa). The second ORF started from 53bp to 703bp, having CDS of 651bp indicating the NS3a protein. The BLASTn analysis revealed 100% identity with bluetongue virus 16 (Access. no. AFO37735) and 99.56 to 97.38 % identity with complete protein sequences of global 100 BTV isolates of different serotypes as depicted in the appendix 6.

The phylogenetic analysis was carried out using the NS3 nucleotide sequence of BTV/NS3/Dhule/MH/India/sheep/2019 in the preent study and the sequences from the Indian and globally available different BTV NS3 sequences in GenBank. The African horse sickness was used as an outliner for the analysis (Accession no. KP009790). The sequences were analyzed by maximum likelihood methods embedded in the MEGA 11 software. Phylogenetic analysis revealed that the BTV/NS3/Dhule/MH/India/sheep/2019 was placed closer to the KF664132/BTV1/India/Ap/sheep/2008 and JX007931BTV16//India/cattle/2010 in single sub clad of the Eastern cluster 1, comprising of BTV from India including BTV1 2, 5, 9, 12, 18, 21 and 23 (Accession no. KF664112, EU131027, EU131022, EU131026, EU131024, MK516729, MK516724, JQ424789, JQ424790, MF615246, JX003696, EU131025, EU131023, and MK516719 respectively). The phylogenetic analysis of NS3 of 27 different serotypes showed that the NS3 sequence of BTV in the present study was closer to BTV-1 and BTV-16; however, the BTV-1 and BTV of the present study were from the sheep origin, but the BTV-16 is from the cattle origin. In Western Maharashtra, to increase milk production, the purchase of good quality cattle breeds from Haryana state is adopted. Therefore, the placing of BTV-16 from the cattle in this sub-clad pointing out the genetic reassortment might be could happened, which requires the full genomic characterization of the BTV of the present study in future. All the different serotypes of BTV Indian isolates were placed in a single clad except the

BTV 2 (JQ904064) from Hissar, India. The phylogenetic tree depicting the BTV evolutionary hierarchy has been shown in Fig. No.4.8 (original tree). The bootstrap consensus tree has also revealed the placing of BTV/NS3 /Dhule/MH/India/sheep/2019 closer to the KF664132/BTV-1/India/AP/Sheep/2008 and JX007931/BTV-16/India/Cattle/2010 (Fig. No. 4.9). The phylogenetic placing of different serotypes in the Eastern and Western, and south African continental placing with some exception has been observed. It showed three major clusters and sub-clusters. Similarly other researchers showed the placing of the different BTV based on segments 10 in Eastern, Western, and European comprising different BTV from the globes. Steyn *et al.* (2016) showed that the phylogenetic analysis of NS3/A indicated three major (Eastern group 1 and 2 and Western group 1) and two minor clusters. The S10 sequence data showed that reassortment of the S10 gene segment (Pierce *et al.,* 1998). The genetic analysis of segment 10 of 19 field isolates of *Bluetongue virus* (BTV) serotypes BTV-1, BTV-4, BTV-9, and BTV-16, showed that regardless of the year of isolation, geographical location, host species, or tissue origin, most BTV-4 isolates had similar NS3/NS3A sequences and clustering of the NS3/NS3A sequences was independent of the viral serotype showing that genome segment reassortment occurred during virus evolution (Nikolakaki *et al.,* 2005). In another study, phylogenetic analysis of 137 BTV strains from Africa, the Americas, Asia, Australia, and the Mediterranean Basin indicated the two main clades that classified the viruses by serotype and isolation year (1900-2003); however, the positive selection based on S10 gene was not detected using phylogenetic reconstruction of ancestral codon states; however, the functional constraints of the NS3 protein are expressed through significant negative (purifying) selection (Balasuriya *et al.,* 2008). In another study, in strains from different origins (topography), the distribution of and the levels of differences in nucleotide identities revealed that VP3, NS2, and NS3 genes were more suitable for topotyping BTVs (Lee *et al.,* 2011). The genetic and phylogenetic characterization of BTV isolates based on segment 10 also showed the four major clusters, where Indian isolates were closer to BTV of Australia and China (Pudupakkam *et al.,* 2017; Biswas *et al.,* 2021). It was also observed that some minor-sub clustering

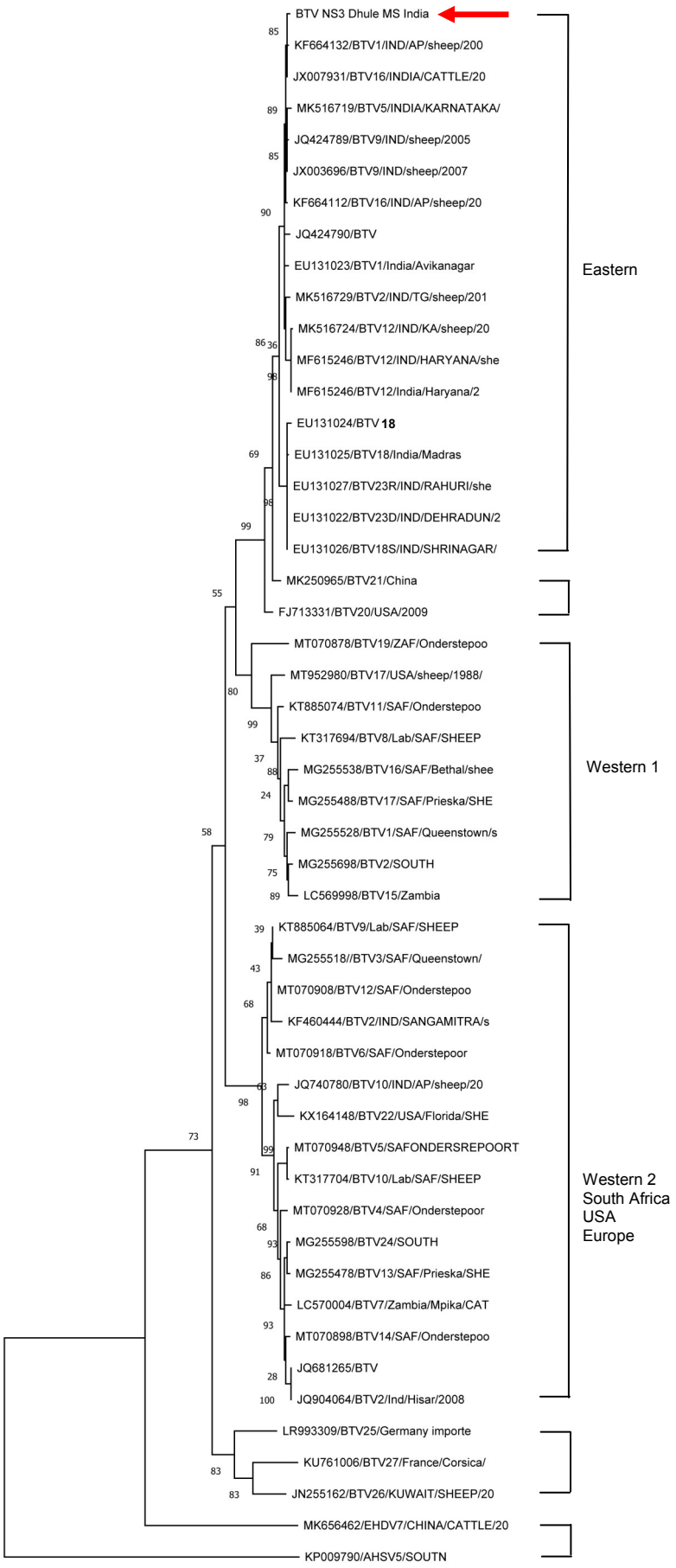


Figure 4.8: Molecular phylogenetic analysis of nucleotide sequences (50) of NS3 genes by Maximum Likelihood method using MEGA 11 (Original tree)

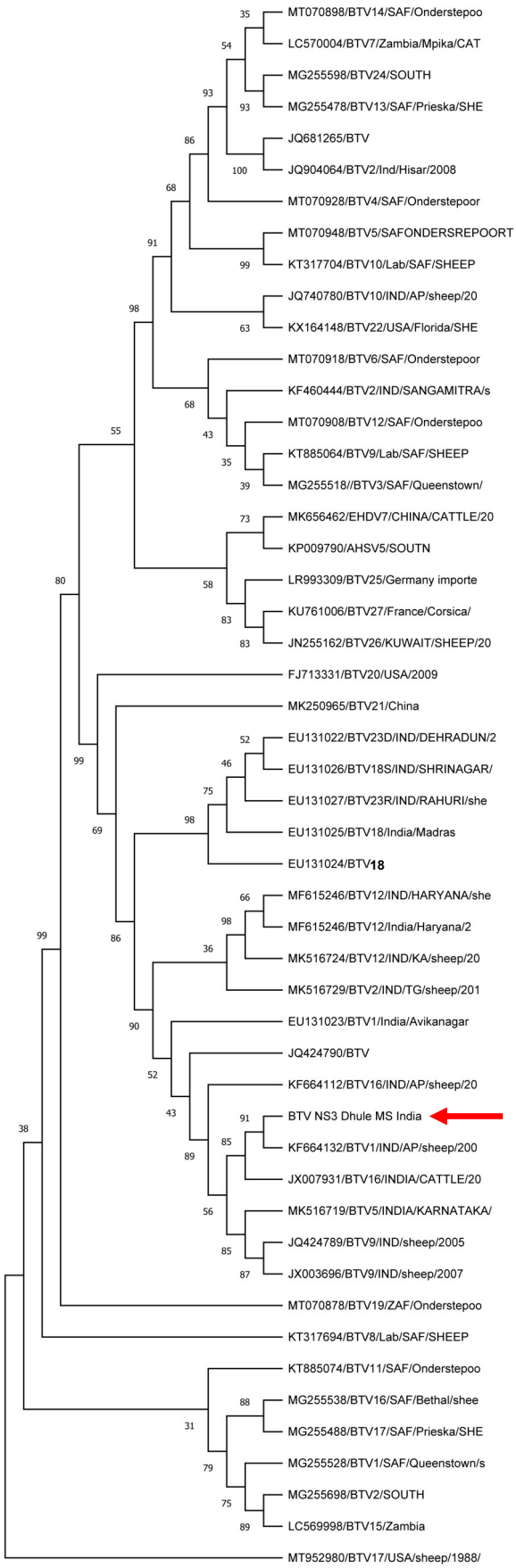


Figure 4.9 Molecular phylogenetic analysis of nucleotide sequences(50) of NS3 genes by Maximum Likelihood method using MEGA 11 (Bootstrap tree)

showed Indian isolates closer to the USA and a few Indian BTV isolates to South African reference and vaccine strains (Biswas *et al.,* 2021).

The phylogenetic analysis of the deduced amino acids sequences of 50 BTV, NS3 protein sequences spanning 27 BTV serotypes, including the sequence of the present BTV strain, revealed the BTV/NS3/Dhule/MH/India/sheep/2019 is closely placed with KF664132/BTV 1/India/AP/sheep/2008 and JX007931BTV16/16/India/cattle/2010 in single subclade. The present study also showed that the EHDV and AHSV are placed as an outliner in the present analysis (Fig no. 4.10). The bootstrap consensus tree inferred from 500 replicates of the taxa was taken to infer the evolutionary history showed that BTV/NS3/Dhule/MH/India/sheep/2019 is closely placed with KF664132/BTV-1/India/AP/sheep/2008 and JX007931BTV16/India/cattle/ 2010 in single sub-clad as well, as there is a certain demarcation of Eastern, Western, and South African isolates placed the geographical positioning of the BTV different serotypes based on their geological location (Fig. No. 4.10 and 4.11) with few exceptions of BTV around globes. In the analysis, it was observed that there is a clustering of strains of different geographical areas into the same group. A similar observation has been reported by Steyn and Venter (2016), where they could show the clustering of strains from a different geographical area into the same group, indicating the spatial spread of the segment 10 gene. The present study observed a similar trend that could be because of the introduction of newer strains from the other geographical area owing to trades. In the present study BTV/NS3/Dhule/MH/India/sheep/2019 is closely placed with KF664132/BTV 1/India/Ap/sheep/2008 and JX007931BTV16/India/cattle/2010, it could be a result of genetic reassortment in caprine BTV-1 serotype from Andra Pradesh and bovine BTV-16 strain from Hissar, Haryana. A similar observation in segment 10 (NS3) showing common ancestry and suggesting BTV-6 Netherland virus emerged owing to genetic reassortment with the possibility of BTV-6 vaccine strain and BTV-2 co-infection that could happen during several replication cycle. before this virus arrived in northern Europe (Maan *et al.,* 2010). In another study, the genetic characterization of the NS3 gene of BTV-2 from India suggested BTV-2 is closely related to Asia and Australia BTV's, suggesting common origin

and evolution pattern, different from other global isolates, and the deduced amino acid shows high molecular stability (Pudupakkam *et al.*, 2017). In the present study, it was observed to be a moderate level of geographic divergence, as all BTV serotypes placing is not homogenous based on serotypes rather represents moderate geographical positioning. A similar observation has been reported by the recent study based on NS3 (segment 10), indicating the global trait association of phylogenetic structure was not a homogenous but moderate level of geographical divergence (Biswas *et al.*, 2021).

Recombinant BTV-NS3 protein expression in the prokaryotic system and its characterization.

The available pET-32a-BTV-NS3 recombinant clones in BL-21(DE3) were utilized for the characterization of the NS3 protein. The revived clones were plated on the LB Ampicillin plate (representative Fig. No.4.12). The single colony was picked up, and overnight grown culture in LB broth was further grown for expression analysis. The mid-logarithmic growth phase of transformed pET-32-a-BTV-NS3 recombinant clone in BL-21(DE3) bacterial cells was induced with 1 mM IPTG, and 3 and 5 hrs of induced bacteria were collected, and the lysate was used for further analysis. The cells lysate was analyzed by SDS-PAGE, showed expression of the rNS3 protein as depicted in Fig. 4.13 The clone showed a protein band of the approximate size of 25.5kDa. The expression of the rNS3 in the prokaryotic system has revealed a unique band of the expected size of CDS deduced size, which also corresponds to the expression rNS3 protein in other studies as shown by Barros *et al.* (2009). The codon-optimized and hydrophobic transmembrane region TM1 and TM2 deleted NS3 was expressed in the prokaryotic system showed a 23kDa protein in SDS-PAGE, which corresponded to the calculated CDS of the optimized gene of the study (Tacken *et al.*, 2015).

Optimization of rNS3 protein expression

The recombinant clone was optimized for protein expression. The protein expression kinetics was studied at different incubation intervals and different IPTG concentrations. The induction with these criteria revealed induction of

Figure 4.10 Molecular evolutionary relationship of BTV based on NS3 protein sequence (50) using Maximum Likelihood method using MEGA 11 (Original tree)

Figure 4.11 Molecular evolutionary relationship of BTV based on NS3 protein sequence (50) using Maximum Likelihood method using MEGA 11 (Bootstrap tree)

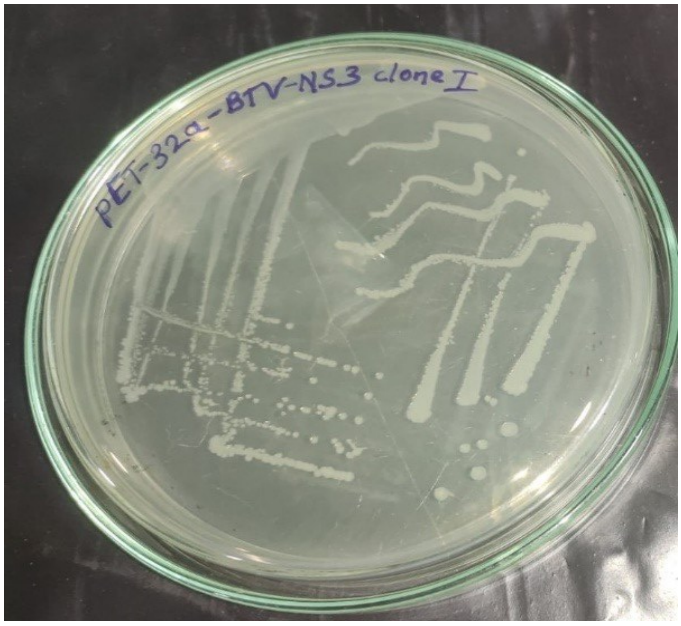


Figure 4.12 : LB Ampicillin plate showing revival of the Recombinant pET-32a-BTV-NS3 clone in BL21(DE3) cells.

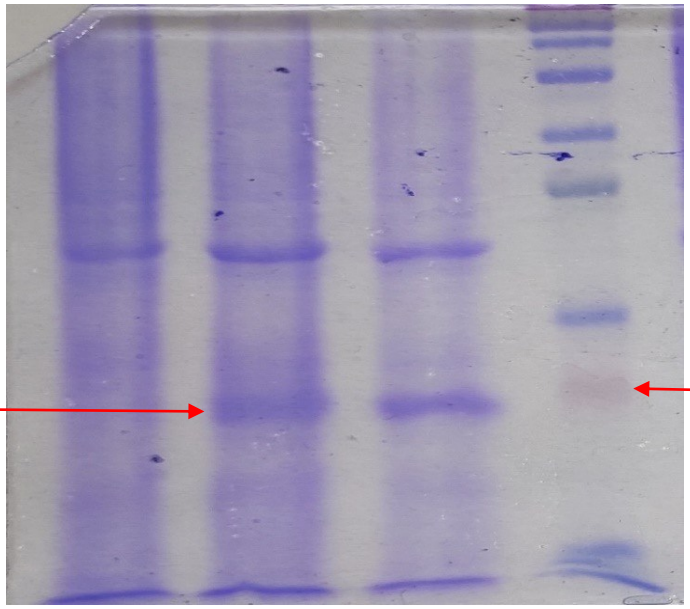


Figure 4.13: SDS-PAGE showing expression of rNS3 protein in time dependent manner

Lane 1-pET32a-BTV-NS3 Uninduced

Lane 2- pET32a-BTV-NS3 post 3 hrs induction

Lane 3- pET32a-BTV-NS3 post 5 hrs induction

Lane 4- Protein ladder (cat # BM003-0500, BR BIO-CHEM, India)

rNS3, expression after 3 and 5 hrs (Fig. 4.13). Therefore, 5 hrs post-induction incubation was used for protein expression. The cultures were induced with different concentrations of IPTG ranging from 0.5mM to 1.5mM. The results indicated similar expression patterns in all the concentrations (Fig. 4.14). Therefore, 1mM IPTG concentration was selected for further induction for purification for small-scale production of rNS3. Many studies use 1mM IPTG for induction of the proteins (Jiang *et al.,* 2016; Wanmakok *et al.,* 2018; Jin *et al.,* 2019).

NS3 protein purification using Ni-NTA chromatography and its analysis:

Overnight grown bacterial culture was inoculated to 10 ml of Luria broth (LB) with Ampicillin and incubated in an orbital shaker incubator to obtain mid logarithmic growth phase. The culture was induced with 1mM IPTG and incubated for 5 hrs. The culture was then centrifuged, and the pellet was stored at - 80°C until further use. SDS-PAGE analysis showed the expression of protein with its expected size.

The protein lysate was sonicated, and recombinant His tagged NS3/A protein was purified using the Ni-NTA resin. The 250µl of Ni-NTA resin were equilibrated, and sonicated supernatant of lysate was allowed to bind and eluted in fractions as per the protocol. All the different fractions collected at different stages of purification were analyzed by SDS-PAGE. The SDS-PAGE analysis showed that the induced rNS3 protein appeared into supernatant after sonication, bound effectively to Ni-NTA agarose and slightly appeared in flow-through and wash while Ni-NTA bound rNS3 showed a purified protein after elution (Fig. 4.15). The 25.5kDa rNS3 protein was confirmed on SDS-PAGE as there was no specific band with the same Rf value in the uninduced but transformed BL21 (DE3) cells.

The recombinant NS3 protein has been of the same size as previously expressed by a researcher who has shown purified segment 10 protein from BTV-1 and found 25.5KDa NS3 (Gould *et al.,* 1989). The NS3 protein purified in the prokaryotic system and analyzed by the western blot showed a 25kDa band, which was further used for the DIVA system (Barros *et al.,* 2009). The purified protein

of 23kDa was found in TM1 and TM2 delated cloned and expressed and purified using Ni-NTA (Tacken *et al.,* 2015). Thus the purification of NS3 in the present study and its optimization pave future research for the development of a diagnostic platform for the BTV.

The present study has shown that the outbreak suspected for BT in the Dhule district of Maharashtra is confirmed by the RT-PCR and sequence analysis as BTV. The primers designed for diagnostic PCR targeting NS3 segment 10 of BTV have shown amplicons of 248bp size in all the positive samples where as large PCR 725bp of NS3 could amplify only one positive sample , indicating its utility of 248bp NS3 RT-PCR being smaller, which is ideal for conventional diagnostic RT-PCR. The sample collection approach of alternative to cold chain maintenance and viral transport media, the FTA card was found suitable likewise the filter paper and newspaper wherein 100% agreement of positivity to the conventional blood sample RT-PCR. Thus in the resource-limited scarce area, the filter paper or ink-free newspaper can be utilized for BTV blood sample collection as a dried spot sample for BTV detection, which can be easily transported to the diagnostic laboratory for nucleic acid-based diagnosis. The alternate sample collection can be adopted in the field easily. The molecular epidemiology based on S10 (NS3) segment showed the possibility that the BTV/NS3/Dhule/MH/India/sheep/2019 strain could be evolved from the genetic reassessment of BTV-16 of cattle origin and BTV-1 of sheep origin based on percent identity and phylogenetic placing. The genetic reassortment could occur due to co-infection of BTV-16 and other BTV in single animal and evolved during virus replication and topologically placed at Maharashtra due to trade of animals.

It is concluded that, irrespective of the serotypes, the topological placing of BTV into eastern (1), western (2), into three clusters, and three subclusters was observed among the 50 sequences analyzed in the present study where the BTV Dhule strain is placed into the eastern cluster. The molecular epidemiology based on NS3 (segment 10) can place the BTV into topographical clustering irrespective of serotypes and origin, with few exceptions that might occur due to reassortment and trade of animals. The recombinant protein of NS3 was expressed in a

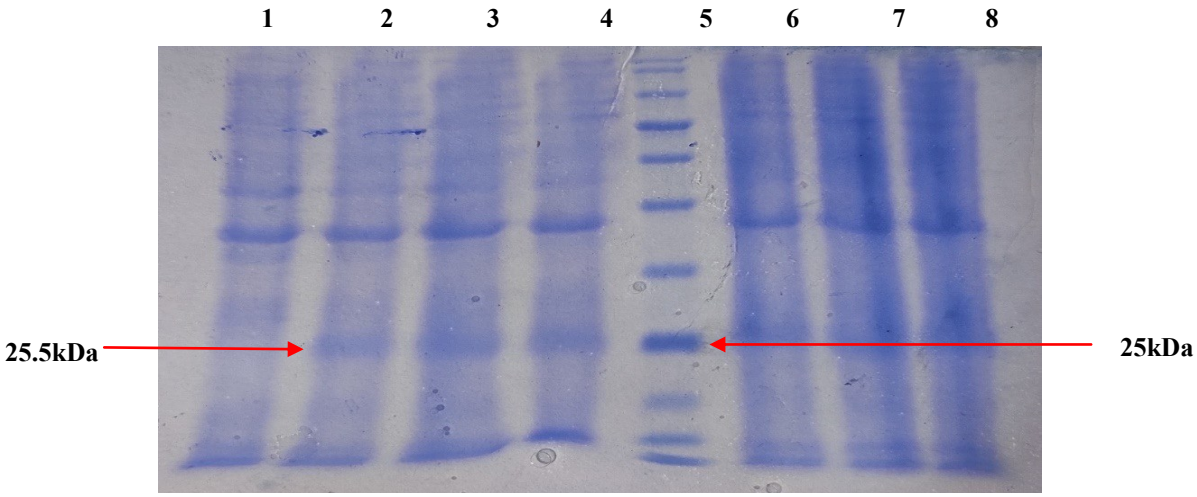


Figure 4.14: SDS PAGE showing optimization of expression of recombinant NS3 protein of BTV at different IPTG concentration

Lane 1- pET32a-BTV-NS3 Uninduced

Lane 2- pET32a-BTV-NS3 induced with 0.5 mM IPTG (3 hrs)

Lane 3- pET32a-BTV-NS3 induced with 1 mM IPTG (3 hrs)

Lane 4- pET32a-BTV-NS3 induced with 1.5 mM IPTG (3 hrs)

Lane 5- Protein ladder (cat # BM019-0500, BR BIOCHEM)

Lane 6- pET32a-BTV-NS3 induced with 0.5 mM IPTG (5 hrs)

Lane 7- pET32a-BTV-NS3 induced with 1 mM IPTG (5 hrs)

Lane 8- pET32a-BTV-NS3 induced with 1.5 mM IPTG (5 hrs)

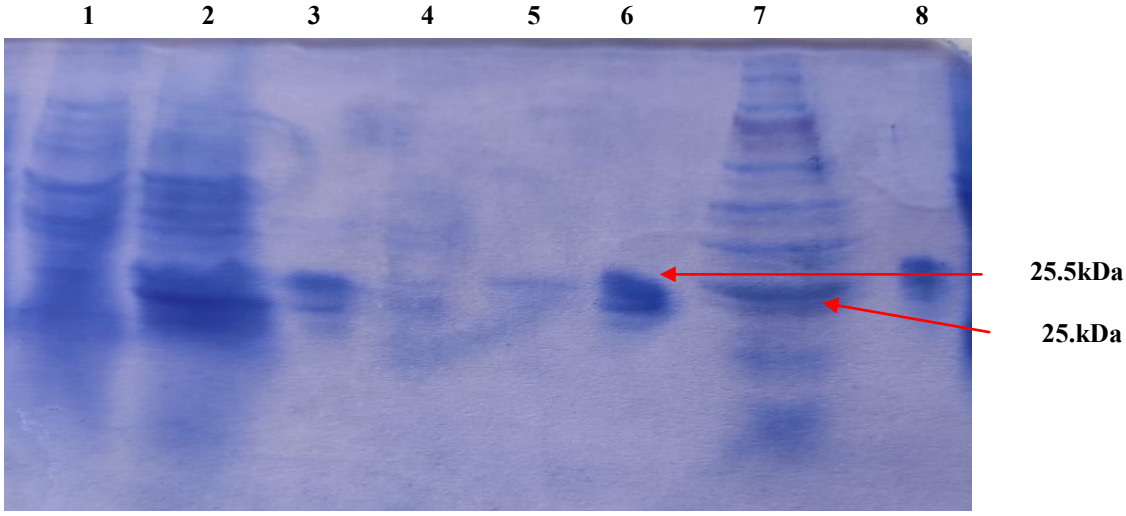


Figure 4.15 : SDS-PAGE showing confirmation of the purified protein by Ni-NTA agarose gel chromatography

Lane 1- pET32a-BTV-NS3 Uninduced

Lane 2- pET32a-BTV-NS3 Induced

Lane 3- Supernatant after sonication

Lane 4- Flow through after binding of rNS3 to Ni-NTA resin

Lane 5- Wash after binding of rNS3 to Ni-NTA resin

Lane 6 Purified NS3 Elute 1

Lane 7- Protein marker(cat # 27839, BioLit®)

Lane 8- Purified NS3 Elute 2

prokaryotic system and could be optimized at 1mM, and 5 hrs of induction and His-Tag BTV NS3 protein showed the unique band of around 25.5 kDa, which was found to be easily purified using Ni-NTA chromatography; thus, it can be utilized for the future diagnostic platform and differentiating infected from vaccinated animals (DIVA) purpose.

The present study showed that the NS3 gene-based diagnostic PCR could be employed for BTV detection, alternative sample collection on newspapers is user friendly, molecular epidemiology can infer the topology of virus origin irrespective of serotypes and origin, and recombinant NS3 can be expressed in the prokaryotic system with optimization for purification and other recombinant protein-based diagnostic platform can be designed.

Further studies need to be designed for utilization of diagnostic PCR based on NS3 based on Dried sample spot or conventional sample and its adaptation over BTV conventional diagnostic platforms. Further, implementation of alternative sample collection at the field need to be taken up. The full genome sequence analysis of BTV circulating in India need to be carried out to decipher the genetic reassortment viruses so futuristic vaccine for particular geography can be designed. The recombinant NS3 protein in diagnostic platform would be implemented to ascertain the active infections and also for designing the DIVA test.

SUMMARY AND CONCLUSIONS

Bluetongue (BT) is an arthropod-borne infectious disease that affects domestic and wild ruminants, especially prominent in sheep, spread by Culicoides biting midges. The causative agent is the *Bluetongue virus* (BTV), having 28 serogroups/serotypes belonging to the genus *Orbivirus* in the *Reoviridae* family. Bluetongue primarily affects native sheep populations causing severe morbidities and mortalities, abortions, fetal deaths, and malformations, as well as milk, meat, and fleece losses.

In India, bluetongue is endemic, owing to favorable climatic conditions and the density of the natural host population that are essential for the survival of the Culicoides vector and the virus. In India, 23 serotypes have been reported as of now; out of 28 serotypes of BTV that exist globally owing to its reassortment ability due to the segmented genome. However, data on the temporal or spatial distribution of a particular serotype/topotype of BTV are not available in India. NS3 is found to be one of the genes which signify the topotyping, and it is expressed in excess in an active stage of infection and helps in the egress of the virus. So it is one of the prime gene to be utilized from the BTV characterization. The diagnosis of BTV is done by serological and molecular tests. The sample in proper conditions signify the outcome of any test. In India, where so many climatic zones, rearing of sheep and goats by marginal farmers in infrastructure lacking regions, makes it difficult to receive the samples in a pristine state to any laboratory. The Dried sample spot is one of the easiest ways, but sample card cost is a limiting factor; hence the present study also plans to address these issues, trying to use the sample card and other cellulose-based materials viz. ordinary filter paper and ink-free area of newspaper which can be readily available to any locations. The molecular epidemiology is of prime importance in the endemic/enzootic area to ascertain the disease prevalence and its emergence or re-emergence owing to the introduction of different serotypes /topotypes in the newer regions; therefore, the BTV molecular epidemiology is also

addressed in the current study. Further information on the use of nonstructural genes for these *Bluetongue virus* characterizations is obscured, topotyping based on nonstructural genes is limited, diagnosis need and difficulty in sample collection and dispatched remains critical in identification and confirmation, which leads to failure in planning the control of the disease. Looking into this, the present study was planned to amplify the NS3 gene by reverse transcription-polymerase chain reaction (RT-PCR), standardize the dried sample spot-polymerase chain reaction (DSS-PCR), and carry out the phylogenetic relationship based on the nonstructural gene.

In the present study, 10 blood samples of sheep from the outbreak in 2019 in Dhule and Nandurbar Districts of Maharashtra were processed for BTV detection. From the positive samples, 100 µl of blood was blotted on the FTA sample card, Filter paper, and newspaper (ink-free area) on around of 1.5 cm circular area. From these, all samples RNA extraction using Tri Reagent, cDNA synthesis, and PCR was carried out. The confirmation of amplicons was done by agarose gel electrophoresis. The small diagnostic PCR (248bp targeting NS3 designed primer) was employed on all blood samples, and five samples were targeted using an NS3 full length (725bp) RT-PCR and out of which one sample was fully characterized by different BTV gene-RT-PCR viz. NS1, NS2, and VP2 and VP7. The 725bp amplified product of NS3 was commercially sequenced. The sequenced data were curated using Chroma software, and the contigue was generated, followed by nucleotide and amino acid sequence analysis. The 49 sequences around the globes taking available 27 serotypes of BTV segment 10 sequences, and the sequence of the present study was analyzed by ClustalW, followed by phylogenetic analysis at the nucleotides and amino acids levels using the MEGA 11 software. The BIC mode for the best model selection and molecular evolutionary inference by Maximum Likelihood method using 1000 bootstrap replicates for the nucleotide and 500 bootstrap replicates for amino acids was carried out in MEGA 11. The NS3 recombinant clone in pET-32a-BTV-NS3 available at the department was also expressed in BL-21(DE3) cells and expressed protein was optimized for time and IPTG concentration and purification were carried

out using the Ni-NTA chromatography technique and analyzed by SDS-PAGE electrophoresis.

The NS3 diagnostic RT PCR showed 248bp amplicons in 5 samples out of 10 samples. The NS3 RT-PCR targeting 725bp was amplified in only one sample. The RT PCR targeting different genes of BTV on one positive sample was analyzed by agarose gel. The agarose gel electrophoresis revealed the expected size of 967bp, 1087bp, and 1154bp for NS1, NS2, and VP7, respectively, but unable to amplify VP2 and VP7nst amplicons. All five positive blood samples blotted on the FTA card, Filter paper, and newspaper on RT-PCR with diagnostic NS3 primers showed 100% positivity in all samples indicating the NS3 RT-PCR could be useful for conventional as well as Dried sample spot RT-PCR. The sequencing of BTV NS3 showed NS3 is 725bp in size, with 55.94% A+T and 44.05% C+G, indicating obtained partial sequenced is AT-rich. The ORF finder revealed that there was two longest open reading frame, the largest first ORF started from 14bp to 703bp having a CDS of 690bp, encoding 229 amino acid protein as translated, indicating NS3 protein of expected size of 25524 daltons (25.52 kDa). The second ORF was started from 53bp to 703, having CDS of 651bp indicating the NS3a protein. The BLASTn analysis revealed 100% identity with bluetongue virus 16 (Access. no. AFO37735) and 98.56 to 97.38% identity with complete protein sequences of global 100 BTV isolates of different serotypes. BTV/NS3 /Dhule/MH/India/sheep/2019 was reanalyzed using BLASTn revealed 99.72 % to 97.97 % identity with global sequences of BTV available in the GenBank with 100 blast hits. Phylogenetic analysis revealed that the BTV/NS3 /Dhule/MH/India/sheep/2019 was placed closer to the KF664132/BTV1/India/Ap/sheep/2008 and JX007931BTV16//India/cattle/2010 in a single subclade of the Eastern cluster 1, comprising of BTV from India. The phylogenetic analysis of the deduced amino acids sequences of 50 BTV, NS3 protein sequences spanning 27 BTV serotypes, including the sequence of present BTV strain, revealed the BTV/NS3/Dhule/MH/India/sheep/2019 is closely placed

with KF664132/BTV1/India/AP/sheep/2008 and JX007931BTV16/16/India/cattle/2018 in single subclade. There is a certain demarcation of Eastern, Western, and South African isolates placed the geographical positioning of the BTV different serotypes based on their geological location. The present study showed a moderate level of geographic divergence as all BTV serotypes placing in non homogenous based on serotypes and rather represents moderate geographical positioning. The expression of the rNS3 in the prokaryotic system has revealed a unique band of the approximate size of 25.5kDa as the expected size of CDS deduced size. The recombinant BL-21(DE3) clone cultures were induced with different concentrations of IPTG ranging from 0.5 mM to 1.5mM. The results indicated similar expression patterns in all the concentrations, So 1mM IPTG was used for further studies. The recombinant protein purification using Ni-NTA chromatography showed a 25.5kDa rNS3 protein on SDS-PAGE as expected from the deduced amino acid sequence.

The present study concludes that the outbreak in sheep of the Dhule district was caused by the bluetongue virus as confirmed by RT-PCR not only by specific amplifications of NS3 gene but also by other genes of BTV viz. NS1, NS2, and VP7 and further reconfirmation by sequence analysis. The study also showed the utility of NS3 based diagnostic PCR of 248bp designed primer targeting NS3 gene for the BTV confirmation as it showed the robust amplification in all types of the sample like blood, dried blood spot sample on FTA card, Filter paper, and on newspapers. The alternative sample collection can be adopted as a replacement for the conventional blood sample collection as it has 100% positivity agreement by nucleic acid detection of BTV. So in a limited resource scenario, for detection of BTV, blood spotted on filter paper or newspaper can be recommended. The study also concludes that BTV/NS3/Dhule/MH/India/sheep/2019 strain could be evolved from the genetic reassortment of BTV-16 of cattle origin and BTV-1 of sheep origin based on percent identity and phylogenetic placing. The genetic reassortment could be occurred due to co-infection of BTV-16 and other BTV in single animals and evolved over time

during virus replication and topologically placed at Maharashtra due to trade of animals.

The future studies can be planned for adoptions and utilization of alternative sample collection methods in fields and diagnostic NS3 PCR. Also studies need to be carried out on full genome sequencing to ascertain the genetic reassortment of BTV for designing the geography based vaccine. The study can be planned for use of recombinant NS3 protein for diagnostic platform.

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APPENDIX -1

I. Removal of RNase contamination

i. Plasticware

Plasticware not resistant to chloroform were thoroughly rinsed with 0.1M NaOH, 1mM EDTA followed by DEPC treated water, while chloroform - resistant plasticware were rinsed with chloroform to inactivate RNases.

ii. Glassware

Properly cleaned glassware for RNA work were filled with 0.1% DEPC (0.1% in water) and allowed to stand (12 hours) at 37°C, and then heated to 100°C for 15 minutes to eliminate any residual DEPC.

iii. Water

DEPC (0.1 ml) was added to 100 ml of the water and shaken vigorously to bring the DEPC into solution and was incubated for 12 hours at 37°C. Then it was autoclaved for 15 minutes to remove any traces of DEPC.

II. Reagents used in RNA extraction

i. TRI-Reagent (Cat#T9424, SIGMA, USA.USA)
ii. Chloroform (Cat# C2432. SIGMA, USA.USA)
iii. Isopropanol (Cat # 19516 , SIGMA, USA.USA)
iv. 5N acetic acid
v. 75% ethanol (SIGMA, USA)
vi. Absolute Ethanol 75 ml
Sterilized distilled water 25 ml
Mix and strore at 4°C and used as a chilled solution

III. Reagents for Agarose Gel Electrophoresis.

i) Tris-borate EDTA (TBE) Buffer (5X stock solution)

Tris-base/ Tris buffer	54 gm
Boric acid	27.5 gm
0.5M EDTA (pH 8)	20 ml
Distilled water	1000 ml

Store at room temperature

For use dilute 1:10 with water for Agarose gel-electrophoresis

iii) Gel loading dye

Bromophenol blue	0.25%
Sucrose in water	40%
Distilled water	100 ml

Store at 4°C

iv)Ethidium bromide solution (10 mg/ml)

Ethidium bromide	0.1 gm
Distilled water	10.0 ml

Reagents used for preparation of protein expression

1X LB

LB Broth (Cat # L3022-250G, SIGMA, USA)	1.5 g
Distilled water	100 ml

Autoclave and store at 4°C.

2X LB

LB Broth (Cat # L3022-250G, SIGMA, USA)	1.5 g
Distilled water	50 ml

Autoclave and store at 4°C.

LB Agar (1X LB)

LB Broth (Cat # L3022-250G, SIGMA, USA)	1.5 g
Agar agar (Cat # A9539-100G, SIGMA, USA)	1.5 g
Distilled water	100 ml

Autoclave, pour in sterile petri plates and store at 4°C.

Ampicillin stock solution (50 mg/ml)

Ampicillin (Cat # MB104-5G, HIMEDIA, INDIA)	100 mg
Distilled water	2.0 ml

LB Agar with Ampicillin (1X LB)

LB Broth (Cat # L3022-250G, SIGMA, USA)	1.5 g
Agar agar (Cat # A9539-100G, SIGMA, USA)	1.5 g
Distilled water	100 ml

Autoclave and cool to 56°C.

Ampicillin (50 mg/ml)	100 µl

Mix well, pour in sterile petriplates and store at 4°C.

LB broth with Ampicillin

LB Broth (Cat # L3022-250G, SIGMA, USA)	1.5 mg
Distilled water	100 ml

Autoclave and cool to 56°C.

Ampicillin (50 mg/ml)	100 µl

Mix well and store at 4°C.

Reagents used for SDS-PAGE

Acrylamide (30%)

Acrylamide (Cat # A9099-100G, SIGMA, USA)	29.2 g
Bisacrylamid (Cat # 172-100G, Amresco)	0.8 g
Distilled water	100 ml

SDS (20%)

SDS (Cat # L4390-100G, SIGMA, USA)	5.0 g
Distilled water	20 ml

Tris (1 M, pH 8.8)

Tris base (Cat # T6066-1KG, SIGMA, USA)	12.11 g
Distilled water	90 ml
Adjust pH with 1N HCl and make	100 ml

Tris (1 M, pH 6.8)

Tris base (Cat # T6066-1KG, SIGMA, USA)	12.11 g
Distilled water Adjust pH with 1N HCl and make 100 ml.	90 ml

APS (10%)

Ammonium per sulphate	100 mg
Distilled water	1 ml

Prepare just before use.

TEMED (10%)

TEMED (Cat # T9281-25ML, SIGMA, USA)	0.1 ml
Distilled water	0.9 ml

Laemmli buffer (2X)

Tris (1 M. pH 6.80)	0.625 ml

SDS (20%)	1.000 ml
Glycerol (Cat # G5516-100ml, SIGMA, USA).	1.000 ml
β-Mercaptoethanol	0.500 ml
Distilled water	1.875 ml

Electrode buffer

Tris base (Cat # T6066-1KG, SIGMA, USA)	3.0 g
Glycine (Cat # G8898-500G, SIGMA, USA)	14.4 g
SDS (Cat # L4390-100G, SIGMA, USA)	1.0 g
Distilled water	1000 ml

Staining solution

Coomassie brilliant blue (Cat # 024218, SRL)	1.0 g
Methanol (Cat # M0120, Rankem)	500 ml
Glacial acetic acid (Cat # AS002-500ml, HIMEDIA, INDIA)	100 ml
Distilled water	400 ml

De-Staining solution

Methanol (Cat # M0120, Rankem)	500 ml
Glacial acetic acid (Cat # AS002-500ml, HIMEDIA, INDIA)	100 ml
Distilled water	400 ml

Resolving Gel

	10%	12.5%	14%
Acrylamide: Bisacrylamide (30%)	10.00 ml	12.50 ml	14.00 ml
Tris (1M, pH 8.8)	11.20 ml	11.20 ml	11.20 ml
SDS (20%)	0.15 ml	0.15 ml	0.15 ml
Distilled water	8.65 ml	6.15 ml	4.65 ml
TEMED (10%)	0.10 ml	0.12 ml	0.15 ml
APS (10%)	0.10 ml	0.12 ml	0.15 ml

Stacking Gel (5%)

Acrylamide: Bisacrylamide (30%)	1.67 ml
Tris (1M, pH 6.8)	1.75 ml
SDS (20%)	0.10 ml
Distilled water	6.48 ml
TEMED (10%)	0.05 ml
APS (10%)	0.05 ml

Reagents used for protein purification

Ni-NTA Superflow Agarose (Cat # 25214, Thermo)

Stock Solutions:

Sodium phosphate NaH_2PO_4, (1 M)

NaH_2PO_4 (Cat # 71505-250G, SIGMA, USA)	1.1998 g
Deionised water	10.00 ml

Sodium chloride (3 M)

NaCl (Cat # S3014-500G, SIGMA, USA)	1.7532 g
Deionised water	10.00 ml

Imidazole (1 M)

Imidazole (Cat # 56743, SIGMA, USA)	0.6808 g
Deionised water	10.00 ml

Guanidine hydrochloride (6 M)

Guanidine hydrochloride (Cat #G3272-25G, SIGMA, USA)	2.8659 g
Deionised water	5.00 ml

MES (1M)

MES hydrate (Cat # M2933-25G, SIGMA, USA)	0.9765 g
Deionised water	5.00 ml

Working Solutions:

Equilibration buffer

NaH_2PO_4 (1 M)	200 µl (final concentration 20 mM)
Sodium chloride (3 M)	1.00 ml (final concentration 300 mM)
Imidazole (1 M)	100 µl (final concentration 10 mM)
Deionized autoclaved water	6.00 ml

Adjust pH to 7.4

Make up final volume to 10 ml with deionized autoclaved water and degas all the reagents before use.

Wash buffer

NaH_2PO_4 (1 M)	200 ul (final concentration 20 mM)
Sodium chloride (3 M)	1.00 ml (final concentration 300 mM)
Imidazole (1 M)	250µl (final concentration 10 mM)
Deionized autoclaved water	6.00 ml

Adjust pH to 7.4

Make up final volume to 10 ml with deionized autoclaved water and degas all the reagents before use.

Elution buffer

NaH_2PO_4 (1 M)	200 µl (final concentration 20 mM)
Sodium chloride (3 M)	1.00 ml (final concentration 300 mM)
Imidazole (1 M)	3.00 ml (final concontration 300 mM)
Deionized autoclaved water	3.00 ml

Adjust pH to 7.4

Make up final volume to 10 ml with deionized autoclaved water and degas all the reagents before use.

5X Binding buffer NaH_2PO4 (1 M)

NaH_2PO_4 (1M)	2.50 ml (final concentration 250 mM)
Deionized autoclaved water	5.00 ml

Adjust pH to 8.0

Make up final volume to 10 ml with deionized autoclaved water and degas all the reagents before use.

Other chemicals:

Montanide oil (Cat # M8819-25g, SIGMA, USA)

Silver nanoparticles (Cat # 576832-5g, SIGMA, USA)

APPENDIX-2

Preparation of dried blood spots on FTA card, Filter paper and Newspaper

	Without blood spot	**With blood spot**
FTA card		
Filter paper		
Newspaper		

APPENDIX-3

Nucleotide Basic Local Alignment Search Tool (BLASTn) analysis of BTV NS3 genomic segment 10 forward nucleotide sequence indicating its identity with nucleotide sequences of different BTV serotypes.

BLAST® » **blastn suite** » results for RID-0M9B9DCS016

Job Title	1021_610_001_PCR_1_BTV_NS3_F_7_G11.ab1 ...
RID	0M9B9DCS016 Search expires on 02-15 16:48 pm
Program	BLASTN
Database	nt
Query ID	lcl|Query_5105
Description	1021_610_001_PCR_1_BTV_NS3_F_7_G11.ab1 ...
Molecule type	dna
Query Length	764

Descriptions

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus 4 isolate WGV103/ABT/HSR segment 10, complete sequence	Bluetongue virus 4	1187	1187	87%	0.0	99.40%	822	KF560426.1
Bluetongue virus 1 isolate NRT37/ABT/HSR segment 10, complete sequence	Bluetongue virus 1	1174	1174	87%	0.0	98.95%	822	KF664132.1
Bluetongue virus 1 isolate BTV01IND2010-VC12 segment 10, complete sequence	Bluetongue virus 1	1169	1169	87%	0.0	98.80%	817	KP339153.1
Bluetongue virus 16 isolate IND2010/cattle/16 segment 10, complete sequence	Bluetongue virus 16	1169	1169	87%	0.0	98.80%	822	JX007931.1
Bluetongue virus isolate K23/08 segment 10, complete sequence	Bluetongue virus	1169	1169	87%	0.0	98.80%	822	JX399157.1
Bluetongue virus 16 segment 10, complete sequence	Bluetongue virus 16	1169	1169	87%	0.0	98.80%	823	JQ924829.1
Bluetongue virus strain IND2015/K13 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1165	1165	87%	0.0	98.65%	820	MK516728.1
Bluetongue virus strain IND2015/40 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1160	1160	87%	0.0	98.50%	820	MK516734.1
Bluetongue virus 9 isolate BTV09IND2003-M11 segment 10, complete sequence	Bluetongue virus 9	1147	1147	87%	0.0	98.05%	812	KP339193.1
Bluetongue virus 9 isolate BTV09IND2003-M10 segment 10, complete sequence	Bluetongue virus 9	1147	1147	87%	0.0	98.05%	812	KP339183.1
Bluetongue virus 16 isolate BTV16IND2010-AP06 segment 10, complete sequence	Bluetongue virus 16	1147	1147	87%	0.0	98.05%	814	KP339213.1
Bluetongue virus 16 isolate BTV16IND2010-AP04 segment 10, complete sequence	Bluetongue virus 16	1147	1147	87%	0.0	98.05%	801	KP339203.1
Bluetongue virus 9 isolate BTV-9/IND2005/03 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 9	1147	1147	87%	0.0	98.05%	822	KP696660.1
Bluetongue virus 9 isolate BTV-9/IND2004/02 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 9	1147	1147	87%	0.0	98.05%	822	KP696621.1
Bluetongue virus 16 isolate K31-08/ABT/HSR segment 10, complete sequence	Bluetongue virus 16	1147	1147	87%	0.0	98.05%	822	KF664112.1
Bluetongue virus isolate Ind-R1-2007 NS3 gene, complete cds	Bluetongue virus	1147	1147	87%	0.0	98.05%	807	JX003696.1
Bluetongue virus 16 isolate BTV16IND2010-VC07 segment 10, complete sequence	Bluetongue virus 16	1142	1142	87%	0.0	97.90%	801	KP339233.1
Bluetongue virus 16 isolate BTV16IND2011-NR82 segment 10, complete sequence	Bluetongue virus 16	1142	1142	87%	0.0	97.90%	799	KP339223.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus 16 isolate G53/ABT/HSR segment 10, complete sequence	Bluetongue virus 16	1142	1142	87%	0.0	97.90%	822	KF664142.1
Bluetongue virus 1 isolate BTV-1/IND1988/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1138	1138	87%	0.0	97.75%	822	KP696525.1
Bluetongue virus 16 isolate PDP2/13/Ind segment 10, complete sequence	Bluetongue virus 16	1137	1137	87%	0.0	97.60%	822	MH395149.1
Bluetongue virus isolate NRT37/07/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1132	1132	84%	0.0	98.91%	690	MN537927.1
Bluetongue virus isolate NLG3/07/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1132	1132	84%	0.0	98.91%	690	MN537926.1
Bluetongue virus 9 isolate BBF segment 10, complete sequence	Bluetongue virus 9	1132	1132	87%	0.0	97.46%	822	JF443165.1
Bluetongue virus strain IND2014/10NLG NS3 protein (NS3) gene, complete cds	Bluetongue virus	1129	1129	87%	0.0	97.46%	814	MK516731.1
Bluetongue virus strain IND2015/V2 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1129	1129	87%	0.0	97.46%	816	MK516720.1
Bluetongue virus strain IND2015/228 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1129	1129	87%	0.0	97.46%	817	MK516714.1
Bluetongue virus strain IND2015/534 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1129	1129	87%	0.0	97.46%	812	MK516712.1
Bluetongue virus strain IND2015/486 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1129	1129	87%	0.0	97.46%	817	MK516708.1
Bluetongue virus strain IND2015/478 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1129	1129	87%	0.0	97.46%	822	MK516704.1
Bluetongue virus strain IND2015/475 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1129	1129	87%	0.0	97.46%	818	MK516701.1
Bluetongue virus strain IND2015/471 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1129	1129	87%	0.0	97.46%	822	MK516697.1
Bluetongue virus strain IND2015/408 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1129	1129	87%	0.0	97.46%	819	MK516695.1
Bluetongue virus strain IND2015/404 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1129	1129	87%	0.0	97.46%	818	MK516693.1
Bluetongue virus strain IND2015/492 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1129	1129	87%	0.0	97.46%	815	MK516691.1
Bluetongue virus strain IND2015/491 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1129	1129	87%	0.0	97.46%	815	MK516690.1
Bluetongue virus 1 isolate BTV-1/IND1985/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1129	1129	87%	0.0	97.46%	822	KP696516.1
Bluetongue virus isolate IND1992/01 segment 10, complete sequence	Bluetongue virus	1129	1129	87%	0.0	97.46%	822	JQ282777.1
Bluetongue virus isolate KAR16/06/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1128	1128	84%	0.0	98.76%	690	MN537937.1
Bluetongue virus isolate PTG13/11/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1128	1128	84%	0.0	98.76%	690	MN537928.1
Bluetongue virus isolate KDP15/07/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1128	1128	84%	0.0	98.76%	690	MN537918.1
Bluetongue virus strain IND2015/6PK NS3 protein (NS3) gene, complete cds	Bluetongue virus	1124	1124	87%	0.0	97.31%	816	MK516730.1
Bluetongue virus strain IND2015/K4 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1124	1124	87%	0.0	97.31%	817	MK516726.1
Bluetongue virus strain IND2015/547 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1124	1124	87%	0.0	97.31%	819	MK516713.1
Bluetongue virus 12 isolate INDAPADBNMO1/11 segment 10, complete sequence	Bluetongue virus 12	1120	1120	87%	0.0	97.16%	812	KC662621.1
Bluetongue virus isolate TN2/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1119	1119	84%	0.0	98.45%	690	MN537955.1
Bluetongue virus 2 isolate BTV02IND1993 segment 10, complete sequence	Bluetongue virus 2	1116	1116	87%	0.0	96.56%	811	KP339163.1
Bluetongue virus 2 isolate BTV-2/IND2000 nonstructural protein 3 (NS3) gene, complete cds	Bluetongue virus 2	1115	1115	87%	0.0	97.01%	762	KX650180.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus isolate EMBOSS_001 segment 10, complete sequence	Bluetongue virus	1115	1115	87%	0.0	97.01%	822	KX424875.1
Bluetongue virus 1 isolate BTV-1/IND2003/05 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1115	1115	87%	0.0	97.01%	822	KP696571.1
Bluetongue virus 1 isolate BTV-1/IND2003/04 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1115	1115	87%	0.0	97.01%	822	KP696561.1
Bluetongue virus 1 isolate BTV-1/IND1999/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1115	1115	87%	0.0	97.01%	788	KP696543.1
Bluetongue virus strain IND2015/14A NS3 protein (NS3) gene, complete cds	Bluetongue virus	1111	1111	87%	0.0	96.86%	815	MK516733.1
Bluetongue virus strain IND2015/K3 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1111	1111	87%	0.0	96.86%	816	MK516725.1
Bluetongue virus 2 isolate BTV02IND2010-KRM08 segment 10, complete sequence	Bluetongue virus 2	1111	1111	87%	0.0	96.86%	815	KP339173.1
Bluetongue virus strain IND1994/01 segment 10, complete sequence	Bluetongue virus	1111	1111	87%	0.0	96.86%	822	KP268783.1
Bluetongue virus 23 isolate 5268/5 segment 10, complete sequence	Bluetongue virus 23	1111	1111	87%	0.0	96.86%	822	JX272408.1
Bluetongue virus 23 strain 23/Labstr/ZAF/2020/Howell_DVTD_ref 23 isolate Ref 23 segment 10, complete sequence	Bluetongue virus 23	1111	1111	87%	0.0	96.86%	822	MT090664.1
Bluetongue virus 1 isolate BTV-1/IND2001/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1110	1110	87%	0.0	96.71%	822	KP696551.1
Bluetongue virus isolate M10/MBN/2005 NS3 (S10) gene, complete cds	Bluetongue virus	1110	1110	84%	0.0	98.14%	690	JQ424788.1
Bluetongue virus isolate K34/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1110	1110	84%	0.0	98.14%	690	MN537953.1
Bluetongue virus isolate K1/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1110	1110	84%	0.0	98.14%	690	MN537952.1
Bluetongue virus isolate K31/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1110	1110	84%	0.0	98.14%	690	MN537940.1
Bluetongue virus isolate MBN/02/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1110	1110	84%	0.0	98.14%	690	MN537934.1
Bluetongue virus isolate M10/03/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1110	1110	84%	0.0	98.14%	690	MN537933.1
Bluetongue virus strain IND2015/K9 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1106	1106	87%	0.0	96.71%	819	MK516727.1
Bluetongue virus 1 isolate BTV01IND2010-KRM07 segment 10, complete sequence	Bluetongue virus 1	1106	1106	87%	0.0	96.71%	817	KP339143.1
Bluetongue virus 2 isolate BTV-2/IND1993/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 2	1106	1106	87%	0.0	96.71%	822	KP696591.1
Bluetongue virus isolate G52/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1105	1105	84%	0.0	97.98%	690	MN537948.1
Bluetongue virus isolate ONG5/06/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1105	1105	84%	0.0	97.98%	690	MN537935.1
Bluetongue virus 2 isolate IND1982/01 segment 10, complete sequence	Bluetongue virus 2	1104	1104	87%	0.0	96.56%	822	JQ713563.1
Bluetongue virus strain IND2015/K14 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1102	1102	87%	0.0	96.56%	819	MK516729.1
Bluetongue virus strain IND2015/V NS3 protein (NS3) gene, complete cds	Bluetongue virus	1102	1102	87%	0.0	96.56%	817	MK516724.1
Bluetongue virus strain IND2015/34 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1102	1102	87%	0.0	96.56%	814	MK516715.1
Bluetongue virus strain IND2015/473 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1102	1102	87%	0.0	96.56%	815	MK516699.1
Bluetongue virus strain IND2015/470 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1102	1102	87%	0.0	96.56%	821	MK516696.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus strain IND2015/494 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1102	1102	87%	0.0	96.56%	821	MK516692.1
Bluetongue virus isolate IND2015/15-22 NS3 protein and NS5 protein genes, complete cds	Bluetongue virus	1102	1102	87%	0.0	96.56%	816	MK507962.1
Bluetongue virus 16 strain BTV-16 85395/2004 NS3 gene, complete cds	Bluetongue virus 16	1102	1102	87%	0.0	96.56%	824	MH990426.1
Bluetongue virus 16 isolate ISR2001/18 segment 10, complete sequence	Bluetongue virus 16	1102	1102	87%	0.0	96.56%	822	KP821956.1
Bluetongue virus 16 isolate RSArrrr/16* segment 10, complete sequence	Bluetongue virus 16	1102	1102	87%	0.0	96.56%	822	JX129386.1
Bluetongue virus serotype 16 segment S10, complete sequence	Bluetongue virus	1102	1102	87%	0.0	96.56%	822	AY775164.1
Bluetongue virus strain 243277 segment S10, complete sequence	Bluetongue virus	1102	1102	87%	0.0	96.56%	777	AY775163.1
Bluetongue virus isolate VJW/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1101	1101	84%	0.0	97.83%	690	MN537939.1
Bluetongue virus isolate DLP36/12/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1101	1101	84%	0.0	97.83%	690	MN537914.1
Bluetongue virus strain prototype 600577 NS3 protein (S10) gene, complete cds	Bluetongue virus	1099	1099	87%	0.0	96.41%	822	FJ713328.1
Bluetongue virus strain IND2015/7A NS3 protein (NS3) gene, complete cds	Bluetongue virus	1097	1097	87%	0.0	96.41%	822	MK516732.1
Bluetongue virus 16 strain BTV-16 87892TR/2006 NS3 gene, complete cds	Bluetongue virus 16	1097	1097	87%	0.0	96.41%	822	MH990396.1
Bluetongue virus 16 strain ITL2002 segment 10, complete sequence	Bluetongue virus 16	1097	1097	87%	0.0	96.41%	822	KF387530.1
Bluetongue virus strain 8054/02 segment S10, complete sequence	Bluetongue virus	1097	1097	87%	0.0	96.41%	814	AY775162.1
Bluetongue virus 16 strain SW nonstructural protein NS3/NS3A (S10) gene, complete cds	Bluetongue virus 16	1097	1097	87%	0.0	96.41%	785	AF135229.1
Bluetongue virus isolate G58/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1096	1096	84%	0.0	97.67%	690	MN537950.1
Bluetongue virus isolate G53/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1096	1096	84%	0.0	97.67%	690	MN537949.1
Bluetongue virus isolate G10/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1096	1096	84%	0.0	97.67%	690	MN537947.1
Bluetongue virus isolate G4/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1096	1096	84%	0.0	97.67%	690	MN537946.1
Bluetongue virus isolate CUN/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1096	1096	84%	0.0	97.67%	690	MN537945.1
Bluetongue virus isolate M11/MBN/2005 NS3 (S10) gene, complete cds	Bluetongue virus	1094	1094	84%	0.0	97.52%	690	JQ424789.1
Bluetongue virus strain IND2015/477 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1093	1093	87%	0.0	96.26%	822	MK516703.1
Bluetongue virus strain BN96/16 segment 10, complete sequence	Bluetongue virus	1093	1093	87%	0.0	96.26%	822	JN671915.1
Bluetongue virus isolate HIS/85/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1092	1092	84%	0.0	97.52%	690	MN537916.1

Graphic Summary

APPENDIX - 4

BLASTn analysis of BTV NS3 genomic segment 10 reverse nucleotide sequence indicating its identity with nucleotide sequences of different BTV serotypes.

COVID-19 Information

Public health information (CDC)

Research information (NIH)

SARS-CoV-2 data (NCBI)

Prevention and treatment information (HHS)

Español

BLAST® » **blastn suite** » results for RID-0M9NS4V4013

Job Title	1021_610_002_PCR_1_BTV_NS3_F_732_G11.ab1 ...
RID	0M9NS4V4013 Search expires on 02-15 16:54 pm
Program	BLASTN
Database	nt
Query ID	lcl|Query_28545
Description	1021_610_002_PCR_1_BTV_NS3_F_732_G11.ab1 ...
Molecule type	dna
Query Length	698

Descriptions

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus 1 isolate BTV-1/IND1985/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1157	1157	97%	0.0	97.94%	822	KP696516.1
Bluetongue virus 1 isolate BTV-1/IND1988/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1166	1166	97%	0.0	98.23%	822	KP696525.1
Bluetongue virus 1 isolate BTV-1/IND1999/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1126	1126	95%	0.0	97.59%	788	KP696543.1
Bluetongue virus 1 isolate BTV-1/IND2001/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1143	1143	97%	0.0	97.49%	822	KP696551.1
Bluetongue virus 1 isolate BTV-1/IND2003/04 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1143	1143	97%	0.0	97.49%	822	KP696561.1
Bluetongue virus 1 isolate BTV-1/IND2003/05 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1143	1143	97%	0.0	97.49%	822	KP696571.1
Bluetongue virus 1 isolate BTV01IND2010-KRM07 segment 10, complete sequence	Bluetongue virus 1	1129	1129	96%	0.0	97.19%	817	KP339143.1
Bluetongue virus 1 isolate BTV01IND2010-VC12 segment 10, complete sequence	Bluetongue virus 1	1187	1187	96%	0.0	99.11%	817	KP339153.1
Bluetongue virus 1 isolate NRT37/ABT/HSR segment 10, complete sequence	Bluetongue virus 1	1197	1197	97%	0.0	99.26%	822	KF664132.1
Bluetongue virus 12 isolate INDAPADBNMO1/11 segment 10, complete sequence	Bluetongue virus 12	1142	1142	96%	0.0	97.63%	812	KC662621.1
Bluetongue virus 16 isolate BTV16IND2010-AP04 segment 10, complete sequence	Bluetongue virus 16	1168	1168	95%	0.0	98.66%	801	KP339203.1
Bluetongue virus 16 isolate BTV16IND2010-AP06 segment 10, complete sequence	Bluetongue virus 16	1175	1175	97%	0.0	98.53%	814	KP339213.1

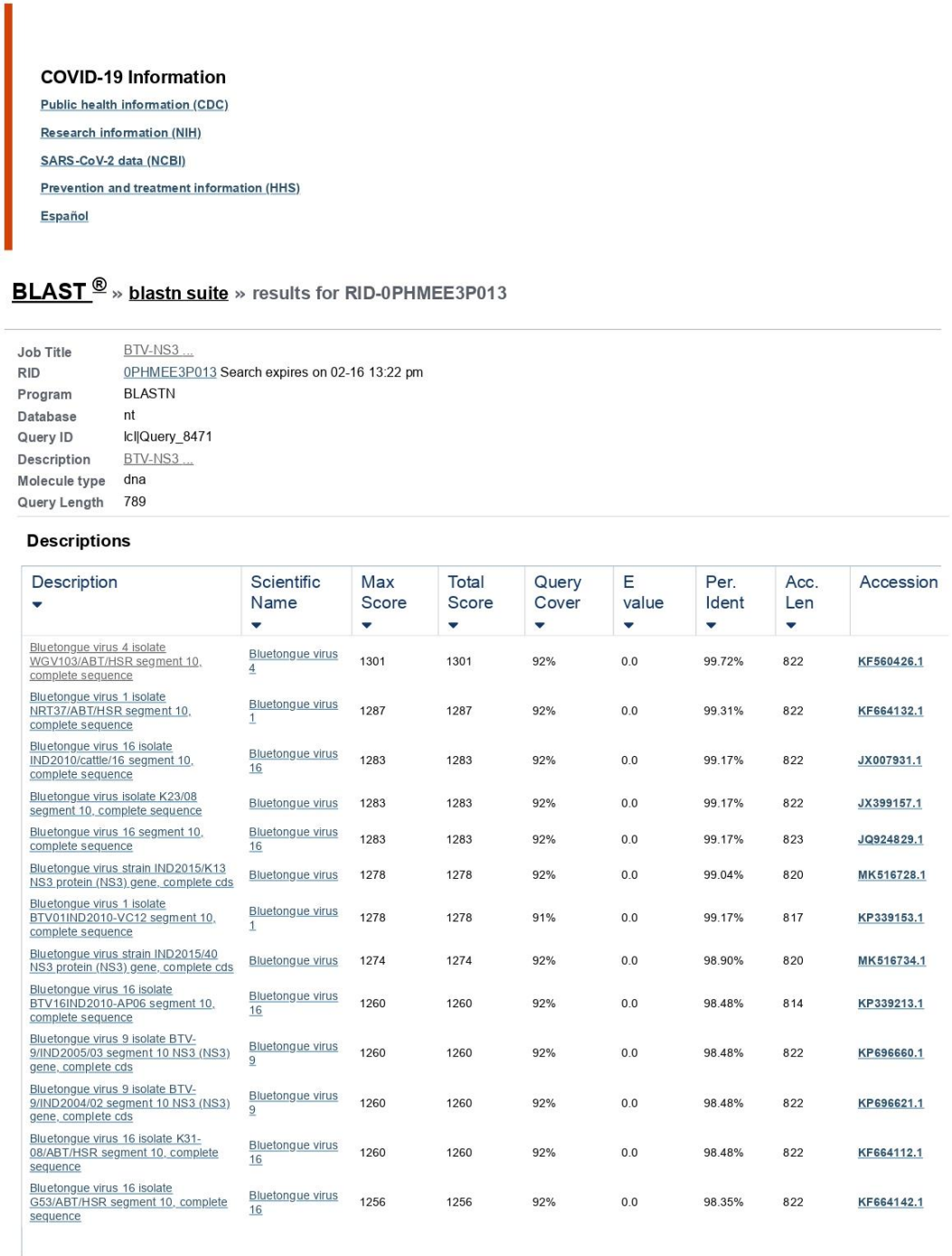
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus 16 isolate BTV16IND2010-VC07 segment 10, complete sequence	Bluetongue virus 16	1164	1164	95%	0.0	98.51%	801	KP339233.1
Bluetongue virus 16 isolate BTV16IND2011-NR82 segment 10, complete sequence	Bluetongue virus 16	1164	1164	95%	0.0	98.51%	799	KP339223.1
Bluetongue virus 16 isolate G53/ABT/HSR segment 10, complete sequence	Bluetongue virus 16	1170	1170	97%	0.0	98.38%	822	KF664142.1
Bluetongue virus 16 isolate IND2010/cattle/16 segment 10, complete sequence	Bluetongue virus 16	1193	1193	97%	0.0	99.12%	822	JX007931.1
Bluetongue virus 16 isolate IND2014/01 segment 10 NS3 gene, complete cds	Bluetongue virus 16	1166	1166	97%	0.0	98.23%	822	KX302643.1
Bluetongue virus 16 isolate ISR2001/18 segment 10, complete sequence	Bluetongue virus 16	1130	1130	97%	0.0	97.05%	822	KP821956.1
Bluetongue virus 16 isolate K31-08/ABT/HSR segment 10, complete sequence	Bluetongue virus 16	1175	1175	97%	0.0	98.53%	822	KF664112.1
Bluetongue virus 16 isolate PDP2/13/Ind segment 10, complete sequence	Bluetongue virus 16	1165	1165	97%	0.0	98.08%	822	MH395149.1
Bluetongue virus 16 isolate RSArrrr/16* segment 10, complete sequence	Bluetongue virus 16	1130	1130	97%	0.0	97.05%	822	JX129386.1
Bluetongue virus 16 segment 10, complete sequence	Bluetongue virus 16	1197	1197	97%	0.0	99.26%	823	JQ924829.1
Bluetongue virus 16 strain BTV-16 85395/2004 NS3 gene, complete cds	Bluetongue virus 16	1130	1130	97%	0.0	97.05%	824	MH990426.1
Bluetongue virus 2 isolate BTV-2/IND1993/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 2	1134	1134	97%	0.0	97.20%	822	KP696591.1
Bluetongue virus 2 isolate BTV-2/IND2000 nonstructural protein 3 (NS3) gene, complete cds	Bluetongue virus 2	1126	1126	95%	0.0	97.59%	762	KX650180.1
Bluetongue virus 2 isolate BTV02IND1993 segment 10, complete sequence	Bluetongue virus 2	1138	1138	95%	0.0	97.16%	811	KP339163.1
Bluetongue virus 2 isolate BTV02IND2010-KRM08 segment 10, complete sequence	Bluetongue virus 2	1134	1134	96%	0.0	97.19%	815	KP339173.1
Bluetongue virus 2 isolate IND1982/01 segment 10, complete sequence	Bluetongue virus 2	1134	1134	97%	0.0	97.20%	822	JQ713563.1
Bluetongue virus 23 isolate 5268/5 segment 10, complete sequence	Bluetongue virus 23	1139	1139	97%	0.0	97.35%	822	JX272408.1
Bluetongue virus 23 strain 23/Labstr/ZAF/2020/Howell_DVTD_ref 23 isolate Ref 23 segment 10, complete sequence	Bluetongue virus 23	1139	1139	97%	0.0	97.35%	822	MT090664.1
Bluetongue virus 4 isolate WGV103/ABT/HSR segment 10, complete sequence	Bluetongue virus 4	1211	1211	97%	0.0	99.71%	822	KF560426.1
Bluetongue virus 9 isolate BBF segment 10, complete sequence	Bluetongue virus 9	1160	1160	97%	0.0	97.94%	822	JF443165.1
Bluetongue virus 9 isolate BTV-9/IND2004/02 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 9	1175	1175	97%	0.0	98.53%	822	KP696621.1
Bluetongue virus 9 isolate BTV-9/IND2005/03 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 9	1175	1175	97%	0.0	98.53%	822	KP696660.1
Bluetongue virus 9 isolate BTV09IND2003-M10 segment 10, complete sequence	Bluetongue virus 9	1168	1168	95%	0.0	98.66%	812	KP339183.1
Bluetongue virus 9 isolate BTV09IND2003-M11 segment 10, complete sequence	Bluetongue virus 9	1169	1169	96%	0.0	98.52%	812	KP339193.1
Bluetongue virus isolate AVK/94/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1131	1131	95%	0.0	97.74%	690	MN537911.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus isolate BTV1A nonstructural protein NS3/NS3A gene, complete cds	Bluetongue virus	1126	1126	95%	0.0	97.59%	690	EU131023.1
Bluetongue virus isolate CHN/03/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1140	1140	95%	0.0	98.04%	690	MN537912.1
Bluetongue virus isolate CUN/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1144	1144	95%	0.0	98.19%	690	MN537945.1
Bluetongue virus isolate DLP36/12/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1149	1149	95%	0.0	98.34%	690	MN537914.1
Bluetongue virus isolate EMBOSS_001 segment 10, complete sequence	Bluetongue virus	1143	1143	97%	0.0	97.49%	822	KX424875.1
Bluetongue virus isolate G10/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1144	1144	95%	0.0	98.19%	690	MN537947.1
Bluetongue virus isolate G4/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1144	1144	95%	0.0	98.19%	690	MN537946.1
Bluetongue virus isolate G52/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1153	1153	95%	0.0	98.49%	690	MN537948.1
Bluetongue virus isolate G53/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1144	1144	95%	0.0	98.19%	690	MN537949.1
Bluetongue virus isolate G58/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1144	1144	95%	0.0	98.19%	690	MN537950.1
Bluetongue virus isolate HIS/85/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1140	1140	95%	0.0	98.04%	690	MN537916.1
Bluetongue virus isolate Ind-R1-2007 NS3 gene, complete cds	Bluetongue virus	1168	1168	95%	0.0	98.66%	807	JX003696.1
Bluetongue virus isolate IND1992/01 segment 10, complete sequence	Bluetongue virus	1157	1157	97%	0.0	97.94%	822	JQ282777.1
Bluetongue virus isolate IND2015/15-22 NS3 protein and NS5 protein genes, complete cds	Bluetongue virus	1130	1130	97%	0.0	97.05%	816	MK507962.1
Bluetongue virus isolate IND2016/118 segment 10, complete sequence	Bluetongue virus	1134	1134	97%	0.0	97.20%	821	MF615246.1
Bluetongue virus isolate K1/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1158	1158	95%	0.0	98.64%	690	MN537952.1
Bluetongue virus isolate K23/08 segment 10, complete sequence	Bluetongue virus	1193	1193	97%	0.0	99.12%	822	JX399157.1
Bluetongue virus isolate K31/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1158	1158	95%	0.0	98.64%	690	MN537940.1
Bluetongue virus isolate K34/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1158	1158	95%	0.0	98.64%	690	MN537953.1
Bluetongue virus isolate KAR16/06/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1171	1171	95%	0.0	99.10%	690	MN537937.1
Bluetongue virus isolate KDP15/07/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1171	1171	95%	0.0	99.10%	690	MN537918.1
Bluetongue virus isolate M10/03/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1158	1158	95%	0.0	98.64%	690	MN537933.1
Bluetongue virus isolate M10/MBN/2005 NS3 (S10) gene, complete cds	Bluetongue virus	1158	1158	95%	0.0	98.64%	690	JQ424788.1
Bluetongue virus isolate M11/MBN/2005 NS3 (S10) gene, complete cds	Bluetongue virus	1150	1150	95%	0.0	98.34%	690	JQ424789.1
Bluetongue virus isolate MBN/02/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1158	1158	95%	0.0	98.64%	690	MN537934.1
Bluetongue virus isolate NLG3/07/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1176	1176	95%	0.0	99.25%	690	MN537926.1
Bluetongue virus isolate NRT37/07/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1171	1171	95%	0.0	99.10%	690	MN537927.1
Bluetongue virus isolate ONG5/06/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1153	1153	95%	0.0	98.49%	690	MN537935.1
Bluetongue virus isolate PTG13/11/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1171	1171	95%	0.0	99.10%	690	MN537928.1
Bluetongue virus isolate TN2/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1162	1162	95%	0.0	98.80%	690	MN537955.1
Bluetongue virus isolate VJW/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1149	1149	95%	0.0	98.34%	690	MN537939.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus NS3 gene, complete cds	Bluetongue virus	1131	1131	97%	0.0	97.05%	690	JQ080436.1
Bluetongue virus serotype 16 segment S10, complete sequence	Bluetongue virus	1130	1130	97%	0.0	97.05%	822	AY775164.1
Bluetongue virus strain 243277 segment S10, complete sequence	Bluetongue virus	1130	1130	97%	0.0	97.05%	777	AY775163.1
Bluetongue virus strain IND1994/01 segment 10, complete sequence	Bluetongue virus	1139	1139	97%	0.0	97.35%	822	KP268783.1
Bluetongue virus strain IND2014/10NLG NS3 protein (NS3) gene, complete cds	Bluetongue virus	1152	1152	97%	0.0	97.79%	814	MK516731.1
Bluetongue virus strain IND2015/14A NS3 protein (NS3) gene, complete cds	Bluetongue virus	1139	1139	97%	0.0	97.35%	815	MK516733.1
Bluetongue virus strain IND2015/228 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1152	1152	97%	0.0	97.79%	817	MK516714.1
Bluetongue virus strain IND2015/34 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1128	1128	96%	0.0	97.05%	814	MK516715.1
Bluetongue virus strain IND2015/40 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1184	1184	97%	0.0	98.82%	820	MK516734.1
Bluetongue virus strain IND2015/404 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1152	1152	97%	0.0	97.79%	818	MK516693.1
Bluetongue virus strain IND2015/408 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1152	1152	97%	0.0	97.79%	819	MK516695.1
Bluetongue virus strain IND2015/470 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1125	1125	97%	0.0	96.90%	821	MK516696.1
Bluetongue virus strain IND2015/471 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1152	1152	97%	0.0	97.79%	822	MK516697.1
Bluetongue virus strain IND2015/473 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1125	1125	97%	0.0	96.90%	815	MK516699.1
Bluetongue virus strain IND2015/475 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1157	1157	97%	0.0	97.94%	818	MK516701.1
Bluetongue virus strain IND2015/478 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1157	1157	97%	0.0	97.94%	822	MK516704.1
Bluetongue virus strain IND2015/486 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1152	1152	97%	0.0	97.79%	817	MK516708.1
Bluetongue virus strain IND2015/491 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1150	1150	95%	0.0	98.06%	815	MK516690.1
Bluetongue virus strain IND2015/492 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1157	1157	97%	0.0	97.94%	815	MK516691.1
Bluetongue virus strain IND2015/494 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1130	1130	97%	0.0	97.05%	821	MK516692.1
Bluetongue virus strain IND2015/534 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1157	1157	97%	0.0	97.94%	812	MK516712.1
Bluetongue virus strain IND2015/547 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1152	1152	97%	0.0	97.79%	819	MK516713.1
Bluetongue virus strain IND2015/6PK NS3 protein (NS3) gene, complete cds	Bluetongue virus	1152	1152	97%	0.0	97.79%	816	MK516730.1
Bluetongue virus strain IND2015/7A NS3 protein (NS3) gene, complete cds	Bluetongue virus	1125	1125	97%	0.0	96.90%	822	MK516732.1
Bluetongue virus strain IND2015/K13 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1188	1188	97%	0.0	98.97%	820	MK516728.1
Bluetongue virus strain IND2015/K14 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1125	1125	97%	0.0	96.90%	819	MK516729.1
Bluetongue virus strain IND2015/K3 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1134	1134	97%	0.0	97.20%	816	MK516725.1
Bluetongue virus strain IND2015/K4 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1152	1152	97%	0.0	97.79%	817	MK516726.1
Bluetongue virus strain IND2015/K9 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1134	1134	97%	0.0	97.20%	819	MK516727.1
Bluetongue virus strain IND2015/V NS3 protein (NS3) gene, complete cds	Bluetongue virus	1130	1130	97%	0.0	97.05%	817	MK516724.1
Bluetongue virus strain IND2015/V2 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1152	1152	97%	0.0	97.79%	816	MK516720.1
Bluetongue virus strain prototype 600577 NS3 protein (S10) gene, complete cds	Bluetongue virus	1127	1127	97%	0.0	96.90%	822	FJ713328.1

APPENDIX -5

BLASTn analysis of BTV NS3 genomic segment 10 consensus sequence indicating its identity with nucleotide sequences of different BTV serotypes.



COVID-19 Information

Public health information (CDC)

Research information (NIH)

SARS-CoV-2 data (NCBI)

Prevention and treatment information (HHS)

Español

BLAST® » **blastn suite** » results for RID-0PHMEE3P013

Job Title	BTV-NS3 ...
RID	0PHMEE3P013 Search expires on 02-16 13:22 pm
Program	BLASTN
Database	nt
Query ID	lcl|Query_8471
Description	BTV-NS3 ...
Molecule type	dna
Query Length	789

Descriptions

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus 4 isolate WGV103/ABT/HSR segment 10, complete sequence	Bluetongue virus 4	1301	1301	92%	0.0	99.72%	822	KF560426.1
Bluetongue virus 1 isolate NRT37/ABT/HSR segment 10, complete sequence	Bluetongue virus 1	1287	1287	92%	0.0	99.31%	822	KF664132.1
Bluetongue virus 16 isolate IND2010/cattle/16 segment 10, complete sequence	Bluetongue virus 16	1283	1283	92%	0.0	99.17%	822	JX007931.1
Bluetongue virus isolate K23/08 segment 10, complete sequence	Bluetongue virus	1283	1283	92%	0.0	99.17%	822	JX399157.1
Bluetongue virus 16 segment 10, complete sequence	Bluetongue virus 16	1283	1283	92%	0.0	99.17%	823	JQ924829.1
Bluetongue virus strain IND2015/K13 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1278	1278	92%	0.0	99.04%	820	MK516728.1
Bluetongue virus 1 isolate BTV01IND2010-VC12 segment 10, complete sequence	Bluetongue virus 1	1278	1278	91%	0.0	99.17%	817	KP339153.1
Bluetongue virus strain IND2015/40 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1274	1274	92%	0.0	98.90%	820	MK516734.1
Bluetongue virus 16 isolate BTV16IND2010-AP06 segment 10, complete sequence	Bluetongue virus 16	1260	1260	92%	0.0	98.48%	814	KP339213.1
Bluetongue virus 9 isolate BTV-9/IND2005/03 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 9	1260	1260	92%	0.0	98.48%	822	KP696660.1
Bluetongue virus 9 isolate BTV-9/IND2004/02 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 9	1260	1260	92%	0.0	98.48%	822	KP696621.1
Bluetongue virus 16 isolate K31-08/ABT/HSR segment 10, complete sequence	Bluetongue virus 16	1260	1260	92%	0.0	98.48%	822	KF664112.1
Bluetongue virus 16 isolate G53/ABT/HSR segment 10, complete sequence	Bluetongue virus 16	1256	1256	92%	0.0	98.35%	822	KF664142.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus 9 isolate BTV09IND2003-M11 segment 10, complete sequence	Bluetongue virus 9	1255	1255	91%	0.0	98.48%	812	KP339193.1
Bluetongue virus 9 isolate BTV09IND2003-M10 segment 10, complete sequence	Bluetongue virus 9	1253	1253	91%	0.0	98.48%	812	KP339183.1
Bluetongue virus 16 isolate BTV16IND2010-AP04 segment 10, complete sequence	Bluetongue virus 16	1253	1253	91%	0.0	98.48%	801	KP339203.1
Bluetongue virus 16 isolate IND2014/01 segment 10 NS3 gene, complete cds	Bluetongue virus 16	1251	1251	92%	0.0	98.21%	822	KX302643.1
Bluetongue virus 1 isolate BTV-1/IND1988/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1251	1251	92%	0.0	98.21%	822	KP696525.1
Bluetongue virus isolate Ind-R1-2007 NS3 gene, complete cds	Bluetongue virus	1251	1251	91%	0.0	98.47%	807	JX003696.1
Bluetongue virus 16 isolate PDP2/13/Ind segment 10, complete sequence	Bluetongue virus 16	1251	1251	92%	0.0	98.07%	822	MH395149.1
Bluetongue virus 16 isolate BTV16IND2010-VC07 segment 10, complete sequence	Bluetongue virus 16	1249	1249	91%	0.0	98.34%	801	KP339233.1
Bluetongue virus 9 isolate BBF segment 10, complete sequence	Bluetongue virus 9	1246	1246	92%	0.0	97.93%	822	JF443165.1
Bluetongue virus 16 isolate BTV16IND2011-NR82 segment 10, complete sequence	Bluetongue virus 16	1245	1245	91%	0.0	98.33%	799	KP339223.1
Bluetongue virus strain IND2015/534 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1242	1242	92%	0.0	97.93%	812	MK516712.1
Bluetongue virus strain IND2015/478 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1242	1242	92%	0.0	97.93%	822	MK516704.1
Bluetongue virus strain IND2015/475 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1242	1242	92%	0.0	97.93%	818	MK516701.1
Bluetongue virus strain IND2015/492 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1242	1242	92%	0.0	97.93%	815	MK516691.1
Bluetongue virus 1 isolate BTV-1/IND1985/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1242	1242	92%	0.0	97.93%	822	KP696516.1
Bluetongue virus isolate IND1992/01 segment 10, complete sequence	Bluetongue virus	1242	1242	92%	0.0	97.93%	822	JQ282777.1
Bluetongue virus strain IND2014/10NLG NS3 protein (NS3) gene, complete cds	Bluetongue virus	1238	1238	92%	0.0	97.80%	814	MK516731.1
Bluetongue virus strain IND2015/6PK NS3 protein (NS3) gene, complete cds	Bluetongue virus	1238	1238	92%	0.0	97.80%	816	MK516730.1
Bluetongue virus strain IND2015/K4 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1238	1238	92%	0.0	97.80%	817	MK516726.1
Bluetongue virus strain IND2015/V2 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1238	1238	92%	0.0	97.80%	816	MK516720.1
Bluetongue virus strain IND2015/228 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1238	1238	92%	0.0	97.80%	817	MK516714.1
Bluetongue virus strain IND2015/547 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1238	1238	92%	0.0	97.80%	819	MK516713.1
Bluetongue virus strain IND2015/486 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1238	1238	92%	0.0	97.80%	817	MK516708.1
Bluetongue virus strain IND2015/471 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1238	1238	92%	0.0	97.80%	822	MK516697.1
Bluetongue virus strain IND2015/408 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1238	1238	92%	0.0	97.80%	819	MK516695.1
Bluetongue virus strain IND2015/404 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1238	1238	92%	0.0	97.80%	818	MK516693.1
Bluetongue virus strain IND2015/491 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1236	1236	91%	0.0	97.79%	815	MK516690.1
Bluetongue virus isolate EMBOSS_001 segment 10, complete sequence	Bluetongue virus	1229	1229	92%	0.0	97.52%	822	KX424875.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus 1 isolate BTV-1/IND2003/05 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1229	1229	92%	0.0	97.52%	822	KP696571.1
Bluetongue virus 1 isolate BTV-1/IND2003/04 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1229	1229	92%	0.0	97.52%	822	KP696561.1
Bluetongue virus 12 isolate INDAPADBNMO1/11 segment 10, complete sequence	Bluetongue virus 12	1228	1228	91%	0.0	97.65%	812	KC662621.1
Bluetongue virus strain IND2015/14A NS3 protein (NS3) gene, complete cds	Bluetongue virus	1224	1224	92%	0.0	97.38%	815	MK516733.1
Bluetongue virus strain IND1994/01 segment 10, complete sequence	Bluetongue virus	1224	1224	92%	0.0	97.38%	822	KP268783.1
Bluetongue virus 23 isolate 5268/5 segment 10, complete sequence	Bluetongue virus 23	1224	1224	92%	0.0	97.38%	822	JX272408.1
Bluetongue virus 23 strain 23/Labstr/ZAF/2020/Howell_DVTD_ref 23 isolate Ref 23 segment 10, complete sequence	Bluetongue virus 23	1224	1224	92%	0.0	97.38%	822	MT090664.1
Bluetongue virus 1 isolate BTV-1/IND2001/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1223	1223	92%	0.0	97.25%	822	KP696551.1
Bluetongue virus isolate NLG3/07/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1223	1223	87%	0.0	99.28%	690	MN537926.1
Bluetongue virus 2 isolate BTV02IND1993 segment 10, complete sequence	Bluetongue virus 2	1221	1221	91%	0.0	97.09%	811	KP339163.1
Bluetongue virus strain IND2015/K9 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1220	1220	92%	0.0	97.25%	819	MK516727.1
Bluetongue virus strain IND2015/K3 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1220	1220	92%	0.0	97.25%	816	MK516725.1
Bluetongue virus isolate IND2016/118 segment 10, complete sequence	Bluetongue virus	1220	1220	92%	0.0	97.25%	821	MF615246.1
Bluetongue virus 2 isolate BTV02IND2010-KRM08 segment 10, complete sequence	Bluetongue virus 2	1220	1220	91%	0.0	97.24%	815	KP339173.1
Bluetongue virus 2 isolate BTV-2/IND1993/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 2	1220	1220	92%	0.0	97.25%	822	KP696591.1
Bluetongue virus isolate KAR16/06/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1218	1218	87%	0.0	99.13%	690	MN537937.1
Bluetongue virus isolate PTG13/11/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1218	1218	87%	0.0	99.13%	690	MN537928.1
Bluetongue virus isolate NRT37/07/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1218	1218	87%	0.0	99.13%	690	MN537927.1
Bluetongue virus isolate KDP15/07/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1218	1218	87%	0.0	99.13%	690	MN537918.1
Bluetongue virus 2 isolate IND1982/01 segment 10, complete sequence	Bluetongue virus 2	1217	1217	92%	0.0	97.11%	822	JQ713563.1
Bluetongue virus strain IND2015/V NS3 protein (NS3) gene, complete cds	Bluetongue virus	1215	1215	92%	0.0	97.11%	817	MK516724.1
Bluetongue virus strain IND2015/494 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1215	1215	92%	0.0	97.11%	821	MK516692.1
Bluetongue virus isolate IND2015/15-22 NS3 protein and NS5 protein genes, complete cds	Bluetongue virus	1215	1215	92%	0.0	97.11%	816	MK507962.1
Bluetongue virus 1 isolate BTV01IND2010-KRM07 segment 10, complete sequence	Bluetongue virus 1	1214	1214	91%	0.0	97.23%	817	KP339143.1
Bluetongue virus strain IND2015/34 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1214	1214	91%	0.0	97.10%	814	MK516715.1
Bluetongue virus strain IND2015/K14 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1211	1211	92%	0.0	96.97%	819	MK516729.1
Bluetongue virus strain IND2015/473 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1211	1211	92%	0.0	96.97%	815	MK516699.1
Bluetongue virus strain IND2015/470 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1211	1211	92%	0.0	96.97%	821	MK516696.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus 16 strain BTV-16 85395/2004 NS3 gene, complete cds	Bluetongue virus 16	1211	1211	92%	0.0	96.97%	824	MH990426.1
Bluetongue virus 16 isolate ISR2001/18 segment 10, complete sequence	Bluetongue virus 16	1211	1211	92%	0.0	96.97%	822	KP821956.1
Bluetongue virus 16 isolate RSArrrr/16* segment 10, complete sequence	Bluetongue virus 16	1211	1211	92%	0.0	96.97%	822	JX129386.1
Bluetongue virus serotype 16 segment S10, complete sequence	Bluetongue virus	1211	1211	92%	0.0	96.97%	822	AY775164.1
Bluetongue virus strain 243277 segment S10, complete sequence	Bluetongue virus	1211	1211	92%	0.0	96.97%	777	AY775163.1
Bluetongue virus isolate TN2/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1209	1209	87%	0.0	98.84%	690	MN537955.1
Bluetongue virus strain prototype 600577 NS3 protein (S10) gene, complete cds	Bluetongue virus	1208	1208	92%	0.0	96.83%	822	FJ713328.1
Bluetongue virus strain IND2015/7A NS3 protein (NS3) gene, complete cds	Bluetongue virus	1206	1206	92%	0.0	96.83%	822	MK516732.1
Bluetongue virus strain IND2015/477 NS3 protein (NS3) gene, complete cds	Bluetongue virus	1206	1206	92%	0.0	96.83%	822	MK516703.1
Bluetongue virus 16 strain BTV-16 87892TR/2006 NS3 gene, complete cds	Bluetongue virus 16	1206	1206	92%	0.0	96.83%	822	MH990396.1
Bluetongue virus 16 strain ITL2002 segment 10, complete sequence	Bluetongue virus 16	1206	1206	92%	0.0	96.83%	822	KF387530.1
Bluetongue virus strain 8054/02 segment S10, complete sequence	Bluetongue virus	1206	1206	92%	0.0	96.83%	814	AY775162.1
Bluetongue virus 2 isolate BTV-2/IND2000 nonstructural protein 3 (NS3) gene, complete cds	Bluetongue virus 2	1205	1205	90%	0.0	97.48%	762	KX650180.1
Bluetongue virus 1 isolate BTV-1/IND1999/01 segment 10 NS3 (NS3) gene, complete cds	Bluetongue virus 1	1205	1205	90%	0.0	97.48%	788	KP696543.1
Bluetongue virus strain BN96/16 segment 10, complete sequence	Bluetongue virus	1202	1202	92%	0.0	96.69%	822	JN671915.1
Bluetongue virus isolate M10/MBN/2005 NS3 (S10) gene, complete cds	Bluetongue virus	1200	1200	87%	0.0	98.55%	690	JQ424788.1
Bluetongue virus isolate K34/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1200	1200	87%	0.0	98.55%	690	MN537953.1
Bluetongue virus isolate K1/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1200	1200	87%	0.0	98.55%	690	MN537952.1
Bluetongue virus isolate K31/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1200	1200	87%	0.0	98.55%	690	MN537940.1
Bluetongue virus isolate MBN/02/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1200	1200	87%	0.0	98.55%	690	MN537934.1
Bluetongue virus isolate M10/03/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1200	1200	87%	0.0	98.55%	690	MN537933.1
Bluetongue virus isolate G52/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1196	1196	87%	0.0	98.41%	690	MN537948.1
Bluetongue virus isolate ONG5/06/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1196	1196	87%	0.0	98.41%	690	MN537935.1
Bluetongue virus isolate VJW/08/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1191	1191	87%	0.0	98.26%	690	MN537939.1
Bluetongue virus isolate DLP36/12/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1191	1191	87%	0.0	98.26%	690	MN537914.1
Bluetongue virus isolate G58/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1187	1187	87%	0.0	98.12%	690	MN537950.1
Bluetongue virus isolate G53/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1187	1187	87%	0.0	98.12%	690	MN537949.1
Bluetongue virus isolate G10/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1187	1187	87%	0.0	98.12%	690	MN537947.1
Bluetongue virus isolate G4/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1187	1187	87%	0.0	98.12%	690	MN537946.1
Bluetongue virus isolate CUN/10/Ind NS3/NS3A gene, complete cds	Bluetongue virus	1187	1187	87%	0.0	98.12%	690	MN537945.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bluetongue virus isolate M11/MBN/2005 NS3 (S10) gene, complete cds	Bluetongue virus	1184	1184	87%	0.0	97.97%	690	JQ424789.1

APPENDIX -6

Protein Basic Local Alignment Search Tool (BLASTp) analysis of BTV NS3 forward genomic segment 10 protein sequence indicating its identity with protein sequences of different BTV serotypes.

BLAST® » **blastp suite** » results for RID-0PK8CDDT01R

Job Title	Protein Sequence ...
RID	0PK8CDDT01R Search expires on 02-16 13:49 pm
Program	BLASTP
Database	nr
Query ID	lcl|Query_89840
Description	lcl|ORF1:14:703 unnamed protein product...
Molecule type	amino acid
Query Length	229

Descriptions

Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
ns3/ns3A protein [Bluetongue virus 16]	Bluetongue virus 16	NA	45029	474	474	100%	1e-168	100.00%	229	AFO37735.1
nonstructural protein NS3/NS3A [Bluetongue virus 3]	Bluetongue virus 3	NA	36423	473	473	100%	2e-168	99.56%	229	AAD34450.1
NS3 [Bluetongue virus 23]	Bluetongue virus 23	NA	45031	473	473	100%	2e-168	99.13%	229	AGJ83630.1
non-structural protein [Bluetongue virus 21]	Bluetongue virus 21	NA	45030	473	473	100%	3e-168	99.13%	229	BAH03440.1
NS3 protein [Bluetongue virus]	Bluetongue virus	NA	40051	473	473	100%	3e-168	99.13%	229	QED41478.1
NS3 [Bluetongue virus 9]	Bluetongue virus 9	NA	45032	473	473	100%	3e-168	99.13%	229	AEZ03684.1
NS3 [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	472	472	100%	4e-168	99.56%	229	AGW27490.1
NS3 [Bluetongue virus 18]	Bluetongue virus 18	NA	197781	472	472	100%	4e-168	99.13%	229	AAM70465.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	472	472	100%	5e-168	99.56%	229	AGL45583.1
NS3 protein [Bluetongue virus]	Bluetongue virus	NA	40051	472	472	100%	5e-168	99.56%	229	QED41486.1
NS3 [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	472	472	100%	5e-168	99.13%	229	ALI51183.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	472	472	100%	5e-168	99.13%	229	ASW41737.1
nonstructural protein NS3/NS3A [Bluetongue virus 9]	Bluetongue virus 9	NA	45032	472	472	100%	6e-168	99.13%	229	AAS02101.1
NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	472	472	100%	6e-168	99.13%	229	QPB69961.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	472	472	100%	6e-168	99.13%	229	AAX13267.1
NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	472	472	100%	6e-168	99.13%	229	QPB69957.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	471	471	100%	7e-168	98.69%	229	AYV65199.1
NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	471	471	100%	7e-168	99.13%	229	QPB69960.1
NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	471	471	100%	7e-168	99.13%	229	QPB69940.1
NS3 [Bluetongue virus 21]	Bluetongue virus 21	NA	45030	471	471	100%	8e-168	99.13%	229	ALI51253.1
NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	471	471	100%	8e-168	99.13%	229	QPB69966.1
nonstructural protein NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	471	471	100%	9e-168	99.13%	229	ABX10965.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	471	471	100%	1e-167	99.13%	229	AYV65210.1
nonstructural protein NS3/NS3A [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	471	471	100%	1e-167	98.69%	229	AAD34448.1
NS3 [Bluetongue virus 16]	Bluetongue virus 16	NA	45029	471	471	100%	1e-167	99.13%	229	AYA95107.1

Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	471	471	100%	1e-167	99.13%	229	QPB69968.1
NS3 protein [Bluetongue virus]	Bluetongue virus	NA	40051	471	471	100%	1e-167	99.13%	229	QED41465.1
nonstructural protein NS3/NS3A [Bluetongue virus 2]	Bluetongue virus 2	NA	35328	471	471	100%	1e-167	99.13%	229	AAD34449.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	471	471	100%	1e-167	99.13%	229	AEP83829.1
NS3 [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	471	471	100%	1e-167	99.13%	229	AKP24119.1
NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	471	471	100%	2e-167	99.56%	229	QPB69959.1
NS3 [Bluetongue virus 2]	Bluetongue virus 2	NA	35328	471	471	100%	2e-167	98.69%	229	AFI73123.1
nonstructural protein NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	470	470	100%	2e-167	98.69%	229	ABU43081.1
NS3 [Bluetongue virus 2]	Bluetongue virus 2	NA	35328	470	470	100%	2e-167	99.13%	229	AFK91790.1
NS3 [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	470	470	100%	3e-167	99.13%	229	AGW27478.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	470	470	100%	3e-167	98.69%	229	ASW41731.1
NS3/3A protein [Bluetongue virus]	Bluetongue virus	NA	40051	470	470	99%	3e-167	99.12%	228	QHJ68772.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	470	470	100%	3e-167	98.69%	229	ASW41789.1
non-structural proteins-3 and -3A [Bluetongue virus 3]	Bluetongue virus 3	NA	36423	470	470	100%	3e-167	98.69%	229	QOL11086.1
NS3/3A protein [Bluetongue virus]	Bluetongue virus	NA	40051	470	470	100%	3e-167	98.69%	229	QHJ68770.1
nonstructural protein NS3/3A [Bluetongue virus 21]	Bluetongue virus 21	NA	45030	470	470	100%	4e-167	98.69%	229	AAM95400.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	470	470	100%	4e-167	98.69%	229	AQZ36544.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	469	469	100%	4e-167	98.69%	229	ASW41747.1
NS3 protein [Bluetongue virus]	Bluetongue virus	NA	40051	469	469	100%	4e-167	98.25%	229	AWL83777.1
non-structural proteins-3 and -3A [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	469	469	100%	5e-167	98.69%	229	QOL11053.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	469	469	100%	7e-167	98.69%	229	ASW41671.1
nonstructural protein NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	469	469	100%	7e-167	98.69%	229	ABU43095.1
unknown [Bluetongue virus]	Bluetongue virus	NA	40051	469	469	100%	7e-167	98.69%	229	AFG72995.1
nonstructural protein NS3/NS3A [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	469	469	100%	7e-167	98.69%	229	AAS02107.1
non-structural proteins-3 and -3A [Bluetongue virus 16]	Bluetongue virus 16	NA	45029	469	469	100%	8e-167	98.69%	229	QPH37486.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	469	469	100%	9e-167	98.69%	229	ASW41761.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	469	469	100%	1e-166	98.69%	229	AAX13265.1
NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	1e-166	98.69%	229	QPB69935.1
NS3 [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	468	468	100%	1e-166	98.69%	229	AIT40484.1
non-structural proteins-3 and -3A [Bluetongue virus 16]	Bluetongue virus 16	NA	45029	468	468	100%	1e-166	98.69%	229	QPA19171.1
NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	1e-166	98.69%	229	QPB69949.1

Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
NS3 protein [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	2e-166	97.82%	234	AUZ97368.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	2e-166	98.25%	229	ASW41767.1
NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	2e-166	98.69%	229	QPB69934.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	2e-166	98.25%	229	ASW41735.1
nonstructural protein NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	2e-166	98.25%	229	ABU43090.1
NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	2e-166	98.69%	229	QPB69951.1
nonstructural protein 3 [Bluetongue virus 5]	Bluetongue virus 5	NA	248909	468	468	100%	2e-166	98.25%	229	AHX56228.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	2e-166	98.69%	229	ASW41695.1
nonstructural protein 3 [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	2e-166	98.69%	229	AAS72881.1
NS3 [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	468	468	100%	2e-166	98.25%	229	AKP24149.1
RecName: Full=Non-structural protein P8; AltName: Full=Non-structural protein NS3; Contains: RecName: Full=Non-structural protein NS3A [Bluetongue virus (serotype 1 / isolate Australia)]	Bluetongue virus (serotype 1 / isolate Australia)	NA	10904	468	468	100%	3e-166	98.25%	229	P13841.1
NS3/3A [Bluetongue virus 4]	Bluetongue virus 4	NA	94967	468	468	100%	3e-166	98.25%	229	AKV61722.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	3e-166	98.25%	229	ALW82964.1
nonstructural protein NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	3e-166	98.25%	229	ABU43093.1
nonstructural protein NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	468	468	100%	3e-166	98.25%	229	ABU43088.1
non-structural proteins-3 and -3A [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	468	468	100%	3e-166	98.25%	229	QPA19235.1
nonstructural protein NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	467	467	100%	4e-166	98.25%	229	ABU43087.1
NS3 [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	467	467	100%	4e-166	97.82%	229	AAM70462.1
non-structural protein NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	467	467	100%	4e-166	97.82%	229	AAC42280.1
non-structural proteins-3 and -3A [Bluetongue virus 7]	Bluetongue virus 7	NA	248911	467	467	100%	5e-166	98.25%	229	QPA19072.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	467	467	100%	5e-166	97.82%	229	ASW41703.1
NS3 [Bluetongue virus 2]	Bluetongue virus 2	NA	35328	467	467	100%	5e-166	98.25%	229	AFK32290.1
non-structural proteins-3 and -3A [Bluetongue virus 16]	Bluetongue virus 16	NA	45029	467	467	100%	5e-166	98.25%	229	QPH37519.1
nonstructural protein NS3/NS3A [Bluetongue virus 15]	Bluetongue virus 15	NA	35331	467	467	100%	5e-166	97.82%	229	AAD34453.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	467	467	100%	6e-166	97.38%	229	AEY77821.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	467	467	100%	6e-166	98.25%	229	ASW41713.1
NS3 [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	466	466	100%	7e-166	97.82%	229	AAM70463.1
non-structural proteins-3 and -3A [Bluetongue virus 4]	Bluetongue virus 4	NA	94967	466	466	100%	8e-166	97.38%	229	QPH37530.1

Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
NS3 [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	466	466	100%	8e-166	98.25%	229	AIT40452.1
nonstructural protein NS3/NS3A [Bluetongue virus 4]	Bluetongue virus 4	NA	94967	466	466	100%	1e-165	97.38%	229	AAU21211.1
NS3 [Bluetongue virus 2]	Bluetongue virus 2	NA	35328	466	466	100%	1e-165	97.82%	229	AEO19833.1
NS3 protein [Bluetongue virus]	Bluetongue virus	NA	40051	466	466	100%	1e-165	97.38%	229	ACX69788.1
nonstructural protein NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	466	466	100%	1e-165	97.38%	229	ABU43096.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	466	466	100%	1e-165	97.38%	229	AYA21869.1
NS3/NS3a [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	466	466	100%	1e-165	97.38%	229	AIF79560.1
nonstructural protein NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	466	466	100%	1e-165	98.25%	229	ABU43080.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	466	466	100%	1e-165	97.38%	229	AJG44866.1
nonstructual protein [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	466	466	100%	1e-165	97.38%	229	ABU48536.1
nonstructural protein NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	466	466	100%	1e-165	97.38%	234	QTZ97134.1
NS3 protein [Bluetongue virus 12]	Bluetongue virus 12	NA	94966	466	466	100%	1e-165	97.38%	229	QKW90219.1
nonstructural protein NS3/NS3A [Bluetongue virus]	Bluetongue virus	NA	40051	466	466	100%	2e-165	97.82%	229	ABU43094.1
nonstructural protein NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	466	466	100%	2e-165	97.38%	235	QTZ97135.1
NS3 [Bluetongue virus 1]	Bluetongue virus 1	NA	35327	466	466	100%	2e-165	97.82%	229	AKP24158.1
NS3 [Bluetongue virus]	Bluetongue virus	NA	40051	466	466	100%	2e-165	97.38%	229	AQR59289.1

»
Graphic Summary

VITA

The author Dr. Dipali Keshaorao Gharat was born on 21 February, 1996 at Sakoli, Dist. Bhandara, Maharashtra. She completed her primary education from Samarth Primary School, Lakhani and passed her Secondary School Certificate (SSC) examination in 2011 from Rani Lakshmibai Kanya Vidyalaya, Lakhani. Then in year 2013 the author passed H.S.C. examination from Nutan Kanya Junior College, Bhandara.

The interest of studying health science attracted author to join the veterinary science. She joined the Nagpur Veterinary College, Nagpur in the academic year 2014-2015 in B.V.Sc & A.H. professional degree course and completed the degree programme successfully in 2019. During the graduation author remained actively engaged in many activities like National Cadet Corps (NCC) and free animal treatment camps organized in and around Nagpur. For further studies, she joined M.V.Sc in Veterinary Microbiology in 2019 at Nagpur Veterinary College, Nagpur.

During post-graduation she participated in national symposium on “Paradigm Transformation of Technological Advancement in Veterinary Science : National Perspective Cum 3rd Alumni Meet held at Akola, Maharashtra, 2019 and won second prize in poster presentation.

Thesis Abstract

a)	Title of thesis	:	**NONSTRUCTURAL GENE BASED MOLECULAR CHARACTERIZATION OF BLUETONGUE VIRUS**
b)	Full Name of Student	:	**GHARAT DIPALI KESHAORAO**
c)	Name & Address of Advisor/ Guide	:	**Dr. P. A. TEMBHURNE** **Assistant Professor,** **Department of Veterinary Microbiology and Animal Biotechnology, T & R Cell,** **Nagpur Veterinary College, Nagpur**
d)	Degree to be awarded	:	**MASTER OF VETERINARY SCIENCE**
e)	Year of award of degree	:	**2022**
f)	Major subject	:	**VETERINARY MICROBIOLOGY**
g)	Total number of pages in the thesis	:	**75**
h)	Number of words in the thesis abstract	:	**300**
i)	Signature of student	:	
j)	Signature, Name & Address of forwarding authority	:	

Associate Dean
Nagpur Veterinary College,
Nagpur

ABSTRACT

Bluetongue (BT) is an infectious arthropod borne viral disease of sheep, domestic and wild ruminants caused by *Bluetongue virus* of genus orbivirus of family *Reovirdae* having 28 serotypes. For BT diagnosis, the sample collection and

dispatched remains critical in identification and confirmation in disease in India owing to lack of infrastructure leads to failure in planning the control of the disease. The study was planned to amplify the NS3 gene by RT-PCR, to standardize the Dried sample spot-PCR and evaluate this gene for the phylogenetic relationship with global BTV virus and also characterized the NS3 encoding protein. In the present study, 10 blood samples from Dhule and Nandurbar District of Maharashtra were analyzed by conventional RT-PCR targeting NS3 gene showed five sample were positive 248bp NS3-RT-PCR and one positive by 726bp NS3-RT-PCR. One positive sample was further genetically characterized by RT-PCR for BTV genes, revealed the expected size of 967bp, 1087bp and 1154bp for NS1, NS2, and VP7 respectively, confirming the BTV infection in outbreak. The blood was also blotted for alternate sample method , all five positive blood sample blotted on the FTA card, Filter paper and NEWS paper , on RT-PCR with diagnostic NS3 primers showed 100% positivity in the all sample. The sequence analysis of 726bp amplicons of NS3 revealed that there were two longest open reading frame, the largest first ORF started from 14bp to 703bp having a CDS of 690bp, encoding 229 amino acid protein of expected size of 25524 dalton (25.52 kDa). The second ORF was started from the 53bp to 703 having CDS of 651bp indicating the NS3a protein. The BLASTt analysis revealed that 100% identitiy with BTV-16 (Access. no. AFO37735) and 98.56% to 97.38 % identity with complete protein sequences of global 100 BTV isolates of different serotypes. The phylogentic analysis of nucleotide and deduced amino acids showed the BTV/NS3/Dhule/MH/India/sheep/2019 is closely placed with KF664132/BTV1/India/Ap/sheep/2008 and JX007931BTV16/India/cattle/2010 in single subclad with forming 3 cluster and 3 subcluster with certain demarcation of eastern, western and south african isolates placed the geographical positioning of the BTV different serotypes based on their geological location irrespective of serotype. The expression and purification the rNS3 in prokaryotic system has revealed a unique band of approximate size of 25.5kDa in SDS-PAGE. The present study concludes that , the outbreak of the Dhule district was caused by Bluetongue virus and NS3 gene RT-PCR (248bp) can be adopted for diagnosis of BTV. The alternative sample

collection dried sample spot on FTA sample card, filter paper and newspaper can be adopted as replacement for the conventional blood sample collection as it showed 100% positivity by diagnostic NS3 RT-PCR. The BTV/NS3/Dhule/MH/India/sheep/2019 strain could be evolved from the genetic reassortment of BTV-16 of cattle origin and BTV-1 of sheep origin based on 100% identity with BTV-16 and its phylogenetic placing owing to trades of animals.

प्रबंध सारांश

अ.	प्रबंधाचे शिर्षक	:	संरचनात्मक नसलेल्या जनकाच्या आधारित निळी जीभ विषाणुचा आण्विक वैशिष्ट्ययीकृत अभ्यास
ब.	विद्यार्थ्याचे पुर्ण नाव	:	घरत दिपाली केशवराव
क.	मार्गदर्शकाचे नाव आणि पत्ता	:	डॉ. प्र. अ. टेम्भूर्णे सहाय्यक प्राध्यापक पशुवैद्यकीय सुक्ष्मजीवशास्त्र व पशुजैवतंत्रज्ञान विभाग, नागपूर पशुवैद्यक महाविद्यालय, नागपूर
ड.	प्रदान करण्यात येणारी पदवी	:	स्नातकोत्तर (एम. व्ही. एस. सी.)
इ.	पदवी प्रदान करण्याचे वर्ष	:	२०२२
फ.	मुख्य विषय	:	पशुवैद्यकीय सुक्ष्मजीवशास्त्र
ग.	प्रबंधातील एकुण पृष्ठे	:	७५
ह.	सारांषातील एकुण शब्द	:	३००
ई.	विद्यार्थ्याची स्वाक्षरी	:	
ज.	अग्रेषित करणाऱ्याची स्वाक्षरी नाव आणि पत्ता	:	

सहयोगी अधिष्ठाता
नागपूर पशुववैद्यक महाविद्यालय, नागपूर

सारांश

निळी जीभ विषाणु हा एक संसर्गजन्य, किटकांमधून होणारा विषाणुजन्य रोग असून तो, मेंढ्याना व इतर पाळीव व जंगली रवंथ करणाऱ्या जनावरांना होतो. रीओविरोडे या कुटुंबातील निळी जीभ विषाणु हा ऑर्बीव्हायरस या वंशामध्ये असुन त्याचे अठ्ठावीस

(२८) रक्तजातीय प्रकार आहेत. निळी जीभ विषाणुच्या निदानासाठी नमुने गोळा करून रोगाची ओळख व पुष्टीकरनासाठी पाठवणे अत्यावश्यक आहे, परंतु पायाभूत सुविधांच्या अभावामुळे रोगाला थांबविण्याचे प्रयत्न अपयशी ठरतात. सदरील अभ्यास एन. एस. ३ जनुकांचे परावर्तन प्रतिलेखन श्रृंखलेद्वारे त्यांचे निदान करण्यासाठी वाढविणे, तसेच शुष्क बिंदु - जनुक परावर्तन प्रतिलेखन श्रृंखला अभिक्रियेद्वारे विश्लेषण, तसेच जगातिल निळी जीभ विषाणूंशी संबंध वंशावळी पद्धतीने अभ्यासने व एन. एस. ३ जनुकाने तयार केलेल्या प्रथिने चे वैशिष्टकृत करून अभ्यासने योजलेले आहे. या अभ्यासात महाराष्ट्रातील धुळे आणि नंदुरबार जिल्ह्यातील दहा (१०) रक्ताच्या नमुन्याचे पारंपारिक जणुक परावर्तन प्रतिलेखन श्रृंखला अभिक्रियेद्वारे एन.एस. ३ जनुक वापरण्यात आले त्यापैकी एका जनुक नमुन्यामध्ये एन.एस.१, एन.एस.२ आणि एन.एस.७ अनुक्रमे ९६७ बी.पी., १०८७. बी. पी. आणि ११५४ बी. पी. अपेक्षित जनुक दिसून आले. त्यामुळे वरील जिल्ह्यातील रोगाच्या उद्रेकात निळी जीभ विषाणुची पुष्टी झाली. पर्यायी नमुना पद्धतीसाठी निळी जीभ रोगाच्या पुष्टीकरनासाठी रक्ताच्या नमुन्याचे एफ. टी. ए. कागदाच्या कपटावर, गाळण्याच्या कागदावर आणि वर्तमान पत्रावर शुष्क ठिपके केले गेले. जनुक परावर्तन प्रतिलेखन श्रृंखला अभिक्रियाने एन.एस.३(२४०) बी. पी. च्या प्रायमरने सर्व नमुन्यांमध्ये १००% सकारात्मकता दाखवली. एन. एस.३ जनुकाच्या (७२६ बी.पी.) अनुक्रमे विश्लेषणातून असे दिसुन आले कि दोन सर्वात लांब ओपन रिडींग फ्रेम असून, पहिली ओ. आर. एफ. १४ बी.पी. ते ७०३ बी.पी. पर्यत असुन त्याला ६९० ची सी. डी. एस. आहे व हे २५५२४ डाल्टन (२५.५१ किलो डाल्टन) च्या अपेक्षित आकाराचे २२९ अमिनो आम्ल कोड करतात. दुसरा ओ.आर. एफ. ५३ बी. पी. ते ७०३ पर्यंत आहे. त्यामुळे एन. एस. ३अ प्रथिने दर्शविनारे ७५१ चे सी. डी. एस. आहे. बी.एल.ए.एस.टी.टी (**BLASTT**) केल्यानंतर असे दिसुन आले कि निळी जिभ विषाणु - १६ रक्तजन्य प्रकार (प्रवेश क्र.ए.एफ ०३७७३५) सोबत १००% आणि जगातील

१०० विविध नीळी जिभ रक्तजातीय प्रकारा सोबत ९८.५६% ते ९८.३८% सारखा आहे. न्यूक्लियोटाइड व अमिनो आम्लच्या वंशावळी विश्लेषनाने नीळी जिभ विषाणु/एन.एस.३/ धुळे /एम. एच./भारत/मेंढी/२०१९ हे के. एफ. ६६४१३२/ नीळी जिभ विषाणु-१/भारत/ए.पी/मेंढी/ २००८ आणि जे.एक्स.००७९३१/नीळी जिभ विषाणु १६/भारत/गाय/२०१० सोबत निगडीत आहेत. तसेच पूर्व, पश्चिम आणि दक्षिण आफ्रिकन विलंगाच्या विशिष्ट सीमांकनासह ३ समुह आणि ३ उपसमुह मध्ये वर्गीकृत झालेले आहेत परंतू ते रक्तजन्य प्रकाराची पर्वा न करता त्यांच्या भौगोलिक स्थानावर आधारित निळी जीभ विषाणुचे समुह ठेवले आहेत. निळी जीभ विषाणु एन्.एस.३ चे जीवाणूमध्ये अभिव्यक्ती व शुद्धीकरण केल्यानंतर त्यांचा रेणूकिय वजन अंदाजे २५.५ किलो डाल्टन एस.डी.एस. पी.ए.जी.ई. मध्ये दिसून आले. सध्याच्या अभ्यासातून असे निष्कर्ष काढण्यात आले कि, धुळे जिल्ह्यातील रोगाचा उद्रेक निळी जीभ विषाणु मुळे झाला, आणि एन.एस.३(२४८ बी. पी.) परावर्तन प्रतिलेखन शृंखला अभिक्रिया निळी जीभ विषाणु च्या निदानासाठी उपयुक्त आहे. एफ.टी.ए कागदाचा कपटा, गाळण्याचा कागद आणि वर्तमान पत्राच्या शाई विरहीत कागदांवरील सुकवलेल्या रक्ताच्या नमुन्यांचा वापर पारंपारिक रक्तांच्या नमुन्याजागी करू शकतो कारण यांनी एन.एस.३ रेणुक परावर्तन प्रतिलेखन श्रृंखला अभिक्रियेमध्ये १००% सकारात्मकता दाखवली आहे. निळी जीभ विषाणु/एन.एस.३/धुळे/एम.एच/भारत/मेंढी/२०१९ हे विषाणु किंबहुना निळी जीभ विषाणु-१६ व निळी जीभ विषाणु-१ यांच्या रेणुकिय अदलाबदला मुळे उत्पत्ती झालेली असावे कारण त्यांच्या वंशावळीतील १००% जवळील ठेव व तसेच जनावरांच्या खरेदी विक्री मुळे हे संभाव्य वाटते.