UNRAVELING THE HOST FACTORS INTERACTING WITH NS3 PROTEIN DURING BLUETONGUE VIRUS REPLICATION

Thesis

Submitted to the
DEEMED UNIVERSITY
ICAR-Indian Veterinary Research Institute
Izatnagar - 243 122 (U.P.), India

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy
(Veterinary Microbiology)

2021

Dedicated To...

All the Speechless Creatures

विषाणु विज्ञान विभाग
भा.कृ.अनु.प.–भारतीय पशु चिकित्सा अनुसंधान संस्थान
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Certificate

This is to be certified that the research work embodied in this thesis entitled **"Unraveling the host factors interacting with NS3 protein during bluetongue virus replication"** *submitted by* **Dr. Chaple Ashwini Rameshrao, Roll No. P-2074,** *for the award of* **Doctor of Philosophy** *Degree in* **Veterinary Microbiology** *at ICAR-Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate herself under my supervision and guidance.*

It is further certified that **Dr. Chaple Ashwini Rameshrao, Roll No. P-2074,** *has worked for more than 34 months in the Institute and has put in more than 300 days attendance under me from the date of registration for the* **Doctor of Philosophy** *Degree in this Deemed University, as required under the relevant ordinance.*

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We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by her for the award of **Doctor of Philosophy** *Degree of this Institute.*

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Doctor of Philosophy** *Degree of the Deemed University, ICAR-Indian Veterinary Research Institute, Izatnagar.*

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ACKNOWLEDGEMENTS

With the fulfillment of the research program, I would like to express my gratitude towards those who have dedicated their time and efforts to my future. With the thought it is next to impossible to put an ocean into a nutshell, still, I am trying to acknowledge people who walked along with me through this journey.

It is my great privilege to have **Dr. D. Muthuchelvan,** Principal scientist, IVRI, Bengaluru, as an advisor who has always supported and motivated me and is the most important person for this accomplishment. I feel blessed under his guidance and accept his holistic contribution as a lifelong debt.

I am highly obliged to **Dr. Sonalika Mahajan**, scientist, IVRI, Izatnagar for designing this work and guiding me at every footstep. Working under her guidance has provided me an opportunity to learn the scientific approach and consistency of work to convert an idea into reality.

I owe my sincere regards to **Dr. Gaurav Sharma**, scientist, IVRI, Izatnagar for his ever-willing help, constructive advice, and encouragement at various phases. His constant support has made it possible for me to conduct my research work at Izatnagar.

I express my heartfelt regards to **Dr. M. A. Ramakrishnan**, Principal scientist, IVRI, Bengaluru, for his immense support and being a constant source of inspiration.

I express my profound gratitude to my Co-advisors, **Dr. Sameer Shrivastva, Dr. Deepak Rawool** and **Dr. Y. P. S. Malik** for their valuable suggestions, and help during the period of my research.

I express my sincere regards to the **Head, Division of Virology** and **all the Scientists from the Division of Virology**, Mukteswar, for fostering the subject knowledge to make the base for research and immense support provided during the Mukteswar stay period.

I offer my gratitude to **Dr. S. Nandi, Dr. Vishal Chandar, Dr. Sunil Jadhav, Dr. Dhara and Dr. Jaydeep Rokade** for their guidance and helping hand during the course.

I am thankful to **the Head, Veterinary BiotechnologyDivision, Joint Director (CADRAD),** and **Dr. A. K. Tiwari** for their help which makes it easy for me to conduct the research work with all the facilities.

I am also thankful to the **Director, Joint Director (Acad.),** and **Scientific Coordinator (Univ.)**, IVRI, Izatnagar for providing the necessary facilities and the financial support to carry out the present work.

I express my heartfelt thanks to **Dr. Damian Vitour**, ENVA, Paris, France for giving me an opportunity to work in his lab for overseas training and providing his kind help to conduct the research.

I am heartily thankful to **ICMR** for providing the fellowship which was a great financial support during this degree program.

I deeply thank my seniors cum labmates, **Drs. Shikha Saxena, Richa Arora, Waseem Malla** for their generous support and ever helping behavior. Their company has provided warmth, friendliness throughout the research work, and memories for a lifetime.

I am so grateful to my colleagues **Drs. Deepak, Anand, Nikunj, Parvaiz, Arfa, Pallavi, Marcia, Varsha, Lahari, Chaynika, Suhashri, Shivashankar and Avadhesh** for their lovely company. The precious moments we spent together as a micro family by name but macro by quantity will always leave a smile on my face. All the masti we did together will remain with me as a collection from IVRI. Time enjoyed together especially at the Mukteswar is the souvenir of our friendship.

I can hardly find any suitable word to acknowledge my dearest friend, **Dr. Mayur Vispute** for his indispensable cooperation and selfless help in my hard times.

I heartily wish to thank my lovely juniors **Drs. Rohit, Rajeshwar, Mukesh, Mahesh, Shubham**, and **Saima** for their love, care, and compassion bestowed on me. Time spent with you people is ever cherishing and blissful.

I would like to extend my heartfelt thanks to my seniors **Drs. Harish, Raja Isaq**, and my beloved juniors **Drs. Tripti, Chyana, Hansmeet, Basit, Muddasir**, and **Muzzamil** for their wonderful company.

I am thankful to **Udit Joshi,** SRF of IVRI, Izatnagar for his help in research work.

I highly appreciate the help of Vivek Bhaiya, Manoj Ji, and the Staff of the Animal Biotechnology Division for their timely help and co-operation.

I express my thanks to all the members of Maharashtra mess for their love and support.

Delicious food at Edwards hotel, Mukteswar can never be forgotten. Bhatt Ji and other staff of Edward hostel deserve special thanks for providing such yummy food and making our stay comfortable.

The love of a mother is the most precious gift to mankind. Whatever I am today I owe it to **my mother**. I am indebted to **my father**, brothers **Shubham and Ishan** for always being with me. Special gratitude and love go to my **Aai and Baba** for their unfailing care, support, and faith.

I must record my sincere thanks to Chachu, Kuldeep bhaiya, and DP bhaiya for their help in compiling the thesis.

Above all, I thank the almighty for blessing such beautiful people and surroundings.

At last, I would like to thanks everyone who has been with me during the study period and I will always be indebted to them.

Date: 13/09/2021
Place: *ICAR-IVRI, Izatnagar*

(Chaple Ashwini Rameshrao)

ABBREVIATIONS

%	:	Percent
µg	:	Microgram
µl	:	Microliter
aa	:	Amino acid
AD	:	Activator domain
Amp	:	Ampicillin
BC	:	Basal control
BD	:	Binding domain
BHK	:	Baby Hamster Kidney
BLAST	:	Basic local alignment search tool
^{o}C	:	Degree celsius
cDNA	:	Complimentary DNA
CPE	:	Cytopathic effects
DAB	:	Diaminobenzidine
DDO	:	Double drop-out
ds	:	Double stranded
DW	:	Distilled water
E. coli	:	*Escherichia coli*
EDTA	:	Ethylene Diamine Tetra-Acetic acid
eIF	:	Eukaryotic translation initiation
EST	:	Expressed sequence tag
EtBr	:	Ethidium Bromide
g	:	Gram
GMEM	:	Glasgow minimum essential medium
GO	:	Gene ontology
GST	:	Glutathione S-transferase
h	:	Hour
IF	:	Immunofluorescence assay
kDa	:	Kilodalton
LB	:	Luria Bertani
LD-PCR	:	Long distance PCR
LiAc	:	Lithium acetate
LiCl	:	Lithium chloride
LN_2	:	Liquid nitrogen
M	:	Molar

min	:	Minute
Na_2CO_3	:	Sodium carbonate
NaCl	:	Sodium Chloride
NC	:	Negative control
NFW	:	Nuclease free water
NSP	:	Non-structural protein
OD	:	Optical density
ONPG	:	Orthonitrophenyl-β-Dgalactopyranoside
ORF	:	Open reading frame
PBS	:	Phosphate buffered saline
PC	:	Positive control
PMSF	:	Phenylmethanesulfonyl fluoride
PPI	:	Protein-protein interactions
QDO	:	Quadruple drop-out
RE	:	Restriction enzyme
RNA	:	Ribonucleic acid
RT	:	Room temperature
s	:	Second
S. cervesiae	:	Saccharomyces cerevisiae
SDO	:	Single drop-out
SEAP	:	Secreted al-kaline phosphatase
SP	:	Structural protein
ss	:	Single-stranded
STB	:	Sample treatment buffer
TAE	:	Tris-acetate-EDTA
TBS	:	Tris base sodium chloride
TBS-T	:	TBS-tween
TSS	:	Transforming and storage solution
vol	:	Volume
Y2H	:	Yeast-two hybrid
β-ME	:	β-mercaptoethanol

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Introduction

1 INTRODUCTION

The livestock sector plays a key role in the livelihood and economy of developing countries. In India, the livestock sector hand out 4.9% to total GDP and 28.4% to agriculture GDP (BAHFS, 2019). According to the 20th livestock census, the entire livestock population in the country is 535.82 million out of which the sheep population is 74.26 million and the goat population is 148.88 million (BAHFS, 2019). In total, sheep and goat contribute ~13.87% and 27.80%, respectively of the total livestock population. However, various infectious and contagious diseases with high mortality and morbidity threaten the health scenario of these domestic small ruminants and subsequently cause huge economic losses. Direct losses occur in terms of death, weight loss, abortions, reduced milk yield or meat quality while indirect losses resulting from export impediments for live animals and their products (Sperlova and Zendulkova, 2011). Amongst several havoc-creating diseases that affect small ruminants, bluetongue is one of the most important diseases with serious economic consequences

Bluetongue (BT) is a viral, infectious, non-contagious, and arthropod transmitted disease of domestic as well as wild ruminants. Sheep is the primary host for the disease while it also affects other species that include cattle, goat, buffalo, antelope, deer, elk, and camel. Being a notifiable disease, it is listed under the category of multiple species diseases by the World Organization for Animal Health (OIE). The disease is marked with fever, facial edema, hemorrhages, oral mucosal ulceration, and coronitis. The clinical presentation of BT differs widely between different ruminant hosts. Pronounced signs are observed in sheep and white-tailed deer ranging from fevered stomatitis, pneumonia, coronitis, hyperemia, cardiac lesions,

and cyanosis of the tongue to death with a mortality rate of up to 80% ((Modrow *et al.*, 2010; Rojas *et al.*, 2019), while the disease usually remains sub-clinical in goats and cattle (Rojas *et al.*, 2019). Historically, BT has been endemic absolutely in temperate and tropical zones (Mellor *et al.*, 2008). In the Indian subcontinent, BT was initially reported in Pakistan as early as in 1958 (Sarwar, 1962) in an imported flock of Rambouillet sheep from the USA and eventually in India from Maharashtra state in the year 1964 (Sapre, 1964). Since then, numbers of outbreaks of BT have been documented from several different parts of the subcontinent and the disease is now found to be endemic throughout the country and particularly in southern states (Sreenivasulu *et al.*, 2004; Rao *et al.*, 2016). Among the 27 serotypes of BTV reported worldwide, 23 serotypes have been reported serologically from India whereas 15 of them are isolated (Maan *et al.*, 2016; Saminathan *et al.*, 2020).

Bluetongue virus (BTV), the causative agent of BT is a non-enveloped, double-stranded (ds) RNA virus within the genus *Orbivirus* of the family *Reoviridae*. So far, 27 distinct serotypes of BTV are reported worldwide (Alkhamis *et al.*, 2020). Apart from these, two putative serotypes i.e. 28 and 29 have also been reported recently (Bumbarov *et al.*, 2016; Bumbarov *et al.*, 2020; Yang *et al.*, 2020). Transmission of BTV mainly occurs through the bite of vector midges of *Culicoides* species (Mellor, 1990). Sporadically, it is transmitted vertically across the placenta, through seminal fluid, by an oral route (De Clercq *et al.*, 2008; Backx *et al.*, 2009) or through direct contact (Batten *et al.*, 2014). BTV genome is approximately 19.2 kb consisting of ten segments of dsRNA encased within a triple-layered icosahedral protein capsid. Because of the segmented RNA genome of the virus genetic variants of BTV keep on emerging on account of the event of genetic recombination and gene re-assortment. Seven structural (SPs; VP1-VP7) and five non-structural proteins (NSPs; NS1-NS5) are encoded by the virus (Belhouchet *et al.*, 2011; Ratinier *et al.*, 2011; Stewart *et al.*, 2015). Two structural proteins, VP2 and VP5 form the outer coat of the particle. VP2 is the serotype determinant thus, involved in serotype-specific virus neutralization activity. The middle layer is composed of VP7 and VP3. VP7 is the major group-specific protein of BTV. The innermost sub-core consist of three smaller structural proteins, VP1, VP4, and VP6, having a role in the replication and transcription of viral RNA (Martyn *et al.*, 1991) . The NS1 protein

is the major protein synthesized during the viral replication (almost 25% of the viral proteins) form the tubules in the virus-infected cells and assist in virus morphogenesis (Owens *et al.*, 2004). The NS2 protein forms the intracellular structures termed viral inclusion bodies (VIBs). NS3 protein has two isoforms i.e, NS3 and NS3a in which NS3a lacks 13 amino acid residues at N-terminal. NS3/NS3a proteins are glycosylated proteins engaged in BTV exit and are only membrane proteins encoded by orbiviruses (Wu *et al.*, 1992). NS3 protein facilitates virus release by inducing membrane permeabilization and thus works as a viroporin. The individual function of NS3a in virus replication or morphogenesis is still unclear**.** Moreover, in all the BTV serotypes the second initiation site in the NS3 gene is totally conserved, signaling towards the particular role of NS3a which has not yet been elucidated. NS4 a newly discovered protein, located in the nucleoli of infected cells. NS4 is not essential for virus replication but confers an advantage in an interferon (IFN)-induced antiviral state (Ratinier *et al.*, 2011; Ratinier *et al.*, 2016). The fifth NSP i.e. NS5 is identified in very recent times as an overlapping ORF in NS3 protein although much vivid information regarding its role is not yet available (Stewart *et al.*, 2015).

The NS3 and NS4 proteins have been identified as INF antagonists and are most likely involved in hampering the cellular innate immune response to BTV infection (Chauveau *et al.*, 2013; Ratinier *et al.*, 2016). Thus, it provides an idea that these non-structural proteins of the virus execute a momentous role in defining host innate immune response towards the infection and play a notable role in viral pathogenesis too. Among the NSPs, the NS3 protein sequence is is considered a potent candidate for diagnostic purposes. Despite these facts, detailed studies are not available on the host-pathogenesis aspect of NS3. Further, the pathogenesis of BTV varies in insect and the mammalian host as it causes severe hemorrhagic disease in sheep and is asymptomatic in the insect host (Mortola *et al.*, 2004). In tissue culture also, BTV exhibits persistent infections in susceptible insect cells without apparent cytopathic effects (CPE) but produces considerable CPE in mammalian cells (Mortola *et al.*, 2004). This clearly indicates that the virus has diversity in replication, packaging, and cell egress depending on the host. Many studies have been undertaken to understand the role of viral proteins in pathogenesis but still, a lot of questions remain unanswered. Precise information on host-virus

interaction during virus trafficking between mammalian and insect vector cells is scanty. The studies focusing on virus and host interaction of BTV remain poorly understood, pointing to the need for a better understanding of BTV pathogenicity in the host which will help to define the molecular determinants of BTV virulence and factors responsible for the subjugation of host machinery (De Clercq *et al.*, 2008). To study the protein-protein interaction (PPI) yeast two-hybrid (Y2H) has been proven as a proficient technique and has been used by many researchers to study the virus-host interaction (Walhout and Vidal, 2001; Brückner *et al.*, 2009; Gladue *et al.*, 2012; Xing *et al.*, 2016; Gladue *et al.*, 2018). Based on the above facts and considering the significance of NS3 protein in BTV pathogenesis, the present study is designed to understand the molecular mechanism of interaction of BTV NSPs (NS3/NS3a) with the mammalian host cells using Y2H screening. Understanding the studies of the interactions will aid in devising the future replication-defective vaccines, a strategy to enhance cellular immunity for therapeutic purposes. The objectives for the present study are as follow:

1. **To construct Y2H compatible mammalian host cDNA library.**

2. **To identify and validate putative host partners for BTV-NS3 protein.**

Review of Literature

2 REVIEW OF LITERATURE

2.1. Bluetongue

Bluetongue (BT) is an infectious, viral, non-contagious, and transboundary disease of ruminants and camelids. It is mainly an arthropod-born disease, transmitted by hematophagous midges of *Culicoides* species. The etiological agent causing BT is the Bluetongue virus (BTV). The clinical presentation of disease varied from inapparent to fatal, determined by various factors which include strain and serotype of the virus, age, species, and breed of the infected animal. BTV has the ability to infect ruminants as well as camelid species but a devastating form of the disease is usually seen in sheep and some species of North American white-tailed deer (Maclachlan, 2010). In total, 27 serotypes have been reported across the world which generates very low levels of cross-protection or no protection, complicating vaccination strategies. Though BT is considered to have emerged from Africa to date it has been distributed in all the continents except the Antarctic continent (Mellor *et al.*, 2008).

Bluetongue was first described by a French biologist Francois de Vaillant as 'tong-sikte', a disease of sheep and cattle (Gutsche, 1979). BT was first reported at the end of the 18th century in South Africa (Spreull, 1905) and was first cited as "epizootic catarrh". It was also called pseudo-FMD, sore-mouth or seerbeck (Vellema, 2008). In South Africa, some farmers noticed cyanosis of tongue in seriously infected animals and coined the term 'bloutong' from which the name 'bluetongue' used these days has been originated (Maclachlan *et al.*, 2009). Before the 1940s, the occurrence of bluetongue was limited to Africa only. The first well-recorded epidemic outside the African continent was dated in sheep in Cyprus in 1943

(Gambles, 1949) and subsequently, the disease was recorded from different countries across the globe including Israel in 1943-44, Texas, the USA in 1948 (Hardy and Price, 1952), Iberian peninsula during 1956-57, Middle East, Asia, and Southern European countries (Gibbs and Greiner, 1994; Mellor and Wittmann, 2002; Calistri *et al.*, 2004). Almost all ruminants are vulnerable to BT, but a clinical form of the disease is mainly exhibited in sheep and white-tailed deer (Johnson *et al.*, 2006). The indigenous sheep are comparatively less prone to BT than the exotic breeds of sheep-like Rambouillet and Merino (Spreull, 1905; Prasad *et al.*, 1992). In cattle and goats disease usually remains sub-clinical, although the immunologically naive population may suffer with an acute infection on virus spread. Clinical sign and case fatality has been reported in cattle by BTV-8 epidemic in Central and Western Europe (Elbers *et al.*, 2008). Under natural condition, the disease may also be present in African antelopes, pronghorn, and other wild ruminants (Howerth *et al.,* 2001) including camelids (Henrich *et al.*, 2007), captive yak (García-Bocanegra *et al.*, 2011), and elephants (Mushi *et al.*, 1990). Antibodies specific to BTV were also recognized in African carnivores including cheetahs, lions, jackals, wild dogs, hyenas, and large-spotted genets (Alexander *et al.*, 1994). These findings can also be linked with the consumption of meat from BTV-infected animals.

2.2. Transmission

BT is usually transmitted by the bite of hematophagous midges belonging to the genus *Culicoides* (Diptera: *Ceratopogonidae*). More than 1400 species of culicoides are reported but hardly 30 of them are documented as BTV transmitters (Meiswinkel *et al.*, 2008). The coexistence of BTV, susceptible hosts, and competent insect vectors boost the spread and development of BT. After getting a blood meal from a BTV-infected animal and completion of an optimal incubation period of around 10 days, culicoides midges can transmit the virus for the rest of their life. Although being an arbovirus, the occasional transmission of BTV via alternative routes has also been reported (Belbis *et al.*, 2013). Transmission of BTV serotype 8 by an oral route has been observed in ruminants, which shows feeding of infected colostrum can cause infection in calves (Menzies *et al.*, 2008). Transmission by crossing the placental barrier under experimental conditions has been noted with field strains of BTV-1 (van der Sluijs *et al.*, 2011) and BTV-2 (Rasmussen *et al.*, 2013) while BTV-8 can cause

transplacental transmission in clinical condition (Coetzee *et al.*, 2012; Belbis *et al.*, 2013). Embryos collected during the viraemic phase from infected donor ewes can also transmit the virus to recipient ewes (Venter *et al.*, 2011). Aerosol transmission between infected ruminants is thought as the major route for the spread of serotypes BTV-25 and BTV-26 (Batten *et al.*, 2014). Besides, there are some reported cases that claim the ingestion of BTV infected ruminant fetuses and meat for infection in carnivores (Jauniaux *et al.*, 2008; Mauroy *et al.*, 2008).

2.3. Pathogenesis

BTV enters within the host with the bite of an infected culicoides vector and then wanders towards the regional lymph nodes where initial replication of the virus occurs. In due course, the virus disseminates via blood and lymph to secondary sites like the spleen and lungs where the virus replicates predominantly in endothelium and mononuclear phagocytes (Sperlova and Zendulkova, 2011). BTV infection comes up with cell death by necrosis and apoptosis. Vascular occlusion and pathology of target tissue result from injury to small blood vessels leading to the release of various vasoactive mediators causing edema and effusions (Sperlova and Zendulkova, 2011). BTV is quantitatively associated with platelets because of its short lifespan. In the late course of infection virus is chiefly associated with erythrocytes as infection of erythrocytes helps it to prolong the period of infection in ruminant hosts (Brewer and MacLachlan, 1994). The characteristic of BTV infection in ruminants is the presence of prolonged cell-associated viremia which does not remain persistent during the presence of high titers of neutralizing antibodies (Schwartz-Cornil *et al.*, 2008). Fetal infection occasionally causes abortion in sheep and cattle, but more commonly it leads to teratogenesis. In cattle, natural fetal infection and abortion are reported only from the countries practicing the use of modified live virus vaccines (Maclachlan *et al*., 2000).

In sheep, the manifestation of illness occurs in an acute, chronic or subclinical form. The incubation period of infection is about 6-9 days; at the end of it, fever (104-108 °F) develops and continues for 5-7 days (Tweedle and Mellor, 2002) followed by, apathy, tachypnea, and hyperemia of the nostrils and lips with massive salivation. Initially, nasal discharge is clear, then convert to mucopurulent sequentially get dried and form crust surrounding the

nostrils. Other visible symptoms include edema of the submandibulum, lips, tongue, and ears and conjunctival petechiae (Maclachlan *et al.*, 2009). At the ending stage of the pyrexia, affected sheep may have erosions, ulcers, and excoriations of the oral mucosa in inner lips, gums, chicks, dental pad, and hard palate; coronitis, laminitis, and necrosis of striated muscles resulting in an arched back and movement reluctance. In some cases, cyanotic tongues, profuse hemorrhagic diarrhea, or vomiting causing aspiration pneumonia are observed. Duration of viremia continues for 14 to 54 days (Barzilai and Tadmor, 1971; Koumbati *et al.*, 1999). Torticollis, dermatitis, and wool breaks can also develop (Darpel *et al.*, 2007).

In goats, an acute fall in milk production, edema of the head and lips, nasal discharge, and erythema of the udder and skin can be manifested (Dercksen *et al.*, 2007). Viraemia may stand for 19 to 54 days (Koumbati *et al.*, 1999). While in cattle, viremia ranged as long as 60 days or, even 100 days. Moreover, a clinical form of the disease has been observed in cattle in the BTV-8 epidemics in Western and Central Europe (Darpel *et al.*, 2007; Elbers *et al.*, 2008).

2.4. Epidemiology

BT was presumed to be originated from the Middle East countries (Sarwar, 1962). In the Indian landmass, it was first reported in Pakistan in 1959 and BTV serotype 16 was isolated during this outbreak from sheep (Sarwar, 1962). In India, Spare (1964) revealed the disease for the first time in sheep and goat from Maharashtra state. Subsequently, the disease was reported from Uttar Pradesh in sheep. The virus from the outbreak was isolated by experimental transmission in sheep. Since that time serological as well as the clinical prevalence of the disease has been recorded throughout the nation (Bhambani and Singh, 1968). Among the 27 serotypes of BTV reported worldwide, 23 serotypes have been accounted in India either by serotype-specific antibodies detection or virus neutralization (Rao *et al.*, 2016). Till now 15 serotypes of BTV have been isolated from different regions of India (BTV- 1, 2, 3, 4, 5, 8, 9, 10, 12, 16, 17, 18, 21, 23, and 24) under All-India Network Program on Bluetongue (AINP-BT) and by other research laboratories in the country (Maan *et al.*, 2017). They are mostly from the southern states of India (Chand *et al*; 2015; Reddy *et al.*, 2018).

2.5. Economic impact

BT is an aliment with severe monetary consequences because it has the capability to spread rapidly creating major barriers in the international trade of animals and their products. It leads to direct financial losses as well as indirect losses by imposing the movement restriction on live animals and limiting the export of its products like the embryo, germplasm from BT-affected to non-affected countries (Velthuis *et al.*, 2010). On average 2% to 30% of the mortality observed in BT infected animals. In highly susceptible sheep, it may achieve 100%. Though exact numbers of economic losses across the world have not been expressed but an average of three billion US$ per year is estimated (Sperlova and Zendulkova, 2011). The greatest loss to the farmers was observed in 2005 which was approximately 231 million rupees (Maan *et al*., 2017).

2.6. Bluetongue virus (BTV)

BTV is the member of the *Orbivirus* genus within the family *Reoviridae*. It is a non-enveloped virus with a diameter of 90 nm and a density of 1.337g/cm^3. The relative molar mass is about 10.8×10^7 of which 12% RNA is genomic RNA (Schwartz-Cornil *et al.*, 2008). The virus shows stability in the presence of proteins and can thrive for many years, for instance, in blood stored at 20 °C but it is sensi-tive to some chemicals, presence which make it susceptible to degradation. For example, 3% NaOH, phenol, organic iodine complex, and β-propiolactone found to be deadly for the virus (Radostits, 1994).

2.6.1. Genome

The genome of BTV encompasses 10 linear dsRNA segments (Seg 1 to Seg 10) of different sizes (Fig. 1). It is encircled by three concentric protein coats containing inner, intermediate, and outer capsids. The genome size of the virus is approximately 19.2 kbp. dsRNA segments incorporate 43% GC and 57% AU along with six conserved nucleotides at terminal sequences of 52 and 32 ends of the positive strand (GUUAAA at 52 , and ACUUAC at 32) (Mertens and Sangar, 1985).

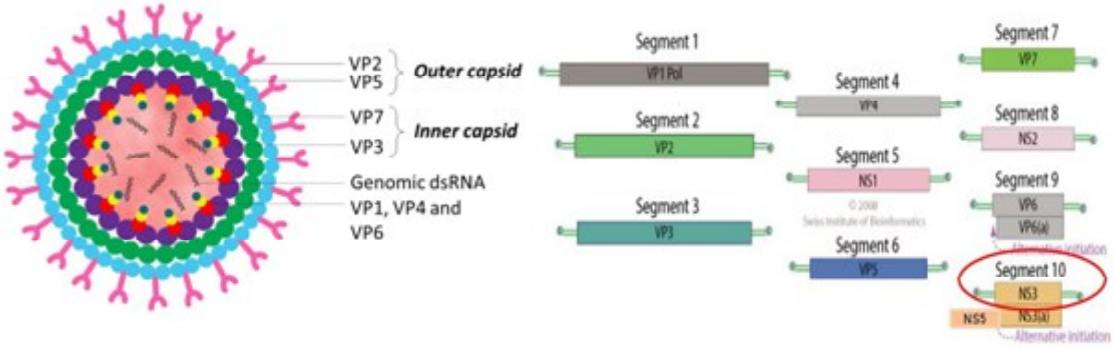


Fig. 1: BTV segmented genome

2.6.2.1. Structural proteins (SPs)

Among the seven SPs of an infectious virion, four are major (VP2, VP3, VP5, and VP7) and three are minor (VP1, VP4, and VP6) proteins.

The outer capsid proteins, VP2 and VP5 are encoded by seg 2 and 6 respectively, to aid the virus entry inside the mammalian host cells. VP2 is a triskelion-shaped large protein with a size of 111 kDa. It binds with the host receptors for virus entry and is involved in hemagglutination and eliciting serotype-specific neutralizing antibodies (Cowley and Gorman, 1989; Zhang *et al.*, 2010). VP2 has an affinity towards a sialoglycoprotein component present on erythrocytes (glycophorin A), suggesting its role in BTV transmission by the *insect* vector to vertebrates during a blood meal. It is the dominant serotype-specific antigen of BTV (Huismans and Erasmus, 1981). Also, it offers defensive immunity against the virulent virus to the animal (Hassan and Roy, 1999).

VP5 (59 kDa) protein exists as a trimmer and is essentially more conserved than VP2. It shows some level of diversity that relates to its geographic origin (Singh *et al.*, 2004). The protein has features similar to class I fusion proteins of many enveloped (Nason *et al.*, 2004), that moderates viral particles release inside the cytoplasm from endosomal compartments depending on the pH. Thus, permits membrane fusion and formation of syncytia (Hassan *et al.*, 2001).

The inner capsid proteins, VP3 and VP7 are encoded by Seg 3 and seg 7 respectively. VP3 (100 kDa) protein has significance in the icosahedral symmetry construction of the virion and is exceptionally conserved in an innermost sub-core (Loudon and Roy, 1991).

It binds with RNA molecules and transcription complexes.VP7 (38 kDa) is a serogroup-specific protein located on the outer core surface.VP7 is important for binding with insect cell receptors and also helps in penetration (Xu *et al.*, 1997). VP3-VP7 complex blocks the production of type 1 IFN through various cytoplasmic sensors and guard the viral dsRNA against degradation (Schwartz-Cornil *et al.,* 2008).

VP1 (149 kDa), VP4 (76 kDa), and VP6 (36 kDa) are the minor core proteins form the transcription complex. The largest viral protein, VP1 is encoded by seg 1 which acts as replicase. It performs optimally at 27 to 37 °C enabling replication in both mammalian as well as insect cells (Boyce *et al.*, 2004). Seg 9 encodesVP4 protein, important for catalyzing the capping of early BTV mRNA in order to provide stability (Sutton *et al.*, 2007). It owes guanylyltransferase activity, linked with the cap formation and uplifting replication efficiency (Ramadevi *et al*., 1998). The VP6 protein is encoded by seg 9 and it operates as an RNA-dependent ATPase and helicase. Additionally, it binds with the ATP and assists in mRNA synthesis by uncoiling dsRNA duplexes (Schwartz-Cornil *et al.,* 2008).

2.6.2.2. Nonstructural proteins (NSPs)

Throughout the past few decades, it has been greatly presumed that the dsRNA genome of BTV is monocistronic but this speculation is altered with the discovery of NS4 protein (Ratinier *et al*., 2011). Researchers are looking towards the putative fifth NSP also (Stewart *et al*., 2015).

NS1

NS1 is a 552 amino acid (aa) protein encoded by seg 5 and synthesized extensively during the early stage of virus replication in virus-infected cells (Huismans, 1979). It is highly conserved across the BTV serotypes. It appears in multimeric form fabricating large tubule-like structures (around a diameter of 50 nm and up to a length of 1000 nm) which are alleged to be linked with producing cytopathogenic effects (Owens *et al.*, 2004).

NS2

NS2 is a 356 aa protein encoded by seg 8 is a principal constituent of peri-nuclear structures in mammalian cells infected with BTV, called "virus assembly factories" or "viral

inclusion bodies" (VIB). NS2 is synthesized in considerable quantities during viral replication and is conjoined with VIBs only, not virions (Butan and Tucker, 2010).

NS3/NS3a

NS3 (229 aa) and NS3a (lacking the N-terminal 13 amino acids of NS3) are encoded by the seg 10 of BTV. They are synthesized in a lesser amount compared to NS1 and NS2 (Patel and Roy, 2014). These are the only glycoprotein yielded by BTV and are expressed in enormous quantity in insect cells, but not in mammalian cells. NS3 protein corresponds as a viroporin and augments virus release from mammalian or insect cells by distorting the cytoplasmic membrane permeability (Han and Harty, 2004). In addition, NS3 binds to the cellular protein Tsg101 (Wirblich *et al.*, 2006), granting BTV particle to bud, similar to retroviruses. NS3 protein of BTV tampers with the induction of the innate immune response in non-hematopoietic cells by interfering with the production of IFN-1 (Chauveau *et al.*, 2013). The exact role of NS3a and how it cross-talk with host proteins to facilitate the exit of BTV from infected cells has not been revealed yet.

NS4

NS4 is a 77 to 79 aa protein discovered by Ratinier and coworkers (2011), encoded by an alternative reading frame in seg 9 of the genome. The protein has a coiled-coil motif and forms aggregates throughout the cytoplasm and nucleus. NS4 is advocated to play a substantive role in viral defense against interferon (Belhouchet *et al.*, 2011). NS3 and NS4 are thought to perform synergistic action to counteract host immune response (Ratinier *et al.*, 2016), and hence both are considered to have an instrumental role in modulating the host-virus interaction.

2.7. Role of NS3 protein in BTV replication

NS3 protein exists in two isoforms i,e. NS3 and NS3a, where NS3a is shortened by 13 aa residues at N-terminal (Han and Harty, 2004; Celma and Roy, 2009). NS3/NS3a protein is a glycosylated protein and is the sole membrane protein drafted by orbiviruses (Wu *et al.*, 1992). The distinct role of NS3a in virus morphogenesis or replication is not yet clear. But among all the BTV serotypes, the second initiation site in the NS3 gene is precisely conserved

implying that NS3a possibly has a meticulous role that remains to be elucidated till date (Celma and Roy, 2009). NS3 and NS3a seem to have an association with smooth intracellular membranes, although they are also present at the plasma membrane (Hyatt *et al.*, 1993). NS3 and NS3a encompass a longer N-terminal domain and small C-terminal cytoplasmic domain, linked by two transmembrane (Mellor and Wittmann, 2002) domains along with a short extracellular domain (Bansal *et al.*, 1998; Belhouchet *et al.*, 2011). It posses two polybasic motifs (92-97 aa and 114-121 aa position) upstream to the TM1 and is common in all orbiviruses (Labadie *et al.*, 2020). BTV NS3 extracellular domain possesses a single glycosylation site which is not found in African horse sickness virus (AHSV) NS3, indicating that it might not be inherent for the protein to function (Wu *et al.*, 1992).

NS3 protein executes multiple functions and has a decisive role in the BTV replication cycle according to host cells. NS3/NS3a presence is also needed for the budding and consequent release of VLPs from infected cells (Hyatt *et al.*, 1993). It communicates with the outer shell protein VP2 and the mutations in the VP2 interaction domain of NS3 protein lead to total disruption in the assembly, trafficking as well as the release of the virus from infected cells (Beaton *et al.*, 2002; Celma and Roy, 2009). Evidence for the viroporin activity of NS3 is convincing and in harmony with former studies, which testify coalition of NS3 with membrane perturbation areas. Studies emphasize that the NS3 protein possesses late domain motifs capable of interacting with the Tsg101 cellular protein, analogous to certain proteins present on enveloped viruses such as filoviruses or HIV which is engrossed in the vacuolar protein sorting pathway. Tsg101 is an element of the endosomal sorting complexes involved in the transport (ESCRT-I) cellular exocytic pathway which is concerned with the release of numerous enveloped viruses (Wirblich *et al.*, 2006). Despite clear understanding regarding the importance of cellular ubiquitination machinery in the budding of several animal viruses, present apprehension about the appropriate targets of the ubiquitin ligases and interplay with other components of the budding machinery is still minimal (Gustin *et al.*, 2011). NS3 protein ubiquitination might accomplish a pivotal role in modulating their intracellular outcome, host-specific stability and, BTV replication kinetics (Ftaich *et al.*, 2015). NS3 protein illustrates cytotoxic effects while expression in mammalian cells (van Staden *et al.*, 1995) and to produce such cytotoxicity, it

requires to associate with membrane. Han and Harty (2004), have shown that membrane association is dependent on the existence of the first transmembrane domain.

Recently, a study discloses that a single aa residue can lead to a hasty and host-specific turnover of NS3 protein in ovine cells. It also highlighted that sequence deviation of NS3 differentially alters the BTV replication mechanism in a host-specific manner (Ftaich *et al.*, 2015). NS3 has been found as major virulence determining factor for BTV-8 to contribute to pathogenicity along with VP2 protein (Janowicz *et al.*, 2015). The absence of NS3/NS3a expression potentially enables demarcation of infected and vaccinated animals representing it as a promising candidate for DIVA (Barros *et al.*, 2009; Feenstra *et al.*, 2014). Lesser CPE in mammalian cells and decreased virus release from mammalian as well as insect cells is observed in absence of NS3/ NS3a proteins. This indicates that the NS3/NS3a knockout virus possibly proves safe in vivo and can be a potential candidate for the BTV vaccine (Feenstra *et al.*, 2014). NS3/NS3a knockout BTV fails to produce viremia also is not able to replicate within the vector but still can induce sterile immunity which makes it a suitable BT Disabled Infectious Single Animal (DISA) vaccine (van Rijn *et al.*, 2017). A very latest study has identified the serine/threonine-protein kinase B Raf (BRAF), an imperative component of the MAPK/ERK pathway, as a new cellular interacting partner of BTV-NS3. BRAF silencing has demonstrated a significant reduction in MAPK/ERK activation by BTV also, the study has described that re-localization of BRAF occurs either at the Golgi apparatus or the cell membrane during the BTV infection (Kundlacz *et al.*, 2019). Evidence are growing with further studies implying that BTV NS3 and NS4 are acting synergistically to disrupt the cellular innate immune response to BTV infection (Ratinier *et al.*, 2011; Chauveau *et al.*, 2013). Conclusively all these studies are highlighting the role of NS3 protein in the pathogenesis of BTV but fail to clarify the exact mechanisms of its crosstalk with the host. In spite of previous studies, there are critical gaps in perception of the molecular pathway of virus lifecycle and, some vital questions remain unanswered. The foremost amongst these is the variations in interaction with the host during virus trafficking between mammalian and insect vector cells. The studies for deducing the interaction map of viral proteins with the host proteins are limited and hence required further detailed studies. In addition, although, majority of the BTV NSPs are conserved

among different serotypes, but the minor role of variants in the NSPs of BTV serotypes of Indian origin is yet to be investigated. Based on this, the present study is designed for understanding the molecular mechanism of viral protein interactions with the host. Understanding the interactions studies will be essential in devising the future strategy to design antivirals for therapeutic purposes. This study will examine the strains of BTV from Indian origin which till date are not been studied in depth.

2.8. Approaches to study virus-host interaction

There are so many robust techniques available to examine the molecular interactions among proteins and thus help to decode their nature and contribution in biology. Mass Spectrometry coupled with Tandom-affinity purification (MS-TAP), Fluorescence Resonance Energy Transfer (FRET), Bio-Layer Interferometry (Darpel *et al.* 2007), Surface Plasmon Resonance (Belbis *et al.* 2013), and Isothermal titration calorimetry (ITC) etc. are the techniques are at hand to study protein-protein interaction but the Y2H system is the most throughput technique recommended by many researchers (Brückner *et al.*, 2009; Rao *et al.*, 2014; Podobnik *et al.*, 2016; Gillen and Nita-Lazar, 2019).

2.8.1. Yeast two-hybrid (Y2H)

Y2H is a dynamic technique available to investigate an *in vivo* PPI, which was first formulated by Fields and Song in 1989. It relies on the redeployment of a functional transcription factor (TF) during the interaction of two proteins or polypeptides of interest (Fields and Song, 1989). A number of eukaryotic transcription components are harmonized into practically distinguishable domains such as the DNA-binding domain (BD) and the activation domain (AD). BD employs the transcription factor on to the particular DNA sequences while AD possesses the transcription machinery accelerating the transcription process. These domains are physically dissociable and do not have to localize in the same protein to show interaction (Paiano *et al.*, 2019). Y2H exploits the transcription factor GAL4 of yeast *Saccharomyces cerevisiae* (*S. cerevisiae*). GAL4 is present in two functionally separable domains in which the N-terminal domain bind with BD and the C-terminal domain with AD. GAL4 BD is fused with a protein called 'bait' and GAL4 AD is fused with a protein called 'prey'. The principle

behind this is upon interaction among the bait and the prey, the DB and AD are brought in close proximity and a functional TF is reconstituted upstream of the reporter gene (Fig. 2). With the advancement of technology protein of interest can be used as bait to identify cellular interacting proteins using diverse cDNA libraries as prey. A key benefit of Y2H is testing of protein interactions most likely occur in their native conformations as it is performed in vivo, resulting an improved sensitivity and accuracy of interaction (Lin and Lai, 2017). Plenty of studies have admitted that Y2H is the most preferred, cost-effective, versatile and throughput approach towards PPI screening (Lentze and Auerbach, 2008; Caufield *et al.*, 2012; Rajagopala, 2015; Mehla *et al.*, 2017; Moosavi *et al.*, 2017).Y2H has been extensively applied to analyze host-virus PPI (Gladue *et al.*, 2012; Silva *et al.*, 2013; Gladue *et al.*, 2018).

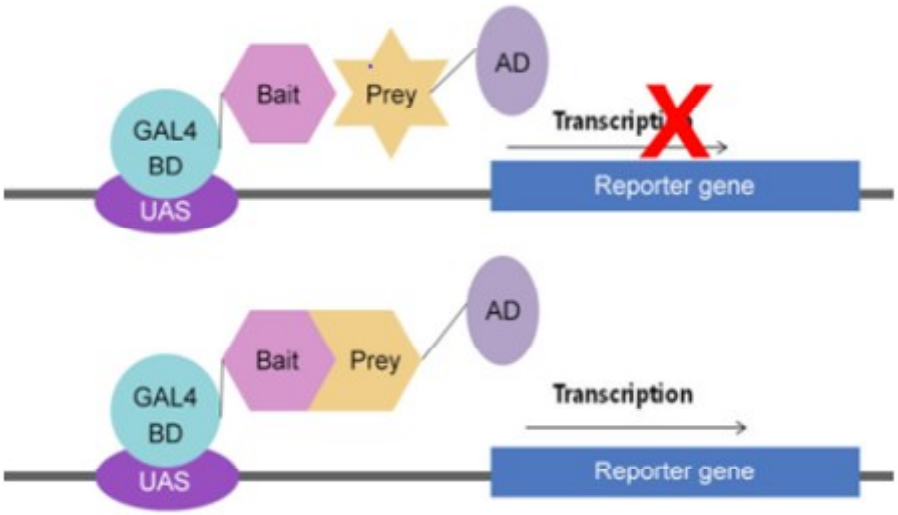


Fig. 2 : Yeast-two hybrid system principle

2.8.2. Application of Yeast two-hybrid in studying virus-host interactions

Y2H helped to identify several candidate partners for the N protein of the measles virus (Surjit *et al.*, 2004). Y2H system has effectively recognized hepatocyte proteins working collaboratively with hepatitis B virus (HBV) S protein (Bai *et al.*, 2005). Interaction of human death domain-attached protein Daxx, a Fas-linked protein with capsid protein of dengue virus (DENV C) has been successfully revealed through Y2H (Limjindaporn *et al.*, 2007). Efficient Y2H screening of envelope proteins (E1 and E2) of chikungunya virus **(**CHIKV**)** spotted five host proteins interacting with E1 and three proteins interacting with E2 (Dudha and Gupta,

2016). For the first time, DEV UL24- interactors were explored using the DEV-CHv strain post-infected DEF cells cDNA library, and their functions were studied (Gao *et al.*, 2017). Y2H screening of nsP2 protein of CHIKV as bait with cDNA library from the human fetal brain has pinpointed seven proteins from the host as putative partners explaining CHIKV nsP2-host interaction (Rana *et al.*, 2017). Murine hepatitis virus (MHV) NSPs have also been screened with Y2H (Vidalain *et al.*, 2015). Using Y2H, RelA(p65) has demonstrated as a novel binding partner for the 2C protein of enterovirus 71 (EV71). Additionally, its innate immunity antagonizing mechanism is also described (Du *et al.*, 2015). In human cytomegalovirus (HCMV), Y2H confirms interaction between the RL13 protein and NUDT14 protein, and that NUDT14 may have the potential to modulate the viral infection (Wang *et al.*, 2016). Caspase-1 in mammalian cells has been identified as the interacting host partner against human parainfluenza virus type 2 (hPIV2) V protein (Ohta *et al.*, 2018).

Along with the viruses of human importance, Y2H has been immensely used for the virus of veterinary relevance. Avian influenza virus protein interactions screening using Y2H elucidates the interaction between virus nucleoprotein and cellular proteins (Wensi *et al.*, 2010). Y2H has also determined that PB2 of the influenza virus is the only subunit that interacts with ANP32A (Wei *et al.*, 2019). Intraviral PPI of Chandipura virus was deciphered with Y2H (Kumar *et al.*, 2012). In the case of porcine reproductive and respiratory syndrome virus (PRRSV) Y2H screening characterized Poly(A)-binding protein (PABP), a host cellular protein as an interactor for nucleocapsid (N) protein in porcine alveolar macrophages (PAMs) (Wang *et al.*, 2012b). Also, Li *et al.* (2018) have stated that nonstructural protein 12 (Nsp12) of PRRSV interacts with Porcine galectin-3 (GAL3) from PAMs cDNA library, claiming Nsp12 role in PRRSV pathogenesis. Y2H applied to identify CSFV NS5A communicating proteins against the swine umbilical vein endothelial cell (SUVEC) cDNA library has unmasked16 interacting partners mostly concerned with gene transcription and protein metabolism (Zhang *et al.*, 2014) (Zhang *et al.*, 2014). Y2H screening of NS4B protein of CSF with porcine alveolar macrophage cDNA library has highlighted 14 interacting proteins (Lv *et al.*, 2018). Apart from this, Gladue *et al.* (2018) employed Y2H for the detection of CSFV p7 interaction with an integral ER transmembrane protein, CAMLG. A study conducted by Wan and coworkers

(2011) exercising the Y2H system underlined that Hdj2 a heat-shock protein 40, from a mouse brain cDNA library interact with JEV nonstructural protein 5 (NS5) which encodes viral RdRp and has a role in JEV replication. Host proteins interacting with the 2C protein of the Foot-and-mouth disease virus (FMDV) were described using Y2H (Gladue *et al.*, 2013; Mahajan *et al.*, 2021). Along with this, N-myc and STAT interactor (Nmi) proteins are identified as FMDV 2C interacting partners explaining the crucial role of Nmi in FMDV 2C-provoked apoptosis (Wang *et al.*, 2012a). A modified version of Y2H used to find interactions involving proteins from vesicular stomatitis virus (VSV). Y2H has proved interaction among VP2 and VP3 protein of Chicken anemia virus (CAV) with different independent interactive domains within the two proteins (Sun *et al.*, 2018). Quite recently, Y2H assay used to study Porcine circovirus type 2 (PCV2) ORF4 protein allied in virus-induced apoptosis and found four interacting host partners (FHC, SNRPN, COX8A, and Lamin C) (Lv *et al.*, 2015). Y2H screening of S1 of Transmissible gastroenteritis coronavirus (TGEV) with porcine intestinal cells cDNA library resulted in 12 intracellular proteins as interactors (Yuan *et al.*, 2019).

2.8.3. Yeast two-hybrid in BTV

In BTV, Y2H brought to light that the NS3 protein C-terminal domain interacts with the VP2 protein of an entirely assembled virion, implying that NS3 may act as a bridge that makes assembled virion communicate with the cellular export machinery (Beaton *et al.*, 2002). The study has additionally turned out that NS3 is unable to interact with VP5 via its cytoplasmic C-terminal domain. Another cellular interacting partner for NS3 identified by Y2H is S100A10/ p11, a cellular trafficking protein subunit of the calpactin complex. But, this protein interacts only with NS3, not with NS3a, and involves the N-terminal of NS3 (Celma and Roy, 2011). Further Y2H has revealed that the first 13 aa of NS3 carries a putative amphiphatic helix having importance in binding with p11, suggesting precision of interaction (Beaton *et al.*, 2002).

From the above-mentioned literature, it is very much clear that although a lot is known about the contribution of viral protein in the course of infection but still the life cycle of BTV and its behavior inside the host cell is fascinating and elusive. BTV has a very small genome and still, it performs a plentiful of activities indicating that the functional capacity of the virus has

been enhanced due to the precursor proteins, coordination between the viral proteins, and most importantly interaction of the viral proteins with the cellular factors. High-throughput Y2H screens have been used extensively to elucidate the PPI and in the recent past, this technique has been fruitfully exploited to identify the interactions between several viral proteins and host proteins. It may be inferred from the literature that NS3 plays a plethora of roles during BTV infection but the exact mechanism of these activities is precisely not known. There is a paucity of reports on interactions between BTV NS3 and host proteins so further studies on similar lines need to be carried out to unravel the mechanism underlying BTV infection. This study aims to firstly, identifying the host proteins interacting with the BTV NS3/NS3a using the Y2H approach and then exploring the role of such interactions in the course of virus infection. The results generated by this study will provide new insights into the investigation of BTV-host interactions, which may help to develop better strategies to control BTV.

Materials and Methods

3 MATERIALS AND METHODS

3.1 Materials

A. Tissue, cell line, bacteria and yeast strains

Tissue, cell line, bacteria and yeast strains used in this study for various applications are described in table 3.1.

B. Cloning and expression vectors

Various vectors have been used in this study for the cloning and expression of BTV-NS3, truncated NS3 ($NS3_t$), and the various host proteins. The vectors used and their application is mentioned in table 3.2.

C. Oligonucleotide primers

The primers used for cloning and expression of BTV-NS3 and $NS3_t$ proteins in different host system were designed using genome sequence of BTV serotype 10. The primers for cloning and expression of prey proteins were designed using corresponding sheep ORFs as template. All the primers used in this study were obtained from commercial manufacturers (Eurofins genomics). The sequence of the primers is given in table 3.3.

D. Antibodies

Different primary and secondary antibodies have been used to determine the expression and to characterize the identified interactions. Detail of the antibodies along with dilutions is mentioned in table 3.4.

3.2 Methodology

Yeast two-hybrid compatible cDNA libraries from sheep lung tissue and naïve BHK-21 cells were constructed.

3.2.1 Construction and characterization of sheep lung cDNA library

3.2.1.1 Tissue sample

A fresh sheep lung was collected from an abattoir at ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar. The tissue was flash frozen in liquid nitrogen (LN_2) and was stored at -80 °C for further use. The tissue sample was screened for the presence of BTV using the real time reverse transcriptase PCR (q-RT-PCR).

3.2.1.2 Isolation of total RNA

1. The frozen tissue was homogenized with tissue homogenizer (Qiagen, Hilden, Germany) and suspension was used for RNA extraction using the RNeasy® mini kit (Qiagen, Hilden, Germany).

    a. The frozen tissue was homogenized in RLT buffer containing β-ME in it (10 µl of β-ME in 1 ml RLT buffer) followed by homogenization using a needle and syringe.
    b. The lysate was centrifuged for 3 min at full speed. The supernatant was carefully removed by pipetting, and transferred to a new microcentrifuge tube. The collected supernatant (lysate) was only used in subsequent steps.
    c. One volume of 70% ethanol was added to the cleared lysate, and mixed immediately by pipetting.
    d. 700 µl of the sample was transferred to a RNeasy spin column placed in a 2 ml collection tube, and centrifuged for 15 s at 13,000 rpm. The step was repeated with the remaining lysate.
    e. The flow-through was discarded and 700 µl buffer RW1 was added to the column, and centrifuged for 15 s at 13,000 rpm to wash the spin column membrane.
    f. 500 µl of buffer RPE was added to the column, and centrifuged for 15 s at 13,000 rpm to wash the spin column membrane.

Table 3.1: Cell lines, bacteria and yeast strains used in this study

Name	Features	Application	Source
Lung tissue	Sheep lung tissue	Library construction	Abbatoir, IVRI
BHK-21	Baby hamster kidney cell line	Propagation of BTV, library construction	ICAR-IVRI
HEK 293 T	Human embryonic kidney	Plasmid transfection	ICAR-IVRI
DH5α	E.coli (F^- 080lacZOM15 O(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk^-, mk^+) phoAsupE44 Z^-thi^- 1 gyrA96 relA1)	General purpose cloning host	Invitrogen, USA
BL21(DE3)pLysS	E. coli (F-ompT hsdSB (rB- mB-) gal dcm (DE3) pLysS(CamR)	Prokaryotic expression of GST-and His-tagged proteins	Novagen, USA
Y2HGold	S. cervesiae (ADE2, HIS3, MEL1, and AUR1-C reporters)	Cloning and expression of bait proteins, co-transformation for confirmation of interacting proteins	Clontech, USA
Y187	S. cervesiae(MATa,ura3-52, his3-200, ade2-lol, trpl) 90l, leu 2-3, ll2, gal4A, , met–, gal80A, URA3: GAL1UAS-GAL1TATA-lacZ, MEL1	Cloning and expression of prey proteins	Clontech, USA

Table 3.2: Vectors used in this study

Name	Characteristics and use	Source
pGBKT7-BD	Kanamycin resistance marker(Kan^R); TRP1 Auxotrophic marker; Expression of GAL4 DNA-BD fusion bait protein; Encodes c-myc tag	Clontech, USA
pGBKT7-Rec	Amp^R; LEU2 Auxotrophic marker; cloning of ADfusion prey cDNA library; Encodes Haemagluttinin (HA) tag	Clontech, USA
pGBKT7-AD	Amp^R; LEU2 Auxotrophic marker; Expression of GAL4 AD fusion prey proteins; Encodes HA tag	Clontech, USA
pGBKT7-53	Positive control bait plasmid that expresses the GAL4 DNA-BD fused with murine p53 protein in pGBKT7 DNA-BD vector	Clontech, USA
pGBKT7-Lam	Negative control bait plasmid that expresses the Gal4 BD fused with lamin in pGBKT7 DNA-BD vector	Clontech, USA
pGBKT7-T	Positive control bait plasmid that encodes the Gal4 AD fused with SV40 large T-antigen in pGADT7 AD vector and is known to interact with p53	Clontech, USA
pM	Amp^R ; DNA-binding domain cloning vector used to express a fusion of a bait protein with the GAL4 DNA-BD in mammalian cells	Clontech, USA
pVP16	Amp^R ; Activation domain cloning vector used to express a fusion of a test protein with VP16 AD, a herpes virus protein that acts as a transcriptional activator in mammalian cells	Clontech, USA
pG5SEAP	Amp^R ; SEAP mammalian reporter plasmid for cotransfection into mammalian cells with recombinant plasmids derived from pM and pVP16	Clontech, USA
pM3-VP16	Amp^R; Positive control plasmid that expresses a fusion of the GAL4 DNA-BD to the VP16 AD	Clontech, USA
pM-53	Amp^R; Positive control plasmid that expresses a fusion of the GAL4 DNA-BD to the mouse p53 protein	Clontech, USA
pVP16-T	Amp^R; positive control plasmid that expresses a fusion of the VP16 AD to the SV40 large T-antigen, which is known to interact with p53	Clontech,USA
pVP16-CP	Amp^R; negative control plasmid that expresses a fusion of the VP16 AD to a viral coat protein, which does not interact with p53	Clontech, USA
pGEX-4T-1	Amp^R; Cloning and expression of GST-tagged fusion proteins in prokaryotic host	GE healthcare, USA
pCMV-Myc-N	Amp^R; Expression of fusion proteins containing the N-terminal c-Myc epitope tag in mammalian cells under human cytomegalovirus immediate early promoter/ enhancer (PCMV IE)	Clontech, USA

Table 3.3: Primers used in this study

Primer code	Name	Sequence (5'-3')	Reference
P1	SMART III oligo	AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG	Clontech, USA
P2	CDS III primer	ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30VN	Clontech, USA
P3	GBK_S10_20F EcoRI	AGAT***GAATTC***ATGCTATCCGGGCTGATC	This study
P4	GBK_S10_709R SalI	GAC***GTCGAC***GTCAGGTTAATGGCATTTC	This study
P5	GBK_NS3_364R BamHI	AGAT***GGATCC***TCTCTTTAAACCACCTAA	This study
P6	NS3n_r XhoI	GACG***CTCGAG***TTCTTTAGACCACCTAAAT	This study
P7	NS3nt_pM_F EcoRI	AGAT***GAATTC***GCATGCTATCCGGGCTGATC	This study
P8	pCMV-NAP1L1_F Sal	ACTG***GTCGAC***GATGGCAGACATCGACAA	This study
P9	pCMV-NAP1L1_R XhoI	GCAT***CTCGAGC***TCACTGCTGCTTGCACTC	This study
P10	NAP1L1_F_ndeI	ACTG***CATATG***GATGGCAGACATCGACAAC	This study
P11	NAPL1L1_R_SalI	GCAT***GTCGAC***TCACTGCTGCTTGCACTC	This study
P12	VP-NAP1L1_F Sal	ACTG***GTCGAC*** GC ATGGCAGACATCGACAAC	This study
P13	VP-NAP1L1_R HindIII	GCAT***AAGCTT*** TCACTGCTGCTTGCACTC	This study
P14	GBK_AD_T7F	GTAATACGACTCACTATAGGGCGA	This study
P15	GBK_DNA_BDR	TTTTCGTTTTAAAACCTAAGAGTCACT	This study
P16	GAD_ADR	AGATGGTGCACGATGCACAGT	This study
P17	GEXF	CCGGGAGCTGCATGTGTCAGAGG	This study
P18	GEXR	CTGGCAAGCCACGTTTGGTG	This study
P19	pCMV_seqF	GAT CCGGTACTAGAGGAACTGAAAAAC	Clontech, USA
P20	pCMV_seqR	TTGTTGTTGTTAACTTGTTTATTGCA	Clontech, USA
P21	M_VP16_seqR	TTT GTG ATG CTA TTG CTT TAT TTG TAA	This study
P22	M_VP16_seqR	TTTGTGATGCTATTGCTTTATTTGTAA	This study

*RE sites are marked in bold italics

Table 3.4: Antibodies used in this study

S. No	Antibody	Type	Dilution	Source
1	Anti-c-Myc antibody	M/1	1:500	Clonech, USA
2	Anti-NS3 antibody	P/1º	1:4000	Kind gift from Dr Damian Vitour, ANSES, Paris,France
3	GAL4 AD antibody	M/1º	1:2000	Clonech, USA
4	Anti-GST Tag Antibody	M	1:1000	Invitrogen, USA
5	Goat anti-Mouse IgG-Hrp	2º	1:1000	SantaCruz, USA
6	Goat anti-Rabbit IgG-HRP	2º	1:4000	SantaCruz, USA

*P= Polyclonal; M=Monoclonal; 1º = Primary; 2º = Secondary

g. Again 500 µl of buffer RPE was added to the column, and centrifuged for 2 min at 13,000 rpm to wash the spin column membrane.

h. The column was subjected to empty spin at full speed for 1 min to remove the residual flow-through.

i. The column was placed in a new 1.5 ml microcentrifuge tube and 30 µl RNAse-free water was added on to the column.

j. The column was centrifuged at 13,000 rpm for 1 min. The eluate was collected and stored at -80 ºC for further use.

2. The concentration and purity of the isolated total RNAs was determined using nanodrop spectrophotometer (Eppendorf, Hamburg, Germany).

3. The isolated total RNA was analyzed on agarose gel electrophoresis for its integrity.

3.2.1.3 Isolation of mRNA

1. Isolation of poly(A) mRNA from total RNA was carried out using MN-*NucleoTraP*® mRNA kit (Machery-Nagel, Germany).

a. 500 µl of buffer RM0 was added to the total RNA sample isolated.

b. Oligo(dT) Latex beads were vortexed and 15/ µL of beads were added per 100/ µg total RNA. The mixture was incubated initially at 68 °C for 5 min and subsequently, at room temperature (RT) for 10 min. The tube was inverted at an interval of 2 mins during incubation and centrifuged for 15 s at 2,000 x g, and then for 2 min at 11,000 x g.

c. The supernatant was discarded and latex beads were re-suspended in 600 µl of buffer RM2.

d. Oligo(dT) latex beads suspension was transferred into the NucleoTrap® Microfilter placed in a microcentrifuge tube and centrifuged for 15 s at 2,000 x g and then for 2 min at 11,000 x g.

e. The flow-through was discarded and washed twice with 500 µl of buffer RM3.

f. The column was centrifuged again for 1 min at 11,000 x g to completely remove the washing buffer.

g. 20/ µL of prewarmed (68 °C) RNase-free H2O was added per 10/ µL Oligo(dT) latex beads and beads were completely re-suspended by pipetting up and down. NucleoTrap® Microfilter was incubated at 68 °C for 7 min and centrifuge for 1 min at 11,000 x g to collect the eluate.

2. The isolated mRNA was vacuum concentrated using Concentrator plus™ (Eppendorf, Germany).

3. The quantity and integrity of the isolated mRNA was detected by nanodrop spectrophotometer and agarose gel electrophoresis, respectively.

3.2.1.4 cDNA synthesis

1. The isolated mRNA was utilized for single stranded (ss) cDNAs synthesis using Make Your Own "Mate & Plate" Library System (Clontech, USA).

2. The following reagents were mixed in a sterile microcentrifuge tube:

mRNA	2.0 µl
CDS III primer (table 3.3)	1.0 µl
NFW	1.0 µl

3. The reaction mixture was incubated at 72 ºC for 2 min followed by snap chill on ice for 2 min, then spun down briefly.

4. Following components were added to the denatured primed RNA sample and was incubated at 42 ºC for 10 min.

5X First strand Buffer	2.0 µl
100 mM DTT	1.0 µl
10 mM dNTP	1.0 µl
SMART MMLV Reverse Transcriptase	1.0 µl

5. Then, 1 µl of SMART III modified oligo (Table 3.3) was added to the mix and the reaction was further incubated at 42 ºC for 1 h.

6. The reaction was terminated by incubation at 75 ºC for 10 min followed by snap-chill.

7. RNase H (1 µl) was added to the mix and incubated at 37 ºC for 20 min.

8. The ss cDNA was then amplified by long distance PCR (LD-PCR) using Advantage 2 PCR kit (Clontech, USA).

   a. Two 100 µl reactions were set up for one ss cDNA as follow:

First-Strand cDNA	2 µl
10X Advantage® 2 PCR Buffer	10 µl
50X dNTP Mix	2 µl
5' PCR Primer	2 µl
3' PCR Primer	2 µl
10X Melting Solution	10 µl
50X Advantage 2 Polymerase Mix	2 µl
NFW	70 µl
Total	100 µl

   b. The PCR was performed under following cycling conditions in thermal cycler (Takara, Japan):

1 cycle	**22 cycles**	**22 cycles**	**1 cycle**
95 ºC	95 ºC	68 ºC	68 ºC
30 s	10 s	6 min*	5 min

*Extension time was increased by 5 s with each successive cycle.

   c. 7 µl aliquote of PCR product was analyzed using 1 % agarose/EtBr gel.

9. The size-fractionation of double stranded (ds) cDNA was carried out using CHROMA-SPIN TE-400 columns (Clontech, USA).

10. The purified ds cDNA was precipitated by adding 1/10 volume of sodium acetate and 2.5 volume of ice-cold ethanol.

11. It was kept at -20 °C for 1 h and then centrifuged at 14000 rpm for 20 min at RT.

12. The supernatant was removed and pellet was air dried for 20 min.

13. The pellet was re-suspended in 20 µl of NFW and was ready for library construction by in vivo recombination in yeast.

14. The quantity and quality of the purified ds cDNAs was analyzed with nanodrop spectrophotometer and 1 % agarose/EtBr gel, respectively.

3.2.1.5 Construction of sheep lung cDNA library

1. The purified ds cDNA and SmaI-linearized pGADT7-rec vector were co-transformed into *S. cerevisiae* Y187 strain by lithium acetate (LiAc) transformation method (Gietz *et al.*, 1995).

2. The competent Y187 yeast cells were prepared using the Yeastmaker Yeast Transformation System 2 (Clontech, USA).

    a. Briefly, one colony of the Y187 was inoculated into 3 ml of YPDA broth (Clontech, USA) in a sterile 15 ml tube and incubated at 30 °C with shaking at 220 rpm for 10 h.
    b. 5 µl of the culture was then added to 50 ml of YPDA in a 250 ml flask.
    c. The culture was incubated for ~18 h at 30 ºC (220 rpm) till OD_{600} reaches 0.15-0.3.
    d. The cells were centrifuged at 700 g for 5 min at RT. The supernatant was removed and pellet was re-suspended in 100 ml of fresh YPDA broth.
    e. The culture was further incubated for ~4 h at 30ºC (220 rpm) till OD_{600} reaches 0.4-0.5.
    f. The culture was divided into two 50 ml sterile tubes and centrifuged at 700 g for 5 min at RT. The supernatant was removed and each pellet was re-suspended in 30 ml of autoclaved deionized water.
    g. The cells were again centrifuged at 700 g for 5 min at RT, supernatant was removed and each pellet was re-suspended in 1.5 ml of 1.1xTE/LiAc (Appendix I).

h. The cell suspension was centrifuged at high speed for 15 s and each pellet was suspended in 600 µl of 1.1xTE/LiAc. The competent cells were ready to use for transformation.

i. The following components were combined in a pre-chilled 15 ml tube:

ds cDNA (2.4 µg)	20 µl
pGADT7-Rec (0.5 µg/µl)	6.0 µl
Denatured Yeastmaker Carrier DNA(10 µg/µl)	20 µl
Competent cells	600 µl
PEG/LiAc (Appendix I)	2.5 ml

3. All the constituents were gently mixed, and incubated at 30 °C for 45 min with intermittent mixing.

4. 160 µl of DMSO was added and the tube was placed in a water bath at 42 °C for 20 min with mixing at every 10 min.

5. The contents were centrifuged at 700 g for 5 min and pellet was re-suspended in 3 ml of YPD plus medium.

6. The cell suspension was incubated at 30 ºC with shaking for 90 min followed by centrifugation at 700 g for 5 min.

7. The supernatant was removed and pellet was re-suspended in 15 ml of 0.9 % NaCl solution.

8. The transformed cells (1 ml) were diluted in YPDA medium and 100 µl of 10^{-1} and 10^{-2} dilutions were grown on 100 mm SD/-Leu (SDO) agar (Clontech, USA) plates at 30 ºC for 3-5 days to calculate the various parameters of transformed library.

9. The rest of the undiluted mixture (14 ml) was spread on 150 mm SDO agar plate (200 µl /plate), and incubated at 30 ºC for 4 days.

10. The resultant transformants were harvested in freezing medium (Appendix I) and stored at -80 ºC in 1 ml aliquots for future use. This pooled mixture constitutes the final cDNA library.

3.2.1.6 Evaluation of cDNA library

1. The constructed cDNA library was evaluated for number of independent clones and transformation efficiency from 10^{-2} dilution SDO agar plate (Gao *et al.*, 2014).

2. The harvested and pooled library was also plated on SDO plates at 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions to calculate cell density, library titer and library quantity (Cao and Yan, 2013).

3. The average insert size and recombination rate of the library were estimated from the randomly selected 50 colonies from each library.

4. The selected colonies were subjected to PCR using Matchmaker InsertCheck PCR Mix 2 (Clontech, USA).

   a. The colonies to be analyzed were touched with the tip and mixed in 12.5 µl of NFW in a 0.2 ml PCR tube.

   b. 12.5 µl of Matchmaker Insert Check PCR Mix was added to each tube, contents were mixed by pipetting and the tubes were placed in a thermal cycler (Takara, Japan) with the following cycling parameters:

Temperature	Time	Cycles
95 °C	1 min	1
98 °C	30 sec	30
68 °C	3 min	

   c. 5 µl of each reaction was analyzed by electrophoresis on a 1% agarose/EtBr gel.

5. The yeast plasmids were extracted from the selected colonies using Easy Yeast Plasmid Isolation kit (Clontech, USA).

   a. The colonies were patched (1 cm^2) on SD/-Leu agar plates and incubated at 30 °C for 3-5 days.

b. The cells were scooped from half of the patch and re-suspended in 500 µl of 10 mM EDTA.

c. The cells were centrifuged at 11000 g for 1 min and pellets were re-suspended in 200 µl of ZYM Buffer.

d. The zymolyase (20 µl) was added to the cell suspension and mixed gently. The contents were incubated with gentle shaking at 30 ºC for 1 h.

e. The spheroplasts were centrifuged at 2000 g for 10 min and pellets were re-suspended in 250 µl of Y1 Buffer/RNase A solution.

f. The Y2 Lysis Buffer (250 µl) was added to the contents and mixed gently.

g. After incubation at RT for 4 min, 300 µl of Y3 Neutralization Buffer was added to the suspension and mixed gently.

h. The mixtures were then centrifuged at 11000 g for 5 min.

i. The supernatants were then loaded onto the columns and centrifuged at 11000 g for 1 min.

j. The column flow-through was discarded and 450 µl of Y4 Wash Buffer (diluted with ethanol) was added to each column. The columns were centrifuged at 11000 g for 3 min.

k. The column flow-through was discarded and columns were centrifuged again to remove any residual Wash Buffer.

l. The spin columns were placed in a 1.5 ml microcentrifuge tube and 50 µl of YE Elution Buffer was added.

m. The columns were incubated at RT for 1 min and the plasmids were eluted by centrifugation at 11000 g for 1 min.

6. The yeast plasmids were re-transformed into competent *E. coli* DH5α cells by chemical transformation method (Sambrook and Russell, 2001; appendix II) and transformed cells were then spread on LB-Amp (Appendix I) agar plates. The plates were incubated overnight at 37 °C.

7. The putative recombinant colonies were then inoculated in LB-Amp broth (180 rpm; 37 °C; overnight) for plasmid extraction using QIAprep® Spin Miniprep Kit (Qiagen, Germany).

a. The overnight bacterial cultures (3 ml) were centrifuged at 12000 rpm for 3 min and supernatants were removed.

b. The pellets were reconstituted with 250 µl of Resuspension Buffer (P1) containing RNase A and mixed to obtain a homogenous suspension.

c. 250 µl of P2 Lysis buffer was added to the tubes. The contents were mixed gently by inverting several times till the lysate became clear.

d. 350 µl of Neutralization Buffer (P3) was added and the contents were mixed immediately by inverting the tubes.

e. The lysate was centrifuged at 12000 rpm for 10 min and the supernatants were loaded onto the spin columns.

f. The flow-through was discarded and 500 µl of Wash Buffer (PB) was added to the each column.

g. The flow-through was discarded and 750 µl of Wash Buffer (PE) with ethanol was added to the columns.

h. The columns were centrifuged at 12000 rpm for 1 min. The flow-through was discarded and columns were again centrifuged at 12000 rpm for 1 minute.

i. The spin columns were placed in a sterile 1.5 ml microcentrifuge tube and 50 µl of Elution Buffer (EB) was added to the center of the column for elution.

j. The columns were incubated at RT for 1 min followed by centrifugation at 12000 rpm for 1 min.

k. The purified plasmids were quantified by nanodrop spectrophotometer and stored at -20 °C till further use.

8. The extracted plasmids were sent for nucleotide sequencing (Eurofins, India)

3.2.1.7 Nucleotide sequencing and analysis

1. 50 clones were randomly selected from sheep lung cDNA library and were nucleotide sequenced with a single pass reading from the 5'end using primer P14 (Table 3.3).
2. Raw sequences were manually trimmed to remove the vector and low-quality sequences.
3. The assembled cDNA sequences were subjected to BLAST search against the non-redundant (nr) GenBank database to compare them with the currently available ESTs and genes.

4. The BLASTn results with bit scores >80 and e-values less than 10^{-10} were considered as significant matches.
5. The ESTs homologous to known proteins were further annotated for GO terms and the GO analysis was carried out using WEB-based GEne SeT AnaLysiS Toolkit (WebGestalt; Zhang *et al.*, 2005; Wang *et al.*, 2013).

3.2.2 Construction and characterization of naïve BHK-21 cells cDNA library

BHK-21 cells cDNA library was constructed and characterized as described in section 3.2.1 except, the total RNA harvested from the uninfected BHK-21 cells was used as a stating material. Nucleotide sequence analysis for naïve BHK-21 cells cDNA library was not done.

3.2.3. Forward yeast two-hybrid library screening

The Y2H assay was performed to preliminary screen the host proteins interacting with the BTV-NS3 protein. The test was carried out using Matchmaker Gold Yeast Two-Hybrid System (Clontech, USA) where a bait protein is expressed as a fusion to the Gal4 DNA-BD in pGBKT7 vector and, while prey proteins are expressed as fusions to the Gal4 AD in pGADT7-vector. This test was based on the activation of four independent reporter genes (AUR1-C, ADE2, HIS3, and MEL1) which were transcribed in case of positive interaction between bait and prey proteins.

3.2.3.1 Generation of pGBKT7-NS3 bait

1. BHK-21 cells were infected with BTV serotype 10 and observed for the development of cytopathic effects (CPE). The viral dsRNA was extracted from BTV-infected BHK 21 cells by TRI reagent, which is basically a modification of acid guanidium thiocyanate-phenol-chloroform method of RNA extraction followed by sequential precipitation with lithium chloride (LiCl) as per the following protocol:
    a. BTV infected BHK-21 cells were harvested after 72 hours post infection, cells were detached gently from the flask (75 cm^2) using the scraper and the content was pelleted out by centrifugation at 1200 x g for 15 minutes at 4 °C.

b. Supernatant was discarded and 200 µl of TE buffer was added to the cell pellet, mixed properly by vortexing and transferred to three small tubes (2ml DNase-RNase free tubes).

c. To the cell suspension 200 µl of lysis buffer (Appendix IV) was added into each tube, mixed properly and kept for 5-10 minutes at 37 °C.

Note: Lysis buffer has to be pre-equilibrated at 37 °C, it should be clear in appearance by means of dissolved SDS.

d. Proteinase K (stock 20 mg/ml) was added in each tube at the rate of 1/20th volume i.e. approximately 15 µl and incubated at 56 °C for 1 h in a water bath.

e. To the cell lysate 1 ml of TRI reagent (Sigma, USA) was added, mixed well and kept at RT for 10 min.

f. 200 µl of chloroform (0.2 volume of TRI reagent) was added to each tube, mixed thoroughly by vigorous shaking for 15 seconds and kept at room temperature for 5-10 mins. The suspension was then centrifuged at 12000 x g for 15 min at 4 °C.

g. The upper aqueous phase containing total RNA was transferred carefully to another tube without disturbing the interphase.

h. Equal volume of isopropanol was added, mixed thoroughly and incubated on ice for 10-20 mins.

i. The mixture was centrifuged at 12000 x g for 15 min at 4 °C. The supernatant was discarded and pellet was washed with 1 ml of 70% ethanol .

j. After discarding the ethanol the pellet was dried briefly in the incubator and then the pellet was dissolved in 200 µl of TE.

k. In each tube equal volume i.e. 200 µl of 4M LiCl was added and incubated at 4 °C overnight, followed by centrifugation at 12,000 x g for 15 min.

l. The supernatant containing viral dsRNA was collected and equal volume i.e 400 µl of 8 M LiCl was added to it and precipitated overnight at 4 °C. It was then centrifuged at 12,000 x g for 15 min at 4 °C.

m. The supernatant was discarded and the pellet containing viral dsRNA was washed with 70% ethanol, air-dried and dissolved in 50 µl of TE/NFW and stored at -80 °C for further use.

2. Electrohoresis of BTV dsRNA in polyacrylamide gel

Polyacrylamide gel electrophoresis of purified dsRNA (RNA-PAGE) was conducted out as described below:

a. Native polyacrylamide gel (without SDS), 10% resolving gel topped with 5% stacking gel (appendix IV) was casted in gel casting assembly.
b. The assembly was mounted in electrophoresis tank.
c. The dsRNA sample (approximately 1 μg) was mixed with 2X RNA gel loading dye and was loaded into the well.
d. The electrophoresis was carried out at 140 V (constant) till the dye comes out.
e. The resolved segments of dsRNA were visualized by sliver nitrate staining.

3. Silver nitrate staining of dsRNA segments

a. The gel was carefully removed after electrophoresis and fixed with fixing solution (appendix IV) for 20 min at RT.
b. The gel was washed thoroughly and gently with DW. Three washings, each for 2 min were given with gentle shaking.
c. Then the gel was transferred in to the staining solution (appendix IV) and incubated at RT for 30 min with intermittent shaking. During this time developing solution was prepared and kept on ice.
d. The gel was washed again with DW and transferred immediately into the ice cold developing solution in a clean reservoir. Washing and transfer of gel has to be done very quickly (within 30 sec) for better signal.
e. The gel was developed slowly at RT by gentle shaking. dsRNA segments were visible approx. after 5 min of incubation. The reaction was stopped immediately by quick washing of gel with DW followed by addition of stopping solution (appendix IV) at 37 °C.
f. The migration pattern of dsRNA segments were observed as electropherogram.

4. The first strand cDNA synthesis was carried out using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

a. The following contents were added in a 0.2 ml PCR tube

Total RNA	8.0 µl
Random hexamer primer	2.0 µl
NFW	2.0 µl

b. The contents were mixed, centrifuged and incubated for 5 min at 65 °C in a thermal cycler.

c. The tube was removed on ice, spun briefly and replaced on ice.

d. To the denatured primed RNA, the following components were added:

5X Reaction Buffer	4.0 µl
RiboLock RNase Inhibitor (20 U/µL)	1.0 µl
10 mM dNTP Mix	2.0 µl
RevertAid M-MuLV RT (200 U/µL)	1.0 µl

e. The contents were mixed and incubated for 5 min at 25 °C followed by 60 min at 42 °C

f. Lastly, the reaction was terminated by heating at 70 °C for 5 min.

5. The NS3 coding region of BTV 10 was amplified using specific primers (P3 and P4; Table 3.3) and DreamTaq DNA Polymerase (Thermo Scientific, USA).

a. The following components were added in 0.2 ml PCR tube:

Components	**Volume (µL)**
10X DreamTaq Buffer	5
10 mM dNTP mix	1.5
FP-P3 (10 iM)	2
RP-P4 (10 iM)	2
Template cDNA (50 ng)	4
DreamTaq DNA Polymerase	0.35
NFW	35.15
Total volume	50

b. The components were mixed, spun briefly and tubes placed in thermal cycler under following cycling parameters

Conditions	Temperature	Time	Cycles
Initial denaturation	95 °C	2 min	
Cyclic denaturation	95 °C	30	
Cyclic annealing	55 °C	30	35
Cyclic extension	72 °C	40	
Final extension	72 °C	10	

6. The amplified NS3 gene product was run on 1.5 % agarose gel and was purified using QIAquick® gel extraction kit (Qiagen, Hilden, Germany).

a. Three volumes of Buffer QG (600 µl) were added to one volume of gel (200 µg) and incubated at 50 °C for 10 min in a water bath to dissolve the gel.

b. 800 µl of sample from above step was applied to the QIAquick column and centrifuged at 13000 rpm for 1 min. The filtrate was discarded and the column was placed back into the clean collection tubes.

c. 500 µl of Buffer QG was added again to the column and centrifugation was done at 13000 rpm for 1 min.

d. The column was washed with 750 µl of wash PE buffer with 3 min hold time at RT followed by centrifugation at 12000 rpm for 1 min.

e. The filtrate was discarded and the empty column was centrifuged at 13000 rpm for 1 min to remove the residual ethanol. The QIAquick column was then placed in the 1.5 ml nuclease free tubes.

f. Elution was done by adding 30 µl of Buffer EB to the centre of the QIAquick column followed by incubation at RT for 1 min and then centrifugation at 13000 rpm for 1 min.

g. The gel purified DNA was quantified by Nanodrop spectrophotometer and stored at - 20 °C till further use.

7. The purified NS3 amplicon and the pGBKT7-BD vector were RE digested using *EcoRI*-HF and *BamHI* HF enzymes (NEB, USA), gel purified and subjected to ligation using T4 DNA ligase (NEB, USA).

   a. The following components were added in 0.2 ml PCR tube:

10X Ligation Buffer	1.0 µl
Double digested vector (50 ng)	2.0 µl
Double digested insert (14 ng)	1.0 µl
T4 DNA ligase enzyme	1.0 µl
NFW	5.0 µl

   b. The reaction contents were mixed, spun briefly and incubated overnight at 4°C.

8. The ligation mixture was transformed in competent *E. coli* DH5α and plated on LB-kanamycin (LB-Kan) agar plates (Appendix I).

9. Positive clones were confirmed by colony PCR and plasmids were extracted from the positive transformants as described in step 8 of section 3.2.1.5 and were subjected to nucleotide sequencing using sequencing primers (P14 and P15; Table 3.3).

10. The sequence confirmed pGBKT7-NS3 (100 ng) was transformed to competent *S.cerevisiae* Y2H Gold cells (sec 3.2.1.5; step 2) and plated on SD/-Trp (SDO) agar plates (Clontech, USA).

3.2.3.2 Detection of pGBKT7- NS3 bait expression

1. A single isolated colony of the transformed pGBKT7- NS3 clone was inoculated in 5 ml of SD/-Trp medium (Clontech, USA) and incubated overnight at 30 °C with shaking (220 rpm). The pGBKT7-53 plasmid and Y2H Gold cells were also grown overnight as positive and negative control, respectively.

2. Each of the overnight cultures were added to 50-ml of YPD medium (Clontech, USA) and further incubated at 30°C with shaking (220 rpm) until the OD_{600} reaches 0.4-0.6. Thereafter, the OD_{600} units (OD_{600} * Volume) were obtained for each sample.

3. The cultures were poured into the pre-chilled tubes and centrifuged at 1000 x g for 5 min at 4 °C. The pellets obtained were re-suspended in 50 ml of ice-cold water and centrifuged at 1000 x g for 5 min at 4 °C.

4. The cell pellets obtained were frozen immediately by immersing the tubes in LN_2 for 3 min and stored at -80 °C till further use.

5. The yeast protein extracts were prepared from the cell pellets following TCA method.
   a. The cell pellets were thawed on ice for 20 min and were re-suspended in 100 µl of ice-cold TCA buffer (Appendix I) per 7.5 OD_{600} units of cells.
   b. Each cell suspension was transferred to a 1.5 ml tube containing 100 µl of glass beads and 100 µl of ice-cold 20% TCA (Appendix I) per 7.5 OD_{600} units of cells.
   c. The cells were disrupted by vortexing at RT for 1 min, process of disruption was repeated for 4 times. Tubes were placed on ice for 30 sec in between each vortexing.
   d. Supernatants above the settled beads (1^{st} supernatants) were transferred to fresh 1.5 ml tubes and placed on ice (beads are heavy so no need of centrifugation at this point).
   e. Then, 500 µl of an ice-cold 1:1 mixture of 20% TCA and TCA buffer was added to the tubes containing beads and again vortexed two times at RT each for 1 min, placing tubes on ice for 30 sec in between each vortexing.
   f. Supernatants above the settled beads (2^{nd} supernatants) were transferred to 1^{st} supernatants tubes placed on ice.
   g. Carryover glass beads in the combined cell extracts were allowed to settle for 1 min, then liquid above the glass beads was transfer to a fresh, pre-chilled 1.5 ml tube .
   h. Proteins were pelleted by centrifugation at 14,000 rpm for 10 min at 4°C and supernatants were carefully remove and discarded.
   i. Each pellet was re-suspended in 10 µl of TCA-Laemmli loading buffer (Appendix I) per OD_{600} unit of cells.

6. The pellets were then boiled for 10 min, centrifuged at 14000 rpm for 10 min at RT and resolved on 12.5 % SDS-PAGE gel (Appendix III).

7. The resolved proteins were subsequently transferred from gel to the nitrocellulose membrane (NCM) via western blotting apparatus. The protocol of western blotting is described in detail in appendix III.

8. The NCM was incubated overnight in 3% blocking buffer (Appendix III) at 4 °C.

9. Thereafter, the membrane was washed thrice with TBS-T (Appendix III) for 10 min each and was divided into two parts. One part was incubated with A1 antibody (Table 3.4) while the other part was incubated with A2 antibody (Table 3.4) for 1 h at RT.

10. Subsequently, the membranes were washed again and incubated with A5 and A6 antibodies (Table 3.4) for 1 h at RT.

11. The protein bands were visualized by developing the membrane with DAB-substrate solution.

3.2.3.3 Testing of bait clone for auto-activation

This was done to confirm that the pGBKT7-NS3 bait protein does not autonomously activate the reporter genes in Y2H Gold cells in the absence of a prey protein.

1. pGBKT7-NS3 plasmid (100 ng) was transformed in competent Y2H Gold cells using LiAc transformation method as described above.
2. The transformation mixture was diluted to 1/10 and 1/100 dilutions, and 100 µl of each dilution was spread on SD/-Trp (SDO), SD/-Trp/X (SDO/X) and SD/-Trp/X/A (SDO/X/A) agar plates (Appendix I).
3. The positive (pGBKT7-53 + pGADT7-T) and negative (pGBKT7-Lam + pGADT7-T) diploid controls were also spread on SDO, SDO/X, SDO/X/A, and SD/-Trp/-Leu/X/A (DDO/X/A) agar plates.
4. All the plates were incubated at 30 ºC for 3-5 days and results were recorded.

3.2.3.4 Testing of bait clone for toxicity

This was done to verify that the pGBKT7-NS3 bait in yeast is non-toxic.

1. pGBKT7-BD (empty vector) and pGBKT7-NS3 plasmids were transformed in competent Y2H Gold cells.

2. 100 µl of the 1/10 and 1/100 dilutions of the transformation mixtures were spread on SDO agar plates.

3. All the plates were incubated at 30 °C for 3-5 days and results were recorded.

3.2.3.5 Generation and characterization of C-terminus truncated NS3 bait (pGBKT7-$NS3_t$)

$NS3_t$ was amplified from the BTV 10 dsRNA using P3 and P5 primers (Table 3.3) and was cloned and characterized similar to pGBKT7-NS3 bait.

3.2.3.6 Forward library screening using yeast mating

The constructed sheep lung cDNA library was screened using both the pGBKT7-NS3 and pGBKT7-$NS3_t$ baits as per the following procedure:

1. A large colony of bait was inoculated in SD/-Trp medium and grown overnight at 30 °C with shaking (220 rpm).

2. The culture was centrifuged at 1000 g for 5 min and the pellet was re-suspended in SD/-Trp medium to a cell density of $>1x10^8$ cells/ml.

3. The re-suspended bait culture was mixed with 1 ml of constructed sheep lung library (section 3.2.1) in a sterile 2 L flask. 45 ml of 2X YPDA-Kan medium were added to it and incubated at 30 °C with minimum shaking (30 rpm) for 22 h.

4. The presence of zygotes was checked after 20 h under a phase contrast microscope (40X).

5. The culture was centrifuged at 1000 g for 10 min and the pellet was re-suspended in 100 ml of 0.5X YPDA-Kan medium (Appendix I).

6. The cells were again centrifuged at 1000 g for 10 min and re-suspended in 10 ml of 0.5X YPDA-Kan medium.

7. The mated culture was diluted to 1/10, 1/100, 1/1000 and 1/10000 dilutions and 100 µl of each dilution was plated on SD/-Trp, SD/-Leu and SD/-Leu/-Trp (DDO; Clontech, USA) agar plates.

8. The remainder of the culture was plated on 150 mm DDO/X/A (Appendix I) agar plates (200 µl per plate).

9. All the plates were incubated at 30 °C for 3-5 days and the following parameters were calculated.

   a. Number of screened clones = cfu/ml of diploids on DDO x Re-suspension volume

   b. Viability of prey library = No. of cfu/ml on SD/-Leu

   c. Viability of diploids = No. of cfu/ml on DDO

   d. $$\text{Mating efficiency} = \frac{\text{No. of cfu/ml of diploids}}{\text{No. of cfu/ml of limiting partner}} \times 100$$

   e. The blue colonies grew on DDO/X/A were patched onto higher stringency SD/-Leu/-Trp/- Ade/-His/X/A (QDO/X/A) agar plates (Appendix I).

3.2.3.7 Identification of redundant interacting prey proteins

1. The representative clones of identified prey proteins were streaked 2-3 times on DDO/X/A agar plates. Every time a single blue colony was used for re-streaking to segregate the positive interactors (blue) from non-interactors as yeast cells could harbour one or more prey plasmids which could be either interacting or non-interacting.

2. The segregated positive prey clones were patched on QDO/X/A agar plates and prey plasmids were extracted using Easy Yeast Plasmid Isolation kit (Clontech, USA).

3. The isolated yeast plasmids were re-transformed in competent *E. coli* DH5α cells and the prey plasmids were rescued by selection on LB-Amp agar plates.

4. The rescued prey plasmids were sequenced (Eurofins, India) using sequencing primers (P14 and P16; Table 3.3).

3.2.4 Confirmation of positive interactions by co-transformation

This was done to confirm that the identified interactions are genuine and thus distinguish authentic positive from the false-positive interactions.

1. The following combination pairs were co-transformed into competent Y2H Gold cells.
   a. pGBKT7-$NS3_t$ + pGADT7-Rec-Prey
   b. Empty pGBKT7 + pGADT7-Rec-Prey
   c. pGBKT7-53 + pGADT7-T (Positive control)
   d. pGBKT7-Lam + pGADT7-T (Negative control)
2. All of the transformation mixtures were diluted (1/10 and 1/100) and 100 µl of each dilution was plated on DDO/X/A and QDO/X/A agar plates.
3. All the plates were incubated at 30 °C for 3-5 days and the results were recorded.

3.2.5 β-Galactosidase assays for confirmation of bait-host protein interactions

β-Galactosidase (β-Gal) assays use X-gal as substrate because of its high degree of sensitivity.

3.2.5.1 Colony-lift filter assay

1. Colonies of the following diploids were patched on DDO and QDO agar plates and incubated at 30°C for 1-2 days.
   a. Y2H Gold- pGBKT7-$NS3_t$ + Y187-pGADT7-Rec-prey
   b. Y2H Gold-pGBKT7 + Y187-pGADT7-Rec-prey (Prey control)
   c. Y2H Gold-pGBKT7-53 + Y187-pGADT7-T (Positive Control)
   d. Y2H Gold-pGBKT7-Lam + Y187-pGADT7-T (Negative Control)
2. A 125 mm sterile grade 40 filter paper (HiMedia, India) was pre-soaked in 5 ml of Z buffer/X-gal solution (Appendix I) in a sterile 150-mm plate.
3. Another sterile filter paper was placed on the surface of the diploids to be assayed and its sides were gently pressed to facilitate the attachment of colonies. The filter paper was pierced at three locations for the purpose of identification.

4. The evenly wetted filter paper was lifted with the colonies facing up and immersed in the LN_2 for 10 sec.

5. The filter paper was thawed and placed on the presoaked filter with the colonies side up without any entrapment of air bubbles.

6. The filter papers were incubated at 30 ºC till the blue colonies appeared (but < 8 h).

7. The positive colonies were identified by aligning the filter paper to the agar using orientation marks.

3.2.5.2 O-nitrophenyl-β-D-galactopyranoside (ONPG) liquid culture assay

This assay was performed to verify as well as quantify two-hybrid interactions. This assay aids in comparison of the relative strength of the protein-protein interactions in terms of % β-galactosidase activity or Miller units.

1. Two Colonies of each of the following diploids were inoculated in DDO medium and grown overnight at 30 ºC with shaking (220 rpm).
   a. Y2H Gold- pGBKT7-$NS3_t$ + Y187-pGADT7-Rec-prey
   b. Y2H Gold-pGBKT7 + Y187-pGADT7-Rec-prey (Prey control)
   c. Y2H Gold-pGBKT7-53 + Y187-pGADT7-T (Positive Control)
   d. Y2H Gold-pGBKT7-Lam + Y187-pGADT7-T (Negative Control)

2. Two ml of each overnight culture was added to 8 ml of YPD medium and incubated at 30 ºC with shaking (230 rpm) for 3-4 h. The OD_{600} of the cultures were recorded at the time of harvest.

3. 1.5 ml of each culture was dispensed in three tubes and centrifuged at 13000 rpm for 45s .

4. The pellets were re-suspended in 1.5 ml of Z buffer (Appendix I) and centrifuged again at 13000 rpm for 45 sec.

5. The pellets were re-suspended in 300 µl of Z buffer for 5-fold concentration of cultured cells and 100 µl of each cell suspensions was transferred to fresh tubes.

6. The tubes were frozen immediately in LN_2 (0.5-1 min) and thaw at 37 ºC in water bath (0.5-1 min). The freeze-thaw cycle was repeated for three times.

7. The blank tubes were set up with addition of 100 µl of Z-buffer. 700 µl of Z buffer + β- ME (Appendix I) was added to the reaction and blank tubes.

8. The timer was started immediately and 160 µl of ONPG-Z buffer (Appendix I) was added to the reaction and blank tubes.

9. The tubes were incubated at 30 ºC and the reaction was stopped after the development of yellow colour by addition of 400 µl of 1 M Na_2CO_3 and the time elapsed was recorded.

10. The tubes were centrifuged for 13 min at 13000 rpm to pellet cell debris.

11. The clear supernatants were transferred to cuvettes (Eppendorf, Germany) and the OD_{420} of the samples was measured in a spectrophotometer (Eppendorf, Germany).

12. β-Gal units were calculated using the following formulae:

$$\beta\text{-Gal} = 1{,}000 \times OD_{420}/(t \times V \times OD_{600})$$

Where,

t = elapsed time (in min) of incubation

V = 0.1 ml x concentration factor

OD_{600} = A_{600} of 1 ml of culture

3.2.6 Generation and characterization of pGADT7-NAP1L1 clone

3.2.6.1 Generation of full-length pGADT7-NAP1L1 plasmid

1. The coding region of the NAP1L1 was amplified from the sheep lung cDNA using Dream Taq DNA Polymerase and primers set P10 and P11 mentioned in table 3.3.

2. The amplified NAP1L1 and pGADT7 vector were RE digested and ligated using T4 DNA ligase.

3. The ligation mixture was transformed in competent *E. coli* DH5α cells and plated on LB-Amp agar plates.

4. Plasmids were extracted from the positive transformants and were subjected to nucleotide sequencing using sequencing primers (P14 and P16; Table 3.3).

5. The sequence confirmed pGADT7-NAP1L1 plasmid (100 ng) was transformed to competent *S. cerevisiae* Y187 cells and plated on SD/-Leu agar plates

3.2.6.2 Detection of pGADT7-NAP1L1 expression

1. A single isolated colony of pGADT7-NAP1L1 clone was inoculated in 5 ml of SD/-Leu medium and incubated overnight at 30 °C with shaking (220 rpm). The pGADT7-T plasmid and untransformed Y187 cells were also grown overnight to be served as positive and negative control, respectively.

2. The yeast protein extracts were prepared as described in section 3.2.3.2 and loaded onto the 12.5 % SDS-PAGE gel.

3. The resolved proteins were transferred to the NCM via western blotting.

4. The primary (A3) and secondary (A5) antibodies were added and the blot was developed using DAB-substrate solution.

3.2.7 Small scale mating of bait and prey construct

1. The mating between $NS3_t$ and NAP1L1 prey proteins was carried out as per the set up mentioned below

Type	Bait	Prey
Test Sample	pGBKT7-$NS3_t$	pGADT7-NAP1L1
Bait control	pGBKT7-BD	pGADT7-NAP1L1
Prey Control	pGBKT7-$NS3_t$	pGADT7-AD
Basal control	pGBKT7-BD	pGADT7-AD
Positive Control	pGBKT7-53	pGADT7-T
Negative Control	pGBKT7-Lam	pGADT7-T

2. One colony of prey and bait was inoculated in 500 µl of 2X YPDA and mixed by vortexing.

3. The mixture was incubated at 30 °C (220 rpm) for 22 h.

4. The mated culture was diluted 1/10 and 1/100 in 0.9 % NaCl and 100 µl of each dilution was spread on DDO and DDO/X/A agar plates.

5. The agar plates were incubated at 30 °C for 3-5 days and the results were recorded.

3.2.8 Glutathione S-transferase (GST) pull-down assay

The GST pull-down assay is an in vitro technique employed to confirm binding partners of GST-tagged bait $NS3_t$ protein. This involves immobilization of GST tagged bait protein to glutathione affinity resin which then serves as the secondary affinity support for confirming a previously suspected protein partner to the bait. The GST pull-down assay was carried out using Pierce™ GST Protein Interaction Pull-Down Kit (ThermoScientific, USA). The $NS3_t$-host PPI was visualized by SDS-PAGE and western blotting.

3.2.8.1 Cloning and expression of GST-tagged $NS3_t$ bait protein

1. The $NS3_t$ coding region was amplified from pGBKT7-$NS3_t$ using Dream Taq DNA Polymerase and the primers (P3 and P6; Table 3.3).

2. The gel purified $NS3_t$ and the pGEX-4T-1 vector was RE digested and subjected to ligation using T4 DNA ligase.

3. The ligation mixture was transformed in competent *E. coli* DH5α cells and plated on LB-Amp agar plates.

4. The plasmids from the positive transformants were subjected to nucleotide sequencing using sequencing primers (P17 and P18; Table 3.3).

5. The orientation and sequence confirmed plasmid was termed as GST-$NS3_t$ bait plasmid.

6. The as GST-$NS3_t$ plasmid was retransformed in *E. coli* BL21(DE3) pLysS cells as described above and plated on LB-Amp-Chl agar plates (Appendix I).

7. The recombinant colonies were propagated in 10 ml of LB-Amp-Chl broth and incubated for overnight at 37 °C with shaking at 180 rpm in a shaker incubator.

8. The overnight culture was inoculated in ratio of 1:100 in 10 ml of LB-Amp-Chl broth and incubated for ~2 h at 37°C with shaking at 220 rpm (until OD_{600} reaches 0.6).

9. The cultures were induced for protein expression by addition of IPTG (Novagen, USA) to the final concentration of 1 mM.

10. The cultures were incubated for 5 h at 28 °C with shaking at 220 rpm.

11. The 1 ml aliquot of induced culture was centrifuged at 12000 rpm for 3 min and the pellet was resuspended in STB (Appendix III) to check the protein expression by SDS-PAGE and western blotting using A4 antibody.

12. The rest of the culture was centrifuged at 3500 rpm for 15 min and the pellet was stored at -20 °C for further analysis.

13. The solubility of the expressed protein was assessed by running both the pellet and supernatant fractions of lysed bacterial pellet on 12.5% SDS-PAGE.

3.2.8.2 Bait protein preparation and immobilization

1. The IPTG induced culture (5 ml) was centrifuged at 5000 g for 5 min.

2. The pellet was re-suspended in 1 ml of TBS and centrifuged at 5000 g for 5 min.

3. The pellet was re-suspended in 200 µL of ice-cold TBS. To this, 200µL of Pull-Down Lysis Buffer was added.

4. The cells suspension was incubated on ice for 30 min with intermittent mixing.

5. The suspension was centrifuged at 12000 g for 5 min and the supernatant was collected in a new 1.5 ml centrifuge tube and labeled as "bait lysate".

6. The gluatathione agarose (50 µl) was added in spin column and equilibrated with 1:1 TBS: pull down lysis buffer.

7. The bait lysate (350 µl) was added to the spin column containing glutathione agarose and incubated at 4 °C for 30 min.

8. The incubated mixture was centrifuged at 1250 g for 1 min and bait flow through was collected and placed on ice.

9. 400 µl of wash solution was added to the columns, mixed thoroughly and centrifuged at 1250 g for 1 min.

10. The step 9 was repeated five times and all the wash fractions were collected.

3.2.8.3 Cloning and expression of Myc-tagged prey protein

1. NAP1L1 was amplified from pGADT7-NAP1L1 using P8 and P9 primers (Table 3.3) and subcloned into pCMV-myc vector.

2. The plasmids from the positive transformants were subjected to nucleotide sequencing using sequencing primers (P19 and P20; Table 3.3).

3. The orientation and sequence confirmed plasmid was termed as pCMV-Myc-NAP1L1 plasmid prey plasmid.

3.2.8.4 Preparation of prey protein from mammalian cell lysate and capture

1. The HEK 293 T cells were seeded on a 6-well culture plate for 24 h and were transfected with pCMV-Myc-NAP1L1 plasmid.

2. The cells were trypsinized, scrapped and collected in a sterile 1.5 ml centrifuge tube.

3. The cells were centrifuged at 500 g for 5 min and 1 ml TBS was added to the pellet.

4. The cell suspension was centrifuged at 500 g for 5 min and re-suspended in ice-cold TBS (2.5 ml per gm wet weight of cells) and protease inhibitor cocktail.

5. The pull-down lysis buffer (2.5 ml per gm wet weight of cells) was added and suspension was thoroughly mixed.

6. The cell suspension was incubated on ice for ~30 min followed by centrifugation at 12000 g for 5 min.

7. The supernatant was collected in a new 1.5ml tube marked 'Cell lysate' and stored in ice.

8. The prepared cell lysate was added to the immobilized GST-tagged bait protein.

9. The prey-bait mixture was incubated at 4 ºC for 2 h with gentle rocking.

10. The mixture was centrifuged at 1250 g for 1 min and prey flow-through was collected and placed on ice.

11. 400 µl of wash solution was added to the columns, mixed thoroughly and centrifuged at 1250 g for 1 min.

12. The step 11 was repeated five times and all the wash fractions were collected.

3.2.8.4 Bait-prey elution and analysis

1. The glutathione elution buffer (250 µl) was added to each of the column and incubated for 5 min with gentle shaking.

2. The columns were centrifuged at 1250 g for 1 min and the elution 1 (E1) was collected.

3. Similarly, two more elution fractions (E2 and E3) were also collected.

4. All the elution fractions were run on SDS-PAGE followed by western blotting.

5. The bait-interacting proteins were detected by the A1, A2 and A4 antibodies followed by addition of corresponding 2º antibodies.

3.2.9 Mammalian two-hybrid (M2H) system

This test was carried out to confirm the Y2H identified BTV $NS3_t$-host interactions in mammalian cells. The assay is an important follow-up to yeast screens because it tests interactions under conditions that allow for post-translational changes to hybrid proteins (i.e. phosphorylation, acetylation, proteolysis) not possible in yeast system. The test was carried out using (Matchmaker™ Mammalian Assay Kit 2, Clontech, USA) that employs pM and pVP16 vector to generate Gal4 DNA-BD-bait and AD-prey constructs, respectively. pG5SEAP that contains the secreted alkaline phosphatase (SEAP) reporter gene downstream was used as the reporter vector. The interaction between BTV $NS3_t$ and prey proteins was determined by measuring SEAP activity using the Great EscAPe™ SEAP Chemiluminescence Detection Kit (Clontech, USA).

3.2.9.1 Generation of pM-bait and pVP16-prey clones

1. The $NS3_t$ coding region was amplified from pGBKT7-$NS3_t$ using Dream® Taq DNA Polymerase and the primers (P5 and P7; Table 3.3).

2. The gel purified $NS3_t$ and the pM vector was RE digested and subjected to ligation using T4 DNA ligase.

3. The ligation mixture was transformed in competent *E. coli* DH5〈 cells and plated on LB-Amp agar plates and plasmids from the positive transformants were subjected to nucleotide sequencing using sequencing primers (P19 and P20; Table 3.3).

4. The orientation and sequence confirmed plasmid was termed as pM-$NS3_t$ bait plasmid.

5. Similarly, NAP1L1 prey coding region was amplified from respective pGADT7-prey clones using Dream® Taq DNA Polymerase and respective primers (P12 and P13).

6. The amplified PCR products and pVP16 vector were RE digested and subjected to ligation using T4 DNA ligase.

7. The ligation mixture was transformed in competent *E. coli* DH5α cells and plated on LB-Amp agar plates and plasmids from the positive transformants were subjected to nucleotide sequencing using sequencing primers (P21 and P22; Table 3.3).

8. The sequence confirmed pM-$NS3_t$ and pVP16-prey plasmids were purified in high concentration using PureLink® HiPure Plasmid Midiprep Kit (Invitrogen, USA).

    a. To the HiPure Filter Midi Column, 15 ml of Equilibration Buffer (EQ1) was added and solution was allowed to drain by gravity flow.

    b. The overnight bacterial culture (50 ml) was centrifuged at 3500 rpm for 15 min. The supernatant was removed and 10 ml of Resuspension Buffer (R3) with RNase A was added to the pellet.

    c. The contents were mixed till homogenous and 10 ml of Lysis Buffer (L7) was added. The contents were mixed gently until the mixture is homogeneous and incubated at RT for 5 min.

d. To the cell suspension, 10 ml of Precipitation Buffer (N3) was dded and the contents were mixed immediately by inverting the tube until the mixture is homogeneous.

e. The precipitated lysate was loaded onto the equilibrated HiPure Filter Midi Column and allowed to pass through the filter by gravity flow.

f. The column was then washed with 10 ml of Wash Buffer (W8) and the buffer was allowed to flow through the HiPure Filter Midi Column.

g. The flow-through was discarded and 5 ml of Elution Buffer (E4) was added to the Midi column to elute the DNA. The eluant thus obtained contains the purified DNA.

h. To the purified DNA, 3.5 ml of isopropanol was added, mixed and incubated for 2 min at RT.

i. The mixture was centrifuged at 4000 rpm for 1 h at 4°C. The supernatant was carefully removed and the DNA pellet was re-suspended in 3 ml of 70% ethanol.

j. The mixture was centrifuged at 4000 rpm for 10 min at 4°C and the supernatant was removed carefully.

k. The pellet was air-dried for ~10 min and was re-suspended in 200 µl of TE Buffer (TE).

l. The extracted plasmid concentration was measured by nanodrop and was stored at -20 °C for further use.

3.2.9.2 Cell transfection

For M2H, bait (0.5 µg) and prey plasmid (0.5 µg) along with pG5SEAP (0.25 µg) reporter were transfected in HEK 293 T cells using Lipofectamine Ltx with plus reagent (Invitrogen, USA). The transfection set up is given in table 3.5

Table 3.5: Experimental set up for M2H transfection

Type	Bait	Prey	Reporter
Test Sample	pM-$NS3_t$	pVP16-NAP1L1	pG5SEAp
Bait control	pM	pVP16-NAP1L1	pG5SEAp
Prey Control	pM-$NS3_t$	pVP16	pG5SEAp
Basal control	pM	pVP16	pG5SEAp
Positive Control	pM	pVP16-T	pG5SEAp
Negative Control	pM	pVP16-CP	pG5SEAp

3.2.9.3 Measurement of SEAP activity

1. The conditioned culture medium from transfected and mock transfected cells was transferred to 0.5 ml tubes and centrifuged at 12000 rpm for 1 min to remove cell debris.
2. Meanwhile, the 1X Dilution Buffer was prepared and the SEAP Substrate Solution was equilibrated to RT.
3. The media supernatant (25 µl) was transferred to 0.2 ml PCR tubes and 75 µl of 1X Dilution Buffer was added to each sample.
4. The samples were incubated at 65 ºC for 30 min to remove endogenous alkaline phosphatase.
5. The samples were cooled on ice for 2–3 min, and then equilibrate to RT.
6. 100 µl of SEAP Substrate Solution was added to each sample and incubated for 45 at RT.
7. The samples were then transferred to 96-well opaque white plate (Nunc, Denmark) and the chemiluminiscent SEAP signal was measured using Tecan Infinite 200 PRO (Tecan, Switzerland).

Results

4 RESULTS

4.1 Construction and characterization of Y2H compatible cDNA libraries

4.1.1 Isolation and analysis of total RNA and mRNA

Sheep lung tissue and uninfected BHK-21 cells were used as for the construction of sheep lung and naïve BHK-21 cDNA libraries, respectively. The concentration of the total RNA extracted from sheep lung tissue and uninfected BHK-21 cells was 738 ng/ul and 960 ng/ul whereas the A_{260}/A_{280} ratio was 2.1 and 2.01, respectively. The agarose gel electrophoresis of both RNAs revealed 28S and 18S rRNA bands and the intensity of 28S band was twice as that of the 18S rRNA, thus indicating the quality and purity of extracted total RNA (Fig. 3a-b). The poly(A) enriched mRNAs were isolated from and had a concentration of 76 ng/ul and 90 ng/ul with the A_{260}/A_{280} ratio of 2.2 and 2.25 for sheep lung and uninfected BHK 21 mRNA, respectively. The agarose gel electrophoresis has shown a smear > 6 kb for both mRNAs (Fig.4a-b) suggesting that mRNAs are of high quality and suitable for preparing the libraries. The extracted mRNAs were vacuum concentrated to ~370 ng/ul using the Concentrator plus™ (Eppendorf, Germany).

4.1.2 cDNA synthesis

Approximately 600 ng of mRNAs (1.7 µl) were used for the ss cDNAs synthesis using oligo- d(T) primer. The ds cDNAs were amplified from ss cDNAs by LD-PCR. The number of optimal cycles for amplification of ds cDNAs was found to be 22. On agarose gel electrophoresis, the ds cDNA smear from sheep lung mRNA ranged from <100 bp to 6 kb (Fig. 5a) before purification and ~400 bp to 6 kb (Fig. 5b), after purification. Whereas, the ds

cDNA smear from BHK-21 cells mRNA ranged from <100 bp to 5 kb (Fig.6a) and ~400 bp to 5 kb (Fig. 6b), pre- and post-purification, respectively.

4.1.3 Construction and evaluation of cDNA libraries

Co-transformation of ds cDNA (sheep lung and BHK 21) and linearized pGADT7-rec vector into Y187 cells led to the generation of Y2H compatible cDNA libraries. The resultant sheep lung and naïve BHK-21 cDNA libraries represented 1.68×10^6 and 1.2×10^6 independent clones, and the transformation efficiency of the constructed libraries was found to be 5.6×10^6 and 4×10^6/µg of pGADT7-rec vector, respectively. 50 colonies were randomly selected from the constructed cDNA libraries and were subjected to insert-check PCR, amongst which 49 were found positive for sheep lung cDNA library and 46 were found positive for naïve BHK-21 cDNA library. The insert size of the constructed cDNA libraries ranged between 350 bp to 2500 bp and 300 bp to 2000 bp (Fig. 7a-b). Other quality parameters of library were evaluated as described in table 4.1. Though both the libraries qualified the threshold parameters, sheep lung cDNA library performed better than naïve BHK-21 cDNA library and hence chosen for downstream screening of interacting host proteins.

Table 4.1. Parameters for qualification of cDNA libraries

Parameter	Sheep lung cDNA library	naïve BHK-21 cDNA library
Independent clones	1.68×10^6	1.2×10^6
Transformation efficiency	5.6×10^6/mg of pGADT7-rec	4×10^6/mg of pGADT7-rec
Cell density (pre-freezing)	1.074×10^9 cells/ml	6×10^8 cells/ml
Library titre (pre-freezing)	1.8×10^8 cfu/ml	1×10^8 cfu/ml
Cell density (post-freezing)	2.45×10^8 cells/ml	5×10^7 cells/ml
Library titre (post-freezing)	1.2×10^8 cfu/ml	6×10^7 cfu/ml
Recombination %	98.0	92.0
Insert size	896 bp (350-2500bp)	700 bp (300-2000)

4.1.4 Nucleotide sequencing analysis

The randomly selected 50 clones from sheep lung library were sequenced with a single pass reading from the 5'end using T7 primer. The sequences were analyzed with the top

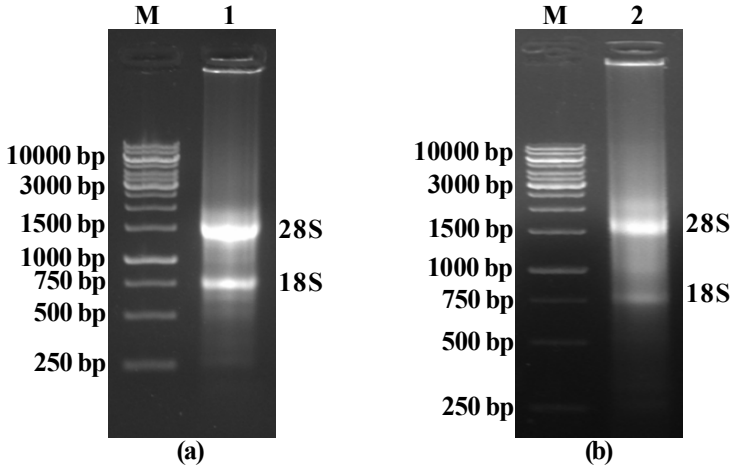


Fig. 3: (a-b) Agarose gel eletrophoresis of total RNA. Lane M: GeneRuler 1kb DNA ladder; **Lane 1:** Total RNA from sheep lung tissue; **Lane 2:** Total RNA from uninfected BHK-21 cells

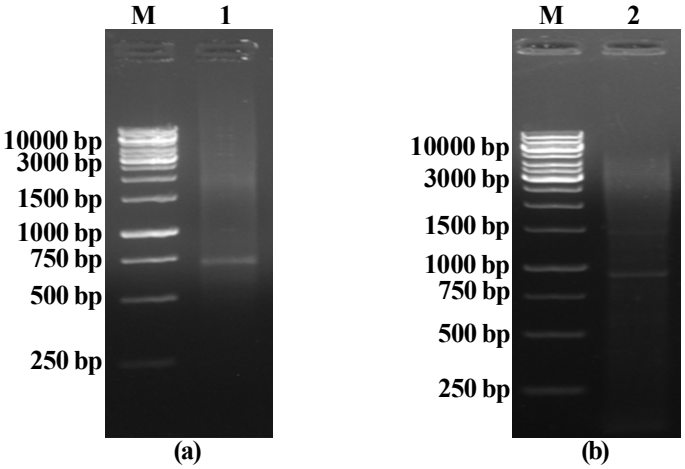


Fig. 4: (a-b) Agarose gel electrophoresis of mRNA. Lane M: GeneRuler 1kb DNA ladder; **Lane 1:** mRNA from sheep lung; **Lane 1:** mRNA from uninfected BHK-21 cells

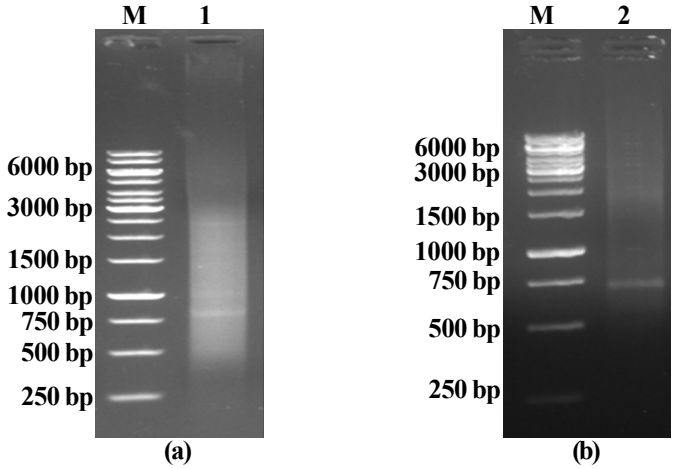


Fig. 5: (a-b) Analysis of ds cDNA of sheep lung on 1% agarose gel. Lane M: GeneRuler 1kb DNA ladder; **Lane 1:** Unpurified ds cDNA; **Lane 2:** Purified ds cDNA

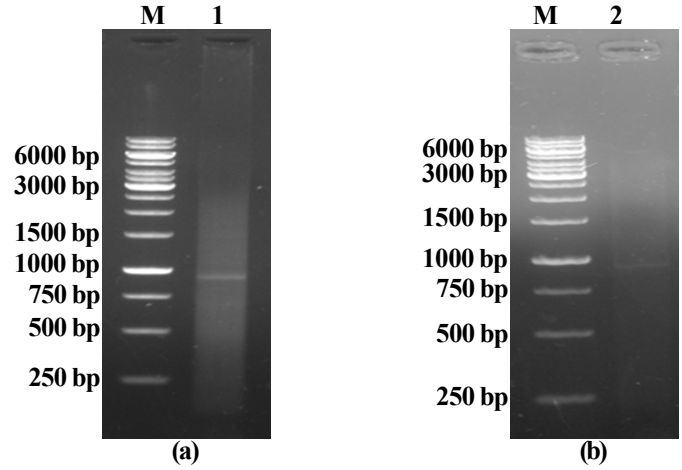


Fig. 6: (a-b) Analysis of ds cDNA of uninfected BHK-21 cells on 1% agarose gel. Lane M: GeneRuler 1kb DNA ladder; **Lane 1:** Unpurified ds cDNA; **Lane 2:** Purified ds cDNA

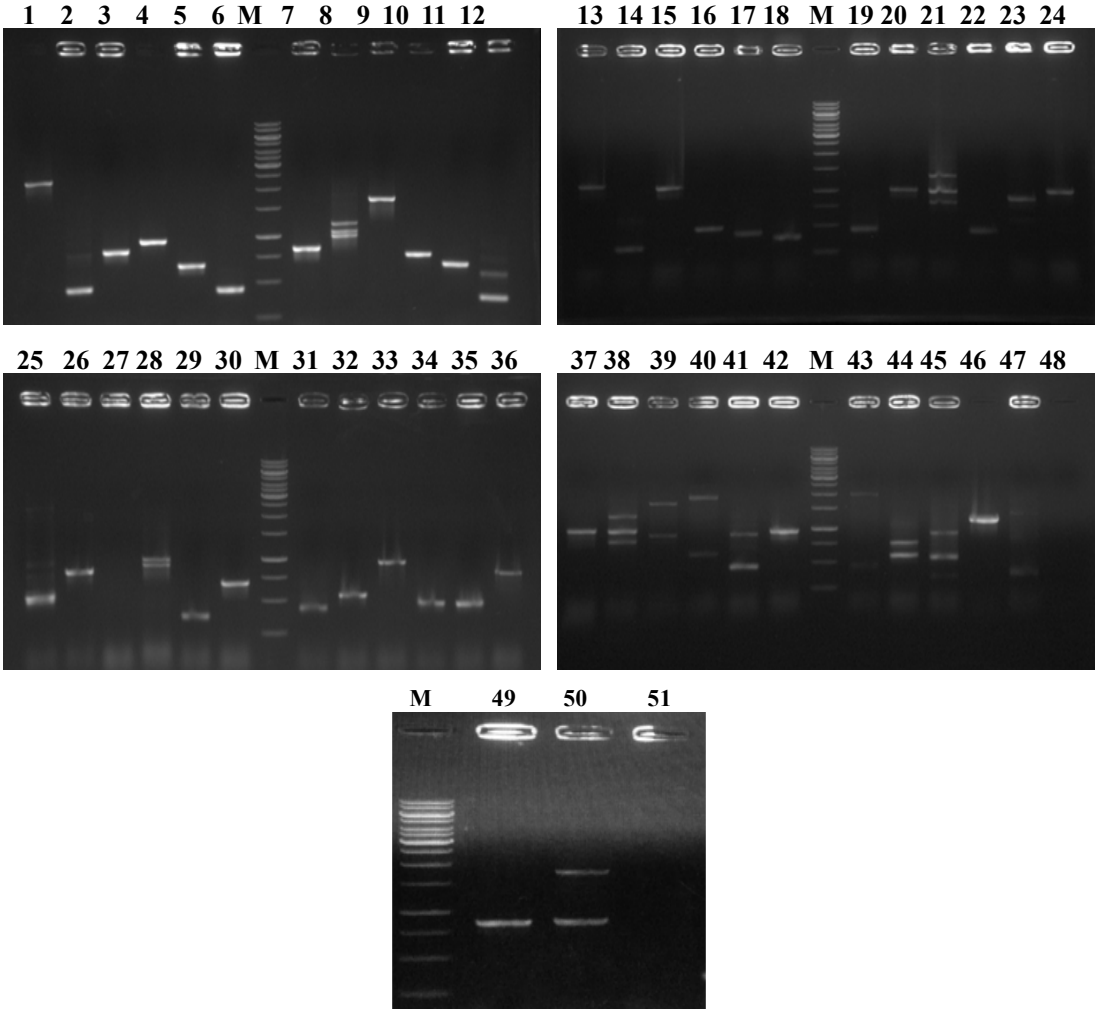


Fig. 7a: Insert-check PCR to identify insert size for sheep lung cDNA library.

Lane M : GeneRuler 1kb DNA ladder

Lanes 1 to 50 : Randomly picked recombinant clones

Lane 51 : NTC

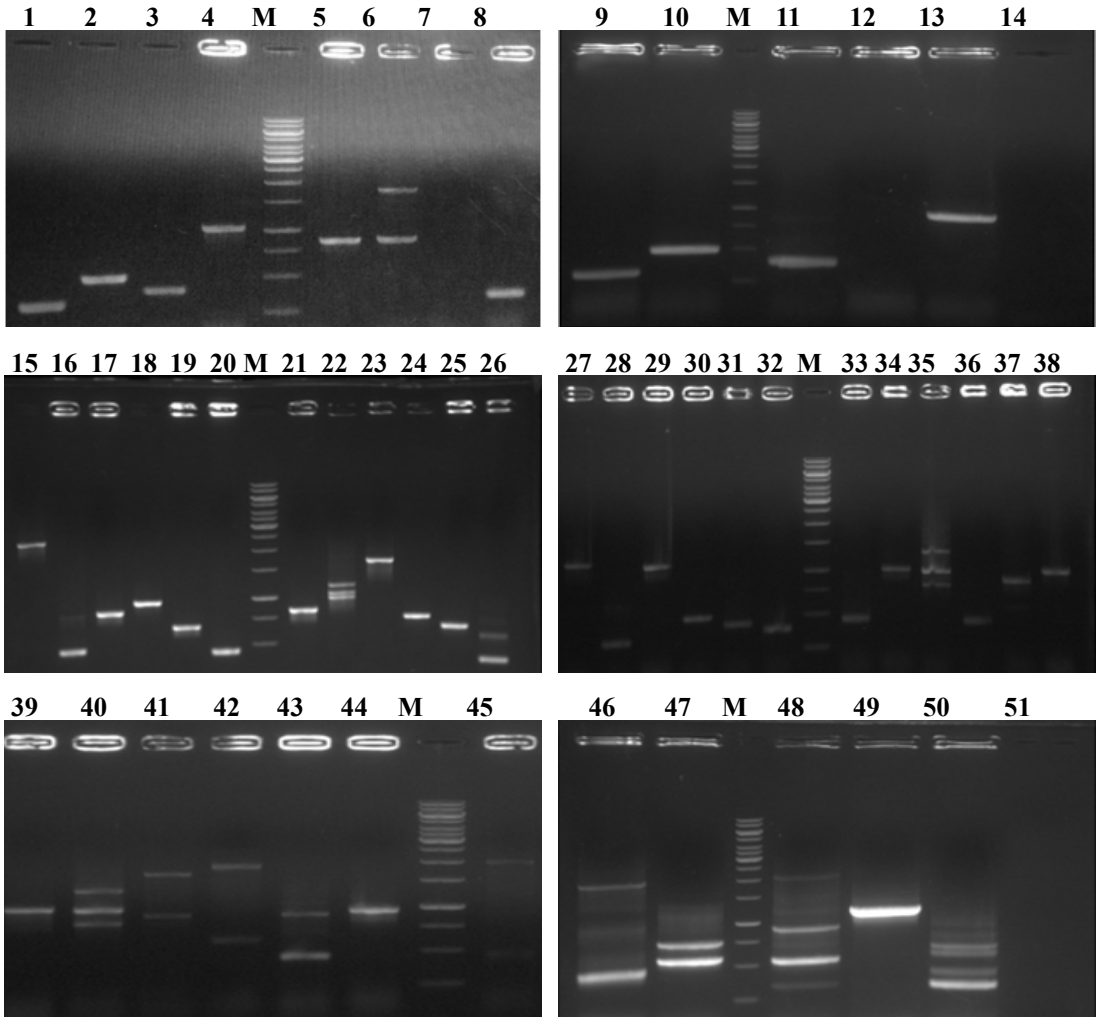
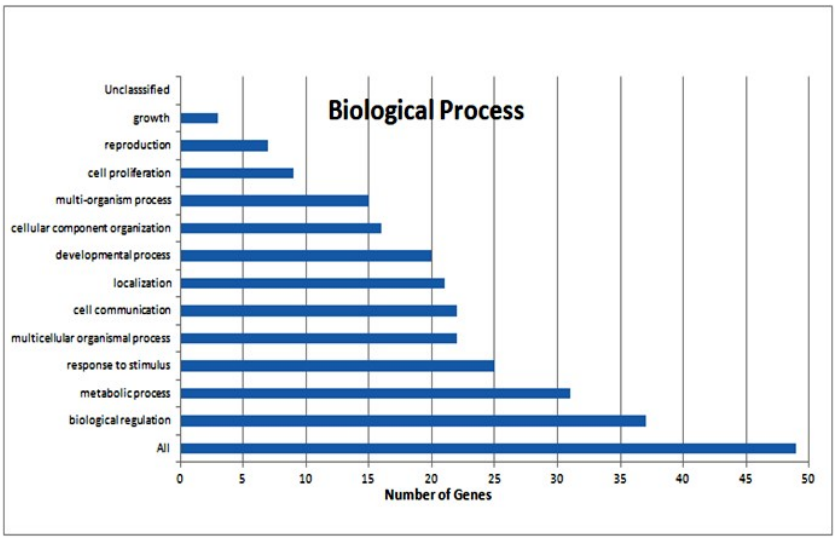


Fig. 7b: Insert-check PCR to identify insert size for uninfected BHK-21 cDNA library.

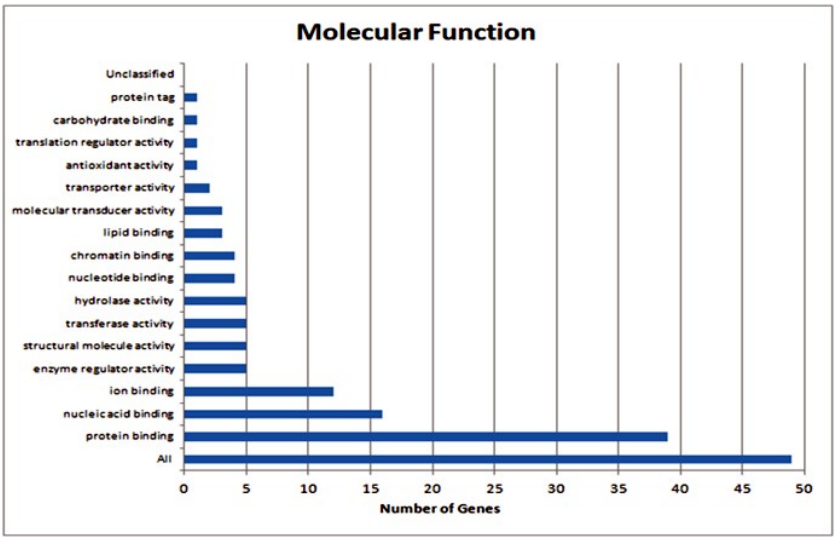
Lane M : GeneRuler 1kb DNA ladder

Lanes 1 to 50 : Randomly picked recombinant clones

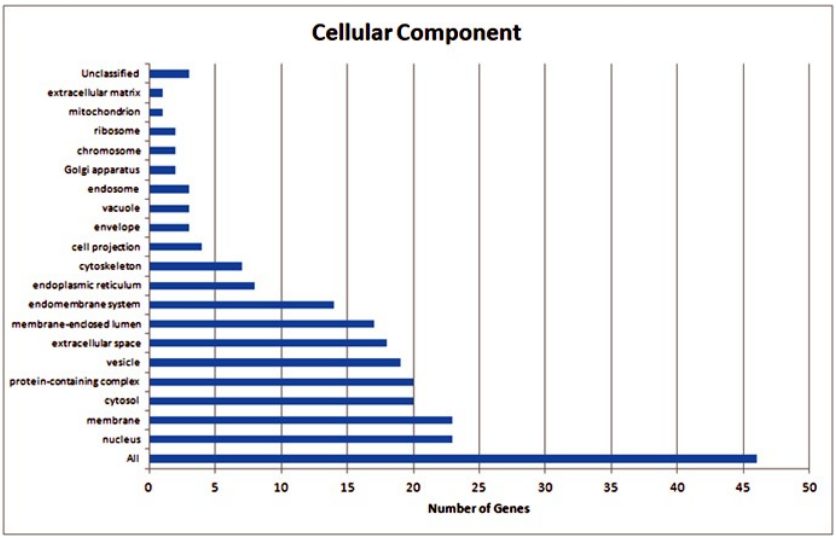
Lane 51 : NTC



(a)



(b)



(c)

Fig. 8: Functional categorization of the ESTs with Gene Ontology (GO) terms under three categories; (a) biological process; (b) molecular function and (c) cellular compartment with respective GO Slim terms

Table 4.2. Putative genes identified in cDNA clones of Y2H sheep lung library on their sequences comparison through BLASTn search engine of NCBI database

Clone No.	GenBank accession	Best hit in the NCBI database mRNA	Accession	Organism	e-value	Identity
CL_3.1_SLY2H_PRDX6	MT518200	Peroxiredoxin 6 (PRDX6)	XM_018060608.1	*Ovis aries*	1e-162	97%
CL_7_SLY2H_ZC3H13	MT518201	Zinc finger CCCH-type containing 13 (ZC3H13) , transcript variant X2	XM_012184401.2	*Ovis aries*	0	99%
CL_5_SLY2H_NPM1	MT518202	Nucleophosmin 1 (NPM1) , transcript variant X2	XM_012096714.3	*Ovis aries*	0	99%
CL_81.1_SLY2H_CCT7	MT518203	Chaperonin containing TCP1 subunit 7 (CCT7)	XM_004006072.3	*Ovis aries*	0	99%
CL_105.1_SLY2H_PIGR	MT518204	Polymeric immunoglobulin receptor (PIGR)	XM_004013573.3	*Ovis aries*	0	99%
CL_80_SLY2H_ring-box	MT518205	Ring-box 1 (RBX1)	XM_004007009.3	*Ovis aries*	0	100%
CL_70_SLY2H	MT682392	60S ribosomal protein L12 (LOC105608827)	XM_012190359.3	*Ovis aries*	4e-131	99%
CL_66_SLY2H_FAIM	MT682394	Fas apoptotic inhibitory molecule (FAIM), transcript variant X2	XM_015092644.2	*Ovis aries*	0	99%
CL_50_SLY2H_MYL6	MT702817	Myosin light chain 6 (MYL6), transcript variant X4	XM_004006589.3	*Ovis aries*	0	99%
CL_99_SLY2H_OSTC	MT702818	Oligosaccharyltransferase complex non-catalytic subunit (OSTC)	XM_004009629.4	*Ovis aries*	0	99%
CL_26_SLY2H_TSPAN4	MT707817	Tetraspanin 4 (TSPAN4), transcript variant X4	XM_027959979.1	*Ovis aries*	0	99%
CL_11_SLY2H_SFTPC	MT707818	Surfactant protein C (SFTPC)	NM_001009729.1	*Ovis aries*	0	99%
CL_9_SLY2H_SPINK1	MT726047	Serine peptidase inhibitor, Kazal type 1 (SPINK1)	XM_004008938.4	*Ovis aries*	0	99%
CL_42_SLY2H_SRSF3	MT726048	Serine and arginine rich splicing factor 3 (SRSF3), transcript variant X1	XM_012100410.2	*Ovis aries*	0	97%
CL_56.1_SLY2H_ phospholipase	MT726049	Phospholipase A2 inhibitor and Ly6/PLAUR domain-containing protein (LOC102185494), transcript variant X2	XM_018062529.1	*Capra hircus*	0	98%
CL_78_SLY2H_RPL 10_ribosomal	MT521866	Ribosomal protein L10 (RPL10)	NM_001112820.1	*Ovis aries*	0	99%
CL_98.1_SLY2H_RASSF4	MT792900	Ras association domain family member 4 (RASSF4), transcript variant X3	XM_027962354.1	*Ovis aries*	0	99%
CL_101_SLY2H_FOXO3	MT796122	Forkhead box O3 (FOXO3), transcript variant X1	XM_027972281.1	*Ovis aries*	0	99%
CL_81_SLY2H_CSNK1G2	MT799834	Casein kinase 1 gamma 2 (CSNK1G2), transcript variant X2	XM_027970242.1	*Ovis aries*	1e-100	99%

Table 4.2. Contd...

Clone No.	GenBank accession	Best hit in the NCBI database mRNA	Accession	Organism	e-value	Identity
CL_76_SLY2H_CMTM8	MT799835	CKLF like MARVEL transmembrane domain containing 8 (CMTM8), , transcript variant X2	XM_027957944.1	*Ovis aries*	1e-73	98%
CL_106_SLY2H_RELA	MT799836	RELA proto-oncogene, NF-kB subunit (RELA)	XM_027959295.1	*Ovis aries*	0	100%
CL_44_SLY2H_CSDE1	MT799838	Cold shock domain containing E1 (CSDE1), transcript variant X4	XM_004002360.3	*Ovis aries*	1e-74	100%
CL_71_SLY2H_BOLA	MT809056	BOLA class I histocompatibility antigen, alpha chain BL3-7-like (LOC101106374)	XM_027958902.1	*Ovis aries*	1e-73	100%
CL_4.1_SLY2H_TEK	MT809057	TEK receptor tyrosine kinase (TEK), transcript variant X2	XM_012127828.3	*Ovis aries*	5e-135	99%
CL_2_SLY2H_HNRNPA3	MT809058	Heterogeneous nuclear ribonucleoprotein A3 (HNRNPA3), transcript variant X2	XM_012132979.2	*Ovis aries*	1e-162	99%
CL_104.1_SLY2H_AQP1	MT809059	Aquaporin 1 (Colton blood group) (AQP1)	XM_018047113.1	*Capra hircus*	0	99%
CL_1_SLY2H_SHISA5	MT809060	Shisa family member 5 (SHISA5), transcript variant X2	XM_027957680.1	*Ovis aries*	7e-71	100%
CL_53_SLY2H_YBX3	MT811537	Y-box binding protein 3 (YBX3), transcript variant X2	XM_027967866.1	*Ovis aries*	4e-47	100%
CL_61_SLY2H_NECTIN2	MT811538	Nectin cell adhesion molecule 2 (NECTIN2), transcript variant X2	XM_027978457.1	*Ovis aries*	2e-134	99%
CL_112_SLY2H_APLP2	MT811539	Amyloid beta precursor like protein 2 (APLP2), transcript variant X6	XM_015103114.2	*Ovis aries*	0	97%
CL_43_SLY2H_CAPS	MT811540	Calcyphosine (CAPS), transcript variant X2	XM_027969799.1	*Ovis aries*	0	100%
CL_48_SLY2H_ALDH1A1	MT811541	aldehyde dehydrogenase 1 family member A1 (ALDH1A1)	NM_001009778.1	*Ovis aries*	0	99%
CL_6.1_SLY2H_SNU13	MT811542	Small nuclear ribonucleoprotein 13 (SNU13)	XM_004007019.3	*Ovis aries*	0	99%
CL_12_SLY2H_HMG20A	MT815844	High mobility group 20A (HMG20A), transcript variant X3	XR_003585729.1	*Ovis aries*	3e-96	99%
CL_17_SLY2H_NFE2L1	MT815845	Nuclear factor, erythroid 2 like 1 (NFE2L1), transcript variant X7	XM_024980275.1	*Bos taurus*	2e-20	100%
CL_8_SLY2H_ sulfotransferase	MT821929	Sulfotransferase 1A1 (LOC101114075), transcript variant X6	XM_012104345.3	*Ovis aries*	3e-75	100%

Table 4.2. Contd...

Clone No.	GenBank accession	Best hit in the NCBI database mRNA	Accession	Organism	e-value	Identity
CL_10_SLY2H_CXCL12	MT835022	C-X-C motif chemokine ligand 12, CXCL12), transcript variant X4	XM_ 027531089.1	*Bos indicus x Bos taurus*	2e-134	99%
CL_49.1_SLY2H_MHC	MT835023	MHC class I antigen clone 2 (BOLA)	NM_001289989.1	*Bos taurus*	0	100%
CL_108_SLY2H_SFTPD	MT835024	Surfactant protein D (SFTPD), transcript variant X2	XM_027962152.1	*Ovis aries*	1e-177	100%
CL_114_SLY2H_TPT1	MT835025	Tumor protein, translationally-controlled 1 (TPT1)	XM_015098053.2	*Ovis aries*	0	100%
CL_27_SLY2H_MLLT10	MW217566	MLLT10 histone lysine methyltransferase DOT1L cofactor (MLLT10), transcript variant X3	XM_027976602.1	*Ovis aries*	0	100%
CL_14_SLY2H_CCDC14	MW217567	Coiled-coil domain containing 14 (CCDC14), mRNA	XM_012095854.3	*Ovis aries*	0	99%
CL_15_SLY2H_BPIFB1	MW217568	BPI fold containing family B member 1 (BPIFB1), transcript variant X2	XM_012189066.3	*Ovis aries*	0	99%
CL_16_SLY2H_SPCS2	MW217569	Signal peptidase complex subunit 2 (SPCS2)	XM_004016323.3	*Ovis aries*	0	99%
CL_18_SLY2H_LAP3	MW217570	Leucine aminopeptidase 3 (LAP3)	XM_012179698.3	*Ovis aries*	0	99%
CL_19_SLY2H_DCTN2	MW346670	Dynactin subunit 2 (DCTN2, transcript variant X3	XM_004006537.4	*Ovis aries*	0	100%
CL_20_SLY2H_ISG15	MW346671	ISG15 ubiquitin like modifier (ISG15)	NM_001009735.1	*Ovis aries*	0	100%
CL_21_SLY2H_KIFA5	MW346672	Kinesin family member 5A (KIF5A)	XM_012174665.3	*Ovis aries*	8e-169	100%
CL_22_SLY2H_ELN	MW346673	Elastin (ELN), transcript variant X8	XM_027961948.1	*Ovis aries*	0	100%

hits and submitted to GenBAnk using BankIt (Table. 4.2). ESTs homologous to known proteins were annotated for GO in terms of biological processes, molecular function and cellular compartment (Fig. 8a-c) using WEB-based GEne SeT AnaLysiS Toolkit (WebGestalt).

4.2 Forward two-hybrid library screening

4.2.1 Construction and characterization of BD-bait clone

The characteristic CPE of BTV i.e. cell rounding, cell lysis, and detachment was observed in BHK-21 cells after 72 h of infection with BTV-10 (Fig. 9). All 10 segments of extracted dsRNA were visible clearly on PAGE by silver nitrate staining (Fig. 10). The full-length NS3 and C-terminus truncated NS3 ($NS3_t$) coding region of BTV was amplified from the RNA of BTV serotype 10 infected cells. The bands for full-length NS3 and $NS3_t$ were observed at the expected sizes of ~690 bp and at ~345 bp, respectively (Fig.11a and 12a). The amplified full-length NS3 and $NS3_t$ regions were subsequently cloned in pGBKT7-BD vector. The positive cloned plasmids were confirmed by colony PCR, RE digestion analysis (Fig. 11b,c and 12b,c) and nucleotide sequencing to check the orientation and reading frame of full-length NS3 and $NS3_t$ coding region. The positive recombinant plasmids pGBKT7-NS3 and pGBKT7-$NS3_t$ were re-transformed into Y2H Gold cells with high transformation efficiency along with the controls. Both the baits revealed white colonies on SDO and SDO/X plates but no colonies on SDO/X/A plates indicating that the pGBKT7-NS3 and pGBKT7-$NS3_t$ baits were not activated autonomously (Table 4.3; Fig. 13). For toxicity analysis, the colony size of chosen baits was compared and found to be at par with the controls indicating that the constructed baits are non-toxic. (Fig.13). Further, both the baits were found to be well expressed and the protein bands were observed at the expected sizes of ~46 kDa (Fig.14a-b) and ~34 kDa (Fig. 15a-b) for pGBKT7-NS3 and pGBKT7-$NS3_t$, respectively.

Table. 4.3: Tesing bait for autoactivation and toxicity

Sampple	Selective agar plates	Presence of colonies	Colony colour
Bait autoactivation test	SDO	Yes	White
Bait autoactivation test	SDO/X	Yes	White
Bait autoactivation test	SDO/X/A	No	N/A
Positive control test	DDO/X/A	Yes	Blue

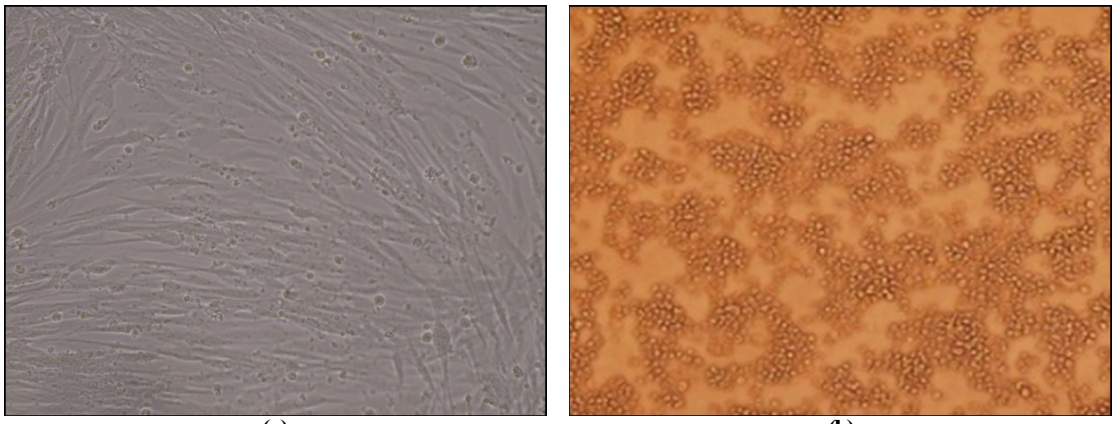


Fig. 9: Cytopathic effect of BTV 10 on BHK-21 cells. (A) Uninfected BHK-21 control cells (72 h); **(B)** Infected BHK-21 cells, 72 h post-infection, showing cell rounding and rupture

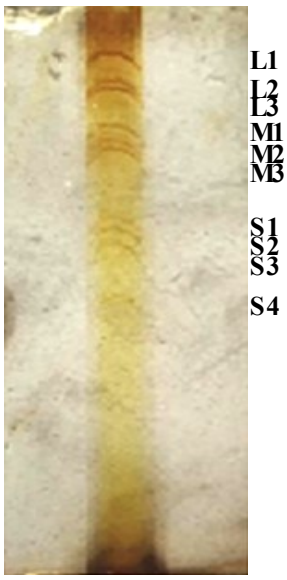


Fig. 10: BTV 10 dsRNA segments- silver nitrate staining

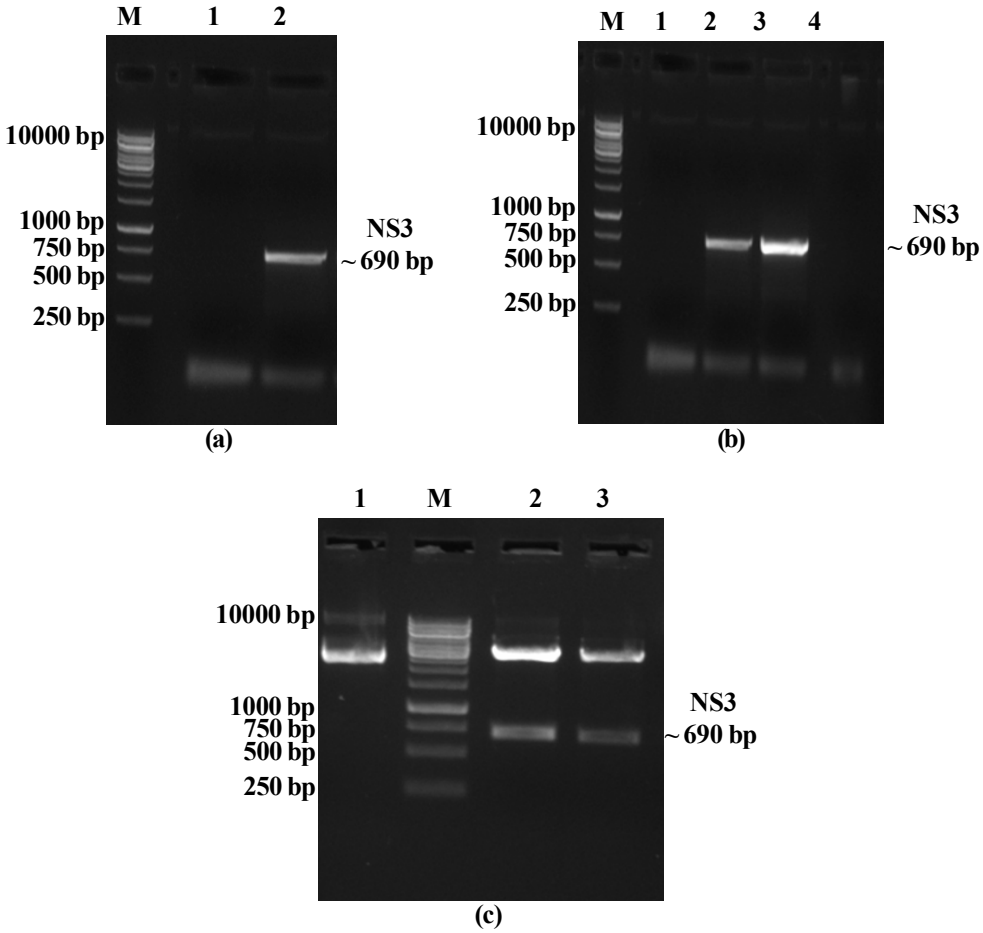


Fig. 11: (a) PCR amplification of full-length NS3 gene. Lane M: GeneRuler 1kb DNA ladder; **Lane 1:** NTC; **Lane 2:** NS3 PCR amplicon. **(b) Colony PCR of pGBKT7-NS3 colonies. Lane M:** GeneRuler 1kb DNA ladder; **Lane 1:** no amplification; **Lanes 2,3:** NS3 amplicon; **Lane 4:** NTC. **(c) RE digestion analysis. Lane M:** GeneRuler 1kb DNA ladder; **Lane 1:** Undigested pGBKT7-BD plasmid; **Lane 2, 3:** pGBKT7-BD plasmid showing release of NS3 (~690 bp).

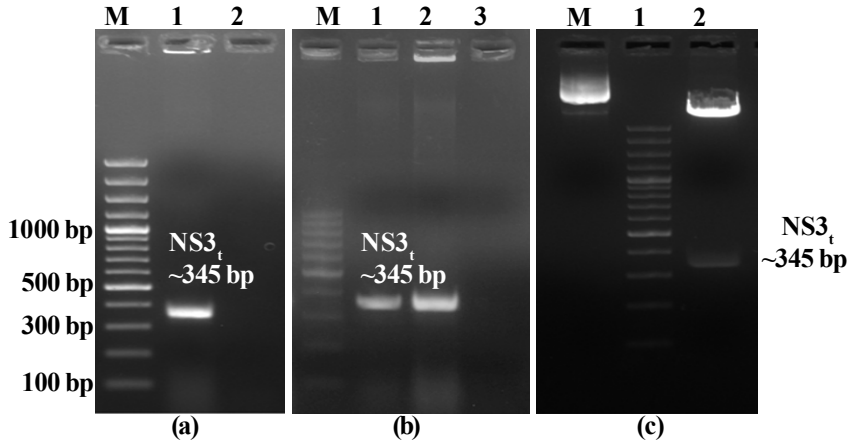


Fig. 12: (a) PCR amplification of $NS3_t$ gene. Lane M: GeneRuler 100 bp DNA ladder; **Lane 1:** $NS3n_t$ PCR amplicon; **Lane 2:** NTC; **(b) Colony PCR of pGBKT7-$NS3_t$ colonies. Lane M:** GeneRuler 100 bp DNA ladder; **Lanes 1-2:** $NS3_t$ amplicon; **Lane 3:** NTC; **(c) RE digestion analysis. Lane M:** GeneRuler 100 bp DNA ladder; **Lane 1:** Undigested pGBKT7-BD plasmid; **Lane 2:** Double digested pGBKT7-$NS3_t$ plasmid showing release of $NS3_t$ (~345bp).

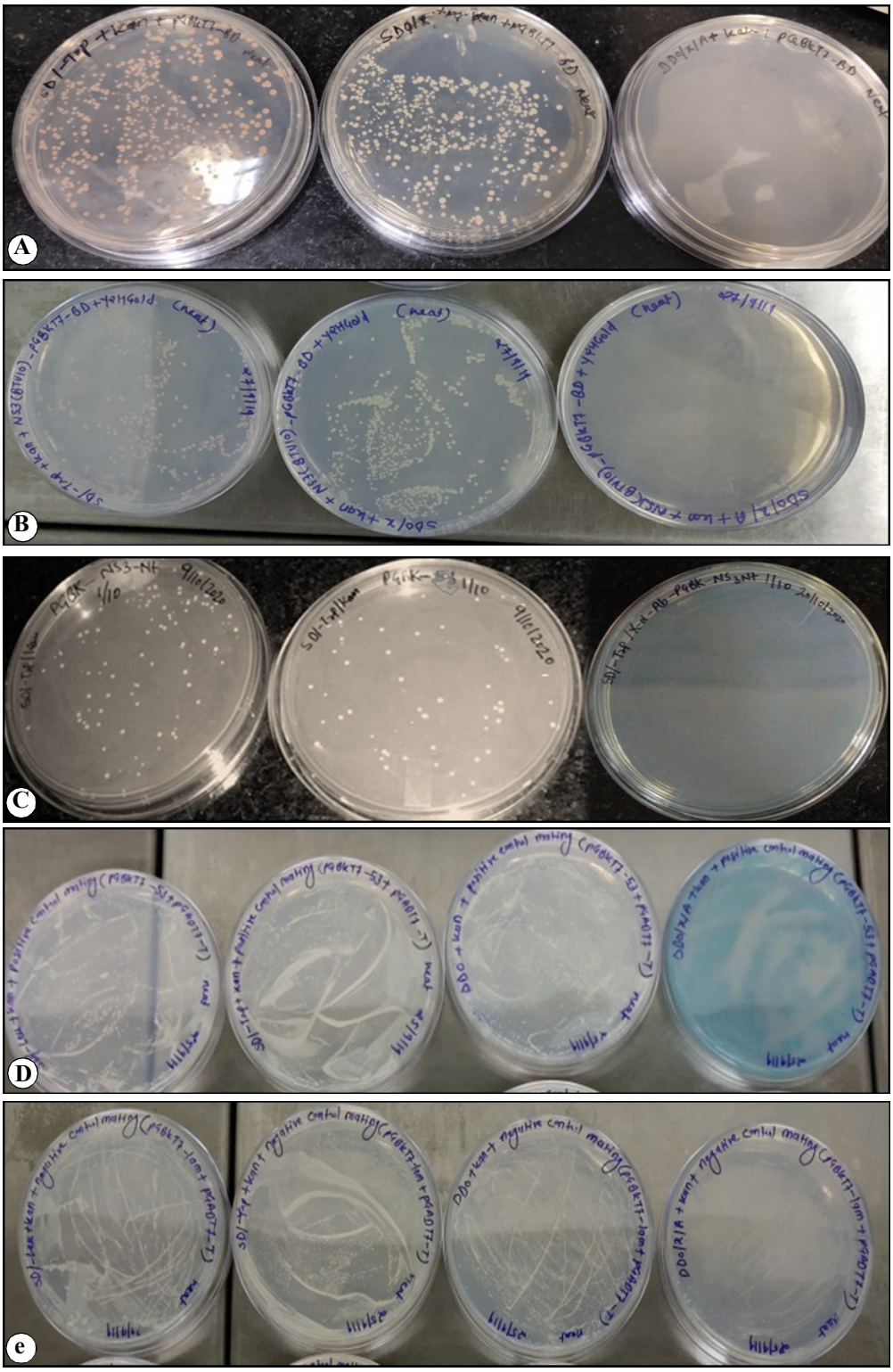


Fig. 13: Auto-activation and toxicity testing of pGKT7-NS3 and pGBKT7-NS3$_t$ bait.
Panel A: Empty pGBKT7-BD colonies on SDO, SDO/X agar plates but no colonies on SDOX/A agar plates; **Panel B:** pGKT7-NS3- colonies on SDO, SDO/X agar plates but no colonies in SDOX/A agar plates; **Panel C:** pGBKT7-NS3$_t$ bait- colonies on SDO, SDO/X agar plates but no colonies in SDOX/A agar plates; **Panel D:** Positive control (pGBKT7-53 + pGADT7-T) showing blue colonies on DDO/X/A agar plates; **Panel E:** Negative control pGBKT7-Lam + pGADT7-T) showing colonies in SDO, DDO/X agar plates but no colonies on DDO/X/A agar plates.

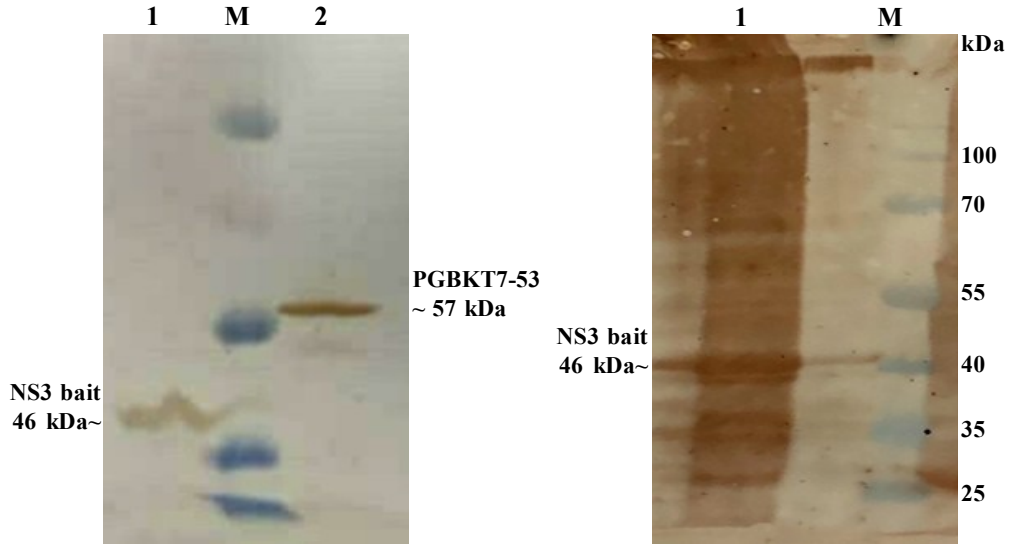


Fig. 14: Expression analysis of pGBKT7-NS3 bait.
(a) Western blot with c-myc monoclonal antibody. Lane-M: PageRuler prestained protein marker; **Lane 1:** pGBKT7-NS3 bait lysate showing recombinant NS3 protein; **Lane 2:** pGBKT7-53 positive control. **(b) Western blot with anti-NS3 polyclonal antibody. Lane-M:** PageRuler prestained Protein marker; **Lane 1:** pGBKT7-NS3 bait lysate showing recombinant NS3 protein.

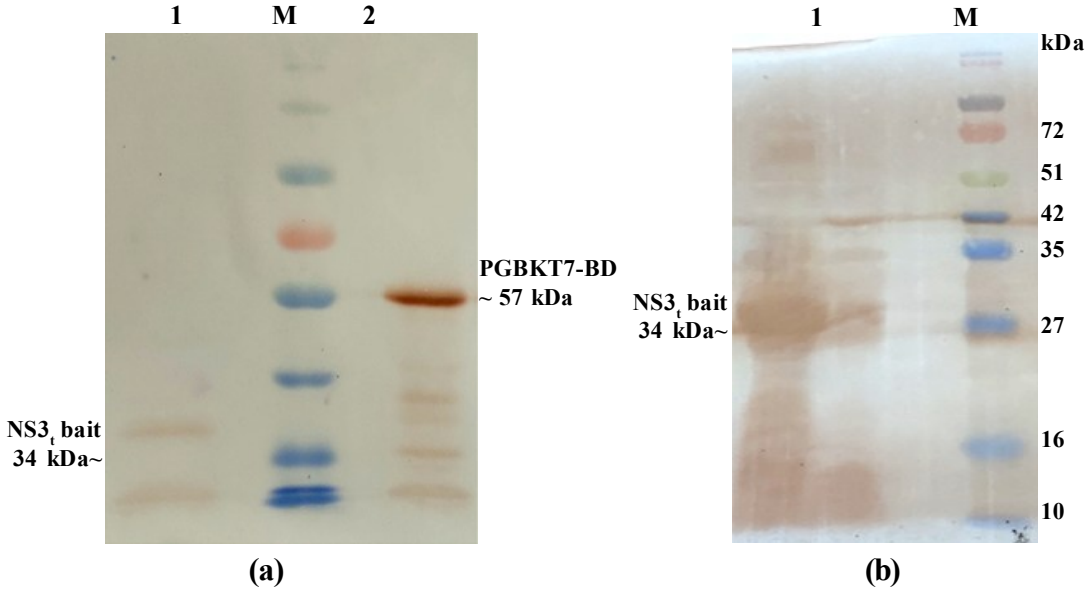


Fig. 15: Expression analysis of pGBKT7-$NS3_t$ bait. (a) Western blot with c-myc monoclonal antibody. Lane-M: PageRular prestained marker; **Lane 1:** pGBKT7-$NS3_t$ bait showing recombinant $NS3_t$ protein; **Lane 2:** pGBKT7-53 positive control; **(b) Western blot with anti-NS3 polyclonal antibody. Lane-M:** Puregene protein marker; **Lane 1:** pGBKT7-$NS3_t$ bait showing recombinant $NS3_t$ protein

4.2.2 Library screening, segregation and rescue of prey plasmids

For preliminary screening, the cell density and titre of the frozen library was calculated and estimated to be 2.4×10^8 cells/ml and $>2 \times 10^7$ cells/ml, respectively. The library was found to be optimal and thus mated with bait having cell density of $>1\times10^8$ cells/ml at 30 ºC with minimum shaking. The three-lobed, clover leaf like zygotes were observed at 20 h post incubation (Fig. 16). The quality parameter of mating were evaluated and noted in table 4.4. All the parameters for the mating between pGBKT7-NS3 bait and sheep lung cDNA library were well above the threshold value. However, no blue colonies appeared on DDO/X/A plates though there were appearance of colonies on SD/-Trp, SD/-Leu and DDO agar plates. The mating experiment was repeated but similar results were obtained.

As there are only two main variables in Y2H i.e. bait and prey, we switched the bait from NS3 to NS4 and repeated the experiment twice. The NS4/prey mating led to the development of blue colonies on DDO/X/A on both the times. So, it was concluded that the reagents and prey library were fine but there must be some domains in NS3 that were hampering its interaction with host protein in Y2H. The amino acid sequence of NS3 was analyzed and TM domains were observed between aa 122 to 139 and aa 166 to 182 (Fig. 17). So, we moved forward with the construction of C-terminus truncated NS3 (pGBKT7-$NS3_t$) bait. The pGBKT7-$NS3_t$ was characterized and mated with the same sheep lung cDNA library. All the parameters of mating were above the threshold values as mentioned in table 4.4. and a total of 65 blue colonies were appeared on DDO/X/A agar plates indicating the positive interaction between $NS3_t$ bait and prey (Fig. 18a). Of these 65 colonies, only 37 colonies were able to grew on QDO/X/A agar plates (Fig. 18b). These 37 colonies were then subjected to amplification followed by sequencing to identify the novel prey proteins and to eliminate the duplicates. A total of 12 proteins were found to be interacting with $NS3_t$, this interacting blue colonies were separated by streaking on DDO/X/A (Fig. 18c) and subsequently patching on QDO/X/A (Fig. 18c). However plasmids could be rescued from only eight colonies (table 4.5). Most of the prey proteins identified were found in more than one colonies.

Table 4.4: Quality estimation of bait and prey library mating

Parameter	pGBKT7-NS3+sheep lung library mating	pGBKT7-NS3$_t$+sheep lung library mating
Viabilty of prey library	1×10^7 cfu/ml	1.05×10^7 cfu/ml
Viabilty of bait	3×10^8 cfu/ml	3.5×10^8 cfu/ml
Viabilty of diploids	3×10^5 cfu/ml	3.3×10^5 cfu/ml
Number of clones screened	4.5×10^6 clones	4.9×10^6 clones
Mating efficiency	3%	3.14%

Table. 4.5: List of prey proteins interacting with pGBKT7-NS3$_t$

S. No.	Gene	Symbol
1	Ovis aries EP300 interacting inhibitor of differentiation 1	EID1
2	Ovis aries ubiquitin conjugating enzyme E2 D4 (putative)	UBE2D4
3	Ovis aries dynactin subunit 2, transcript variant X3	DCTN2
4	Ovis aries protein disulfide isomerase family A member 4	PDIA4
5	Ovis aries nucleosome assembly protein 1 like 1, transcript variant X9	NAP1L1
6	Ovis aries Golgin subfamily A member 2	GOLGA2
7	Ovis aries tumor susceptibility gene 101	Tsg101
8	Ovis aries actinin alpha 1	ACTN1

4.3 Confirmation of positive interactions by alternative yeast-based assays

4.3.1 Co-transformation

All the identified prey plasmids were co-transformed with the NS3$_t$ bait and empty pGBKT7 vector (bait control), to check the auto-activation on part of prey protein. Of the eight screened prey proteins, seven gave rise to blue colonies on both DDO/X/A and QDO/X/A plates and were marked as positive. While, DCTN2 developed very pale blue colonies on both DDO/X/A and QDO/X/A plates and considered as negative (Fig. 19). Further, none of the prey proteins were found to be self activating as there were no coloured colonies on mating with empty bait plasmid (Fig. 19).

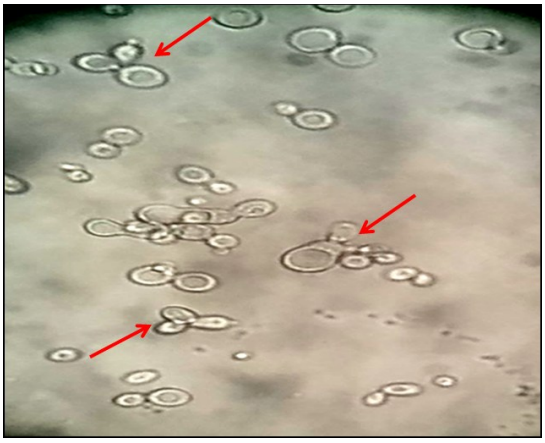


Fig. 16: Yeast zygotes 3-lobed clover leaf structure

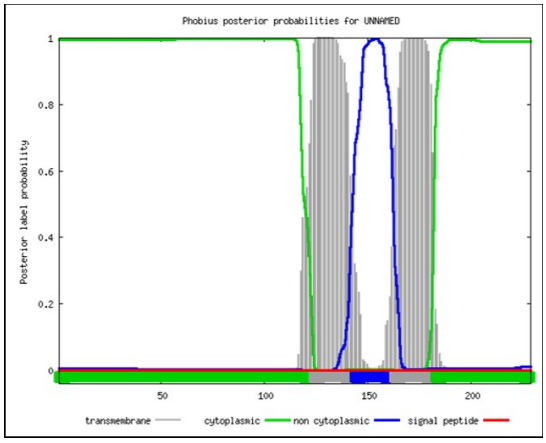
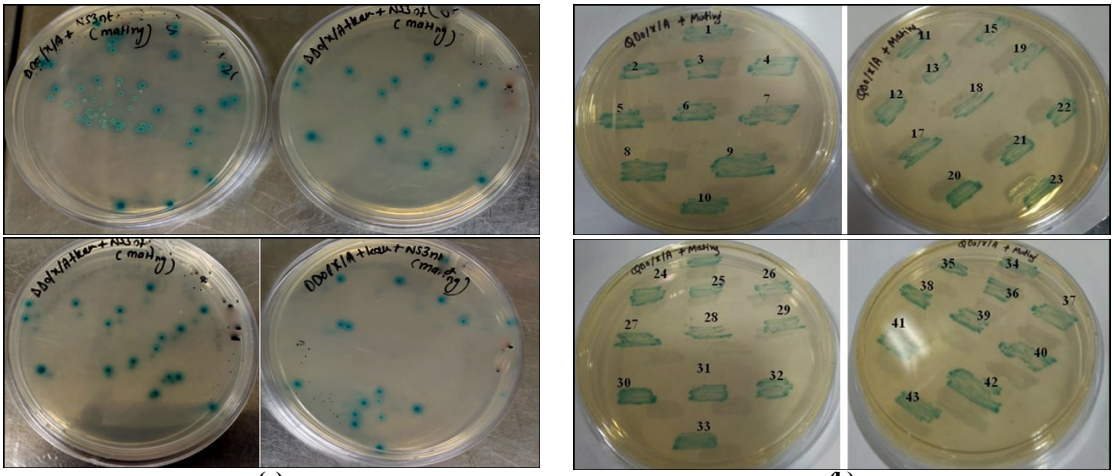
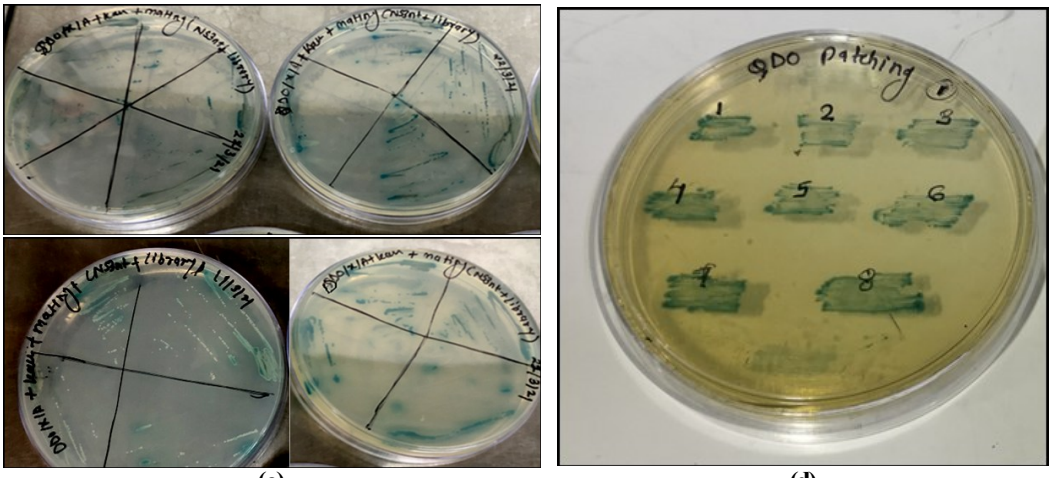


Fig. 17: Transmembrane domain of BTV NS3 sequence

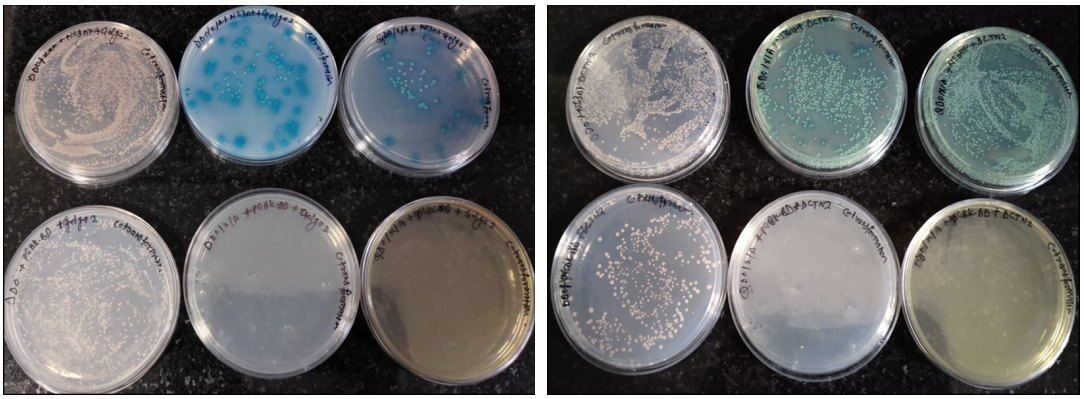


(a) (b)

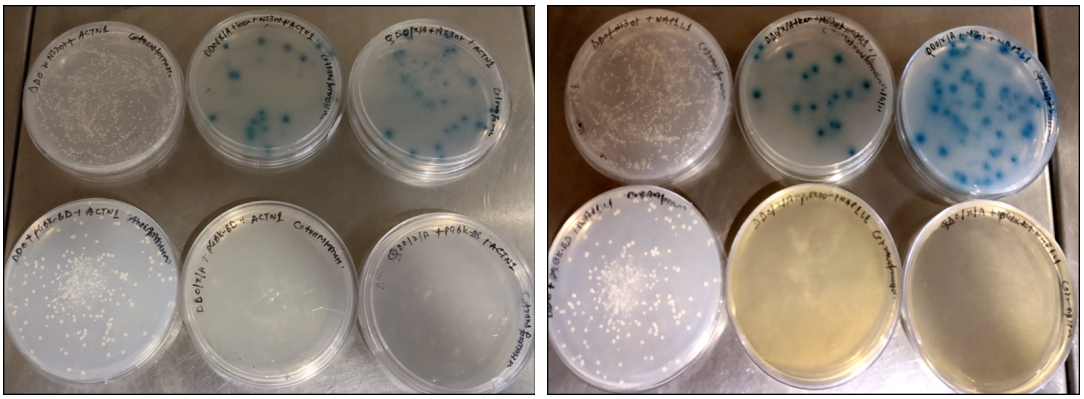


(c) (d)

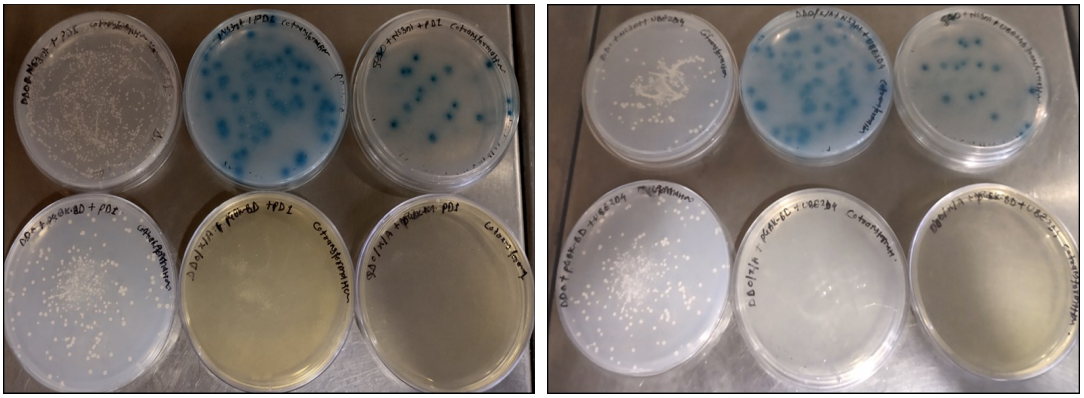
Fig. 18: Forward library screening of pGBKT7-NS3$_t$ with ovine library - a representative figure; (a) Large scale mating; **(b)** Selection on high stringent QDO/X/A media; **(c)** Segregation and rescue of interacting prey plasmids on DDOX/A and QDO/X/A



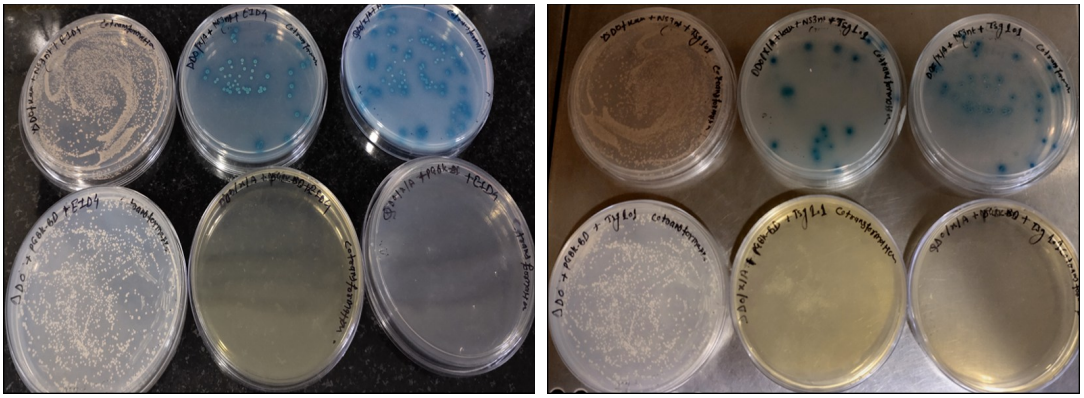
GOLGA2 DCTN2



ACTN1 NAP1L1



PDI UBE2D4



EID1 Tsg101

Fig. 19: Co-transformation of pGBKT7-NS3$_t$ (Bait) and pGBKT7-BD (control) with prey proteins on DDO, DDO/X/A and QDO/X/A agar plates

4.3.2 β-Galactosidase assays

The isolated prey plasmids were mated individually with the pGBKT7-$NS3_t$ bait on small-scale and the resultant diploids were subjected to β Galactosidase (β-Gal) assays to confirm the PPI. The development of the blue (+++), pale blue (+) and white colour on the treated filter papers are indicative of a strong positive, moderate and negative interaction reaction, respectively. Out of the eight prey proteins, only seven colonies lead to the development of blue colour on treatment with X-gal. These seven colonies were termed positive while DCTN2 was tagged as negative as it fails to develop blue colour even after 8 h of incubation (Fig. 20; Table 4.6). For ONPG liquid culture assay, the time taken for development of the yellow color was recorded and the relative strength of the prey-$NS3_t$ and prey-pGBKT7-BD interaction were measured in terms of miller units and relative % β-Gal activity was calculated (Table 4.6). The test was performed thrice in triplicates and the results along with the standard deviation are plotted in fig. 21. The Positive and negative controls were included for each run. All the prey protein has shown significant relative % β-Gal activity except DCTN2. Amongst these, NAP1L1 revealed the highest relative % β-Gal activity.

So, post screening and confirmation with yeast based assay, seven host proteins were considered as the putative interaction partners of $NS3_t$. However, these interactions need to be further validated. Thus, GST pull-down and mammalian two-hybrid assay were performed. However with the limitation of time and reagents, these tests were carried out only for NAP1L1 keeping the functional significance of NAP1L1 and yeast assay results in mind.

4.4 confirmation of NAP1L1/$NS3_t$ interaction

4.4.1 Small scale mating

The NAP1L1 coding region was amplified with the expected size of ~1179 bp (Fig. 22a). The NAP1L1 was cloned into pGADT7-AD vector, RE checked (Fig. 22b) and successfully re-transformed into Y187 cells. The NAP1L1 was well expressed in yeast and the band was observed at the expected sizes of ~64 kDa on detection with Gal4AD antibody (Fig. 22c). The pGADT7-NAP1L1 and pGBKT7-$NS3_t$ were mated along with controls and blue colonies appeared on DDO/X/A and QDO/X/A plates corroborating the NAP1L1/NS3t interaction (Fig. 23).

Table 4.6: Confirmation of BTV $NS3_t$ interacting prey proteins

S. No.	Prey protein	Growth on DDO/X/A		Growth on QDO/X/A		β-galactosidase assay			Interpretation
		pGBkT7-prey +pGBKT7-$NS3_t$	pGBkT7-prey+pGBKT 7-BD	pGBkT7-prey+pGBKT 7-$NS3_t$	pGBkT7-prey+pGBKT 7-BD	Colony lift filter assay	ONPG assay		
		Miller units	Relative % β-Gal						
1	EID1	Blue	White	Blue	Nil	Blue	2.716	25.94	+
2	UBE2D4	Blue	White	Blue	Nil	Blue	4.087	39.03	+
3	**DCTN2**	**Pale blue**	**White**	**Pale blue**	**Nil**	**White**	**0.507**	**2.18**	**-**
4	PDIA4	Blue	White	Blue	Nil	Blue	4.713	45.02	+
5	NAP1L1	Blue	White	Blue	Nil	Blue	6.422	61.45	+
6	GOLGA2	Blue	White	Blue	Nil	Blue	2.203	21.04	+
7	Tsg101	Blue	White	Blue	Nil	Blue	5.214	49.80	+
8	ACTN1	Blue	White	Blue	Nil	Blue	1.140	10.89	+

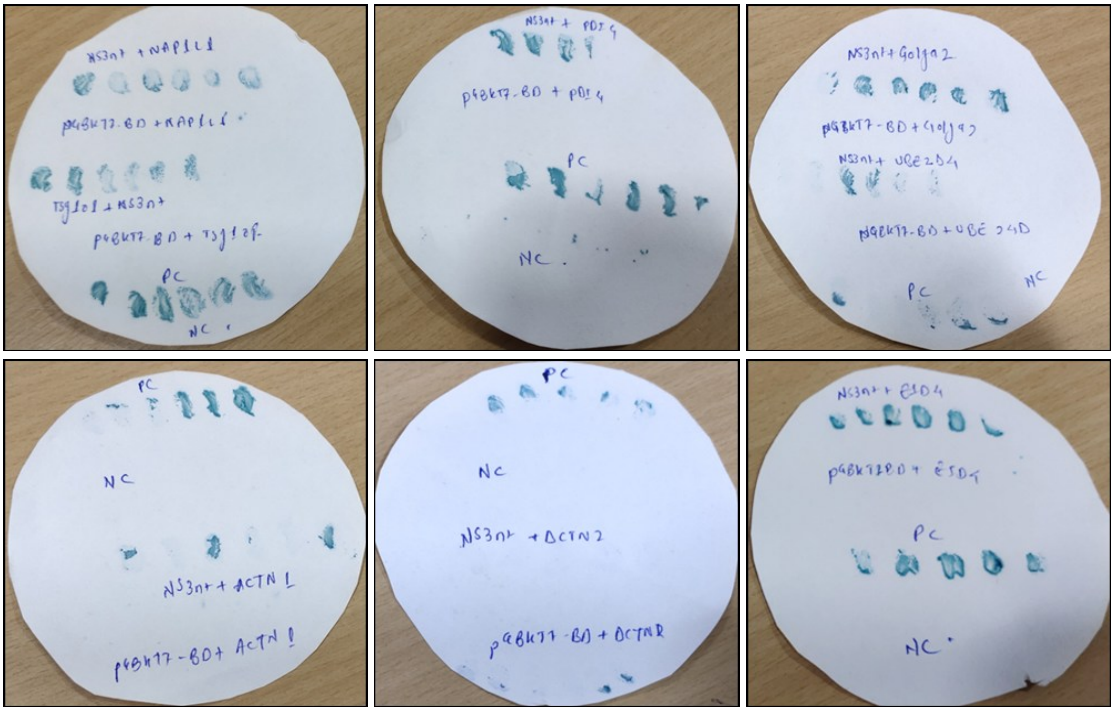


Fig. 20: Colony-lift filter assay performed on pGBKT7-NS3$_t$+prey and pGBKT7-BD+prey diploids

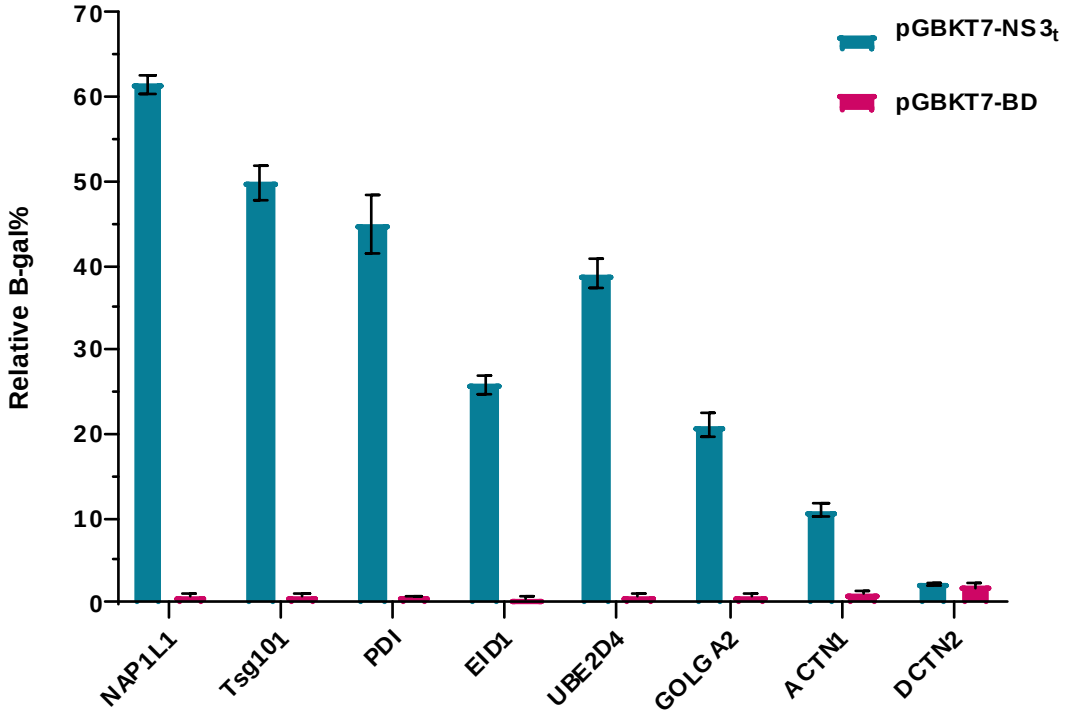


Fig. 21: ONPG liquid culture assay for confirmation of interaction between BTV NS3$_t$ and putative host proteins

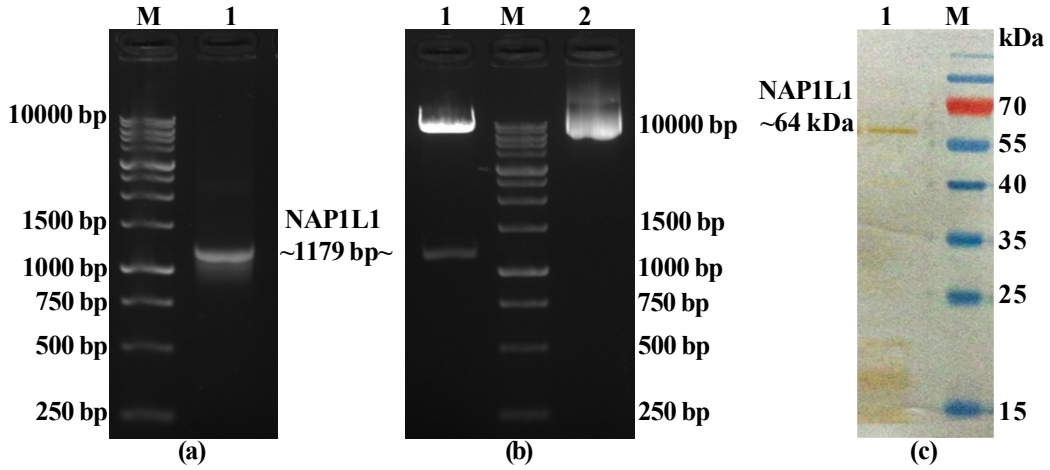


Fig. 22: (a) PCR amplification of NAP1L1 gene. Lane M: GeneRuler 1 kb DNA ladder; **Lane 1:** NAP1L1 PCR amplicon; **(b) RE digestion analysis. Lane M:** GeneRuler 1 kb DNA ladder; **Lane 1:** pGADT7-AD plasmid showing release of NAP1L1 (~1179 bp); **Lane 2:** Undigested pGADT7-NAP1L1 plasmid; **(c) Expression analysisof pGADT7-NAP1L1 with GAL4 AD antibody. Lane M:** Prestained protein ladder; **Lane 1:** pGADT7-NAP1L1 lysate

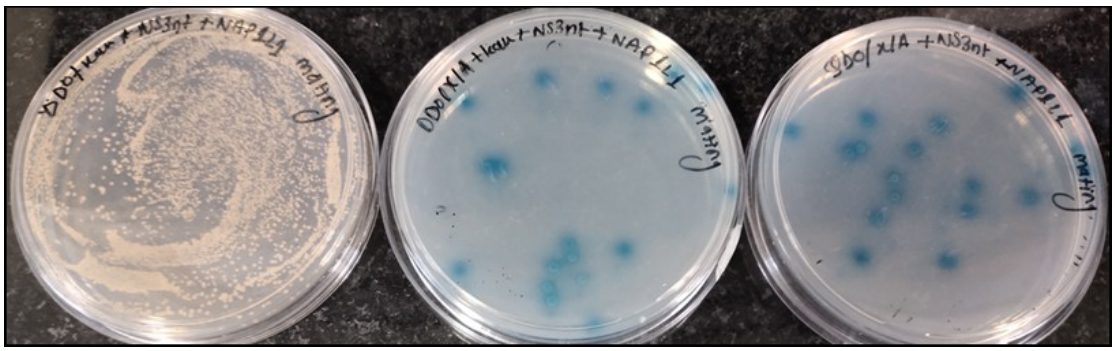


Fig. 23: Confirmation of NAP1L1/NS3$_t$ interaction -Small-scale mating

4.4.2 GST pull-down assay

The $NS3_t$ region was sub-cloned into pGEX-4T-1 vector as confirmed by RE digestion and sequencing. The positive recombinant plasmid was re-transformed in BL21(DE3)pLysS cells and induced with 1 mM of IPTG at 30 ºC for 5 h. The expression of GST- NS3t protein was detected at the expected size of ~39 kDa (12.6 kDa+ 26 kDa GST tag) by SDS-PAGE and western blotting (Fig. 24a-c). The NAP1L1 was sub-cloned into pCMV-Myc-N vector and the sequence confirmed pCMV-Myc-NAP1L1 was transfected into HEK 293 T cells. The NAP1L1-transfected HEK 293 T cells were trypsinized and cell lysate was prepared using pull-down lysis buffer. The expression of NAP1L1 was probed by anti-c-myc antibody and the band was observed at the expected size of ~45 kDa (Fig. 25a).

For GST pull down, the $NS3_t$ protein was incubated with glutathione agarose and thereafter, the bait-agarose complex was incubated with the mammalian prey cell lysate. The bound GST agarose-$NS3_t$-Myc-NAP1L1 complex was eluted and elutes were run on SDS-PAGE and subsequently western blotting was done to detect the GST-$NS3_t$-Myc-NAP1L1 protein. The target band of ~39 kDa was observed with anti-GST, and anti-NS3 antibody while the band of ~45 kDa was observed with anti-c-myc antibody (Fig. 25b) which validates the interaction between $NS3_t$ and NAP1L1 in vitro.

4.4.3 Mammalian two-hybrid (M2H) assay

M2H system is used to analyze PPIs in transiently transfected mammalian cells and is more similar to the real time virus infection in host. For this, the $NS3_t$ region was sub-cloned into the pM vector and the NAP1L1 cloned into the pVP16 vector. The bait and prey plasmids along with controls were transfected into HEK 293 T cells together with pG5SEAP reporter plasmid in a 24-well plate. The positive interaction was characterized by the secretion of alkaline phosphatase in the medium which was detected by chemiluminiscence using CSPD as the substrate. The amount of SEAP activity was measured in terms of relative light units using plate luminometer, after 48 h of transfection. The relative SEAP activity was calculated from RLU. The $NS3_t$/NAP1L1 interaction gave 16- fold higher relative SEAP activity than the BC. The PC generated 21- fold higher relative SEAP activity than the BC while comparatively no relative SEAP activity was observed in case of NC and BC (Fig. 26). The test was repeated thrice in duplicates.

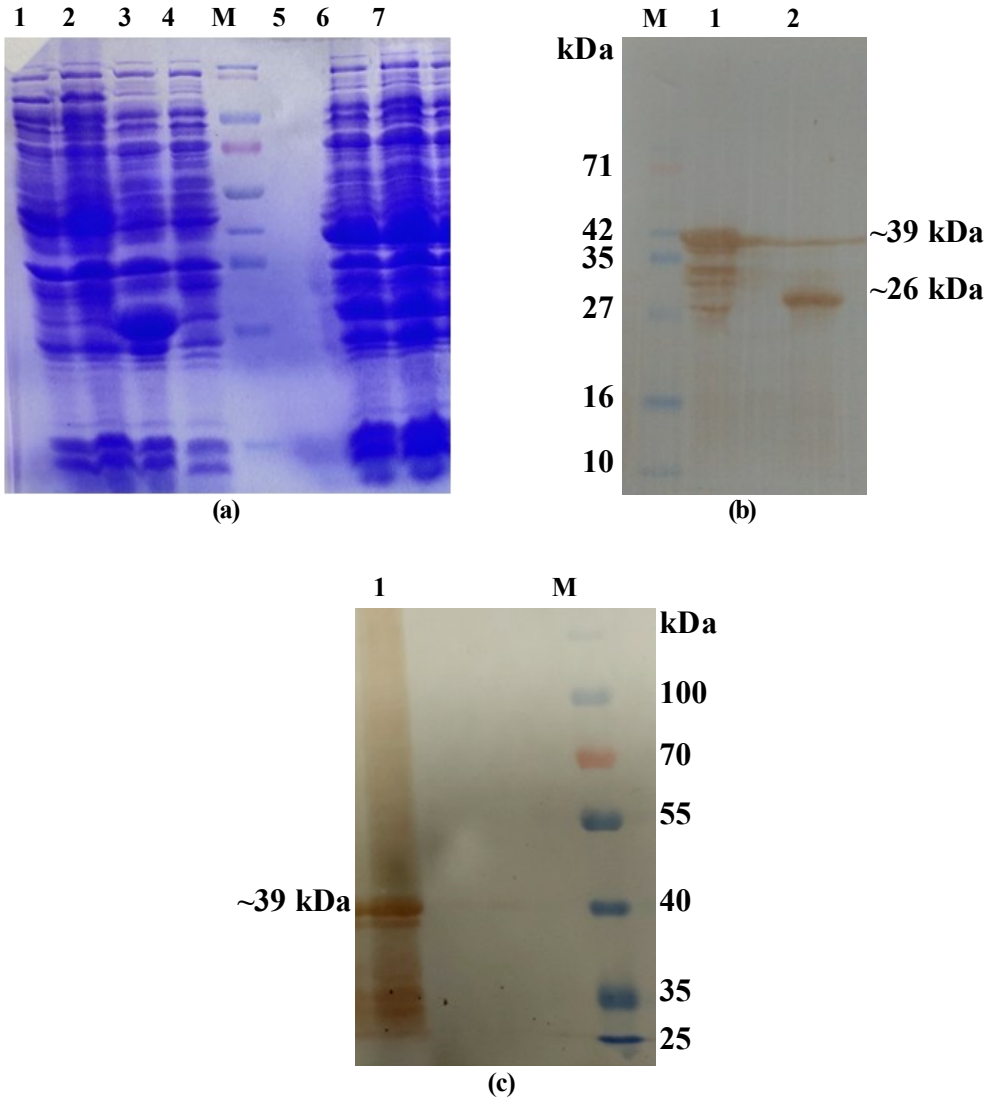


Fig. 24: **(a) GST-tagged NS3$_t$ (pGEX-NS3$_t$) protein expression in prokaryotic host system for GST pull-down assay. Lane 1:** Uninduced *E.coli* BL21(DE3) pLysS lysate (negative control); **Lane 2:** Uninduced pGEX-4T-1; **Lane 3:** Induced pGEX-4T-1 (~26 kDa); **Lane 4:** Uninduced pGEX-NS3$_t$; **Lane 6, 7:** Induced pGEX-NS3$_t$; **Lane M:** Puregene prestained protein ladder; **(b) Western blot analysis of pGEX-NS3$_t$ protein with anti-GST antibody. Lane M:** Puregene Prestained protein ladder; **Lane 1:** pGEX-NS3$_t$ protein lysate (~39 kDa); **Lane 2: Induced** pGEX-4T-1; **(c) Western blot analysis of pGEX-NS3$_t$ protein with anti-NS3 antibody. Lane M:** PageRular Prestained protein ladder; **Lane 1:** pGEX-NS3$_t$ protein lysate (~39 kDa)

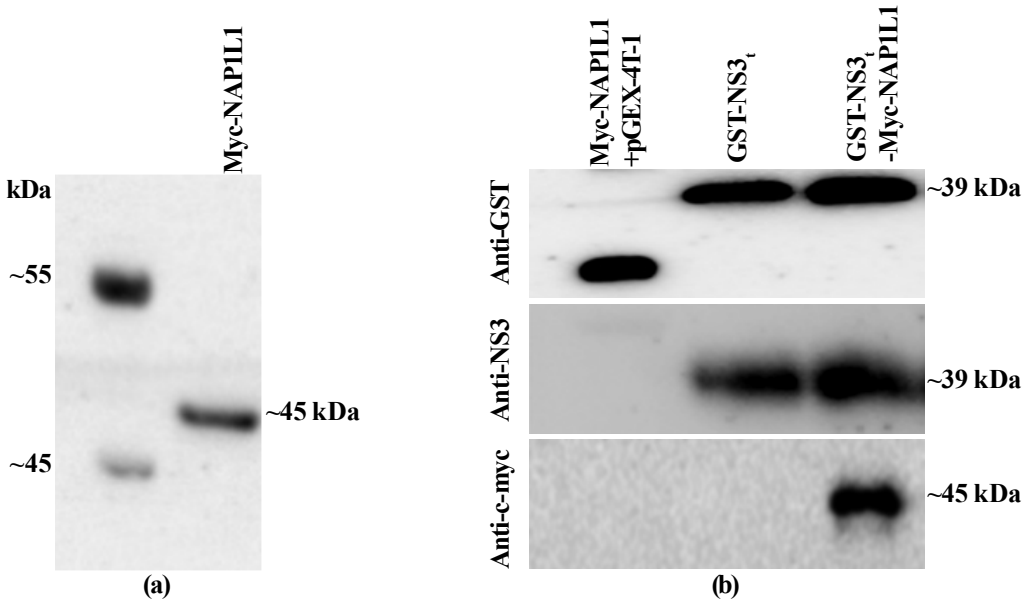


Fig. 25: Confirmation of NAP1L1/NS3$_t$ interaction by GST pull-down assay. (a) Expression of c-Myc fused NAP1L1 protein in transfected HEK 293 T cells (~45 kDa); **(b)** GST Pull-down assay confirming NAP1L1/NS3$_t$ interaction

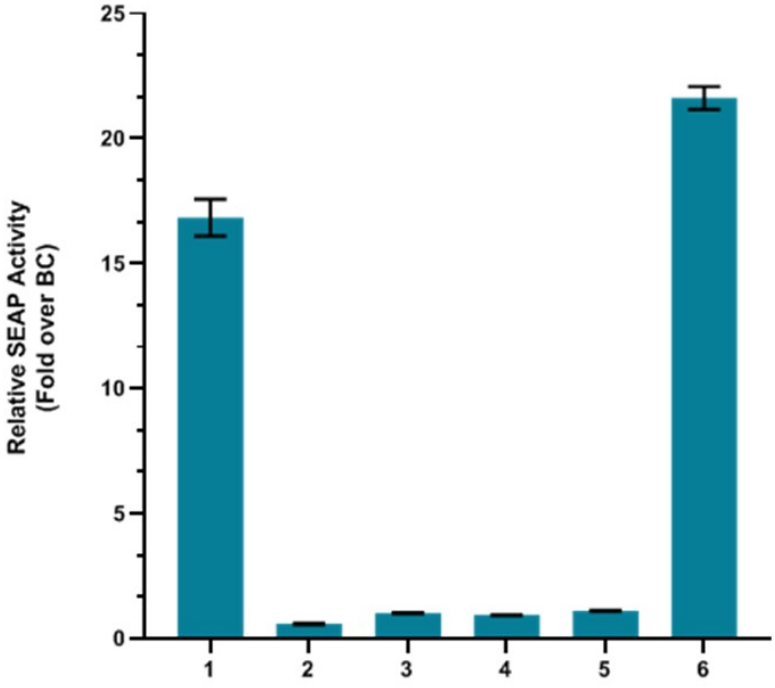


Fig. 26: Confirmation of NAP1L1/NS3$_t$ interaction by Mammalian Two-Hybrid assay. 1: Test sample- pM-NS3$_t$+pVP16-NAP1L1; **2:** None; **3:** Basal Control- pM-+pVP16; **4:** Bait Control- pM-NS3$_t$+pVP16; **5:** Prey Control- pM-+pVP16-NAP1L1; **6:** Positive Control- pM53+pVP16

Discussion

5 DISCUSSION

BT is an important OIE-listed viral disease that affects multiple animal species. It poses huge economic consequences on the livestock sector directly through reduced productivity, mortality, fetal abnormality, and indirectly by affecting the trade for animal products. The highest direct losses to farmers due to BT occurred in 2005, costing approximately 231 million rupees (Rushton and Lyons, 2015). An average estimate of 3 billion USD per annum losses across the globe has been reported due to BT (Sperlova and Zendulkova, 2011; Rushton and Lyons, 2015). Although there are no precise reports for economic losses particularly due to BT in India, a study shows that BT resulted in heavy economic devastation in India between 1991 and 2005 (Singh and Shive, 2009). Due to the losses and economic importance, BT becomes a disease of paramount significance to study and develop control measures.

BT is caused by the BTV, a member of orbivirus genus belonging to the family *Reoviridae*. BTV exists in multiple immunologically distinct serotypes and till date, 27 serotypes are reported worldwide. In India, a total of 23 serotypes have been accounted by serotype-specific antibodies detection (Rao *et al.*, 2016) and 15 of them are isolated from different regions of the country either under All-India Network Program on Bluetongue (AINP-BT) or by other research laboratories in the country (Maan *et al.*, 2017). Most of the serotypes are isolated from the southern states of India (Chand *et al*., 2015; Reddy *et al.*, 2018). BTV is a non enveloped, segmented, dsRNA virus that has a genome of approximately 19.2 kbp. The genome segments are numbered 1-10 and categorized as Large: L1-3; medium: M4-6; and small: S7-10 in the order of decreasing size on agarose gel (Schwartz-Cornil *et al*., 2008). The BTV virions possess a diameter of 90 nm and have a relative molar mass of about 10.8×10^7 g/mol.

BTV is mainly transmitted through biting midges (*Culicoides* spp.) leading to severe hemorrhagic disease. The virus is capable to replicate within both insect and mammalian hosts; however, insects remain asymptomatic while severe pathogenesis is observed in mammalian host results in. The same is reflected in cell culture, where distinctive CPE is observed in mammalian cells but no CPE in insect cells in spite of productive virus replication (Mortola *et al.*, 2004). This differential host response upon virus infection strongly correlates with the amount of apoptosis that occurs in different cells. BTV life cycle requires a congregation of complicated biochemical and biological interactions between viral and host cellular components (Patel and Roy, 2014). To perform all these interactions and to make capital out of host machinery BTV segments encode different proteins which contribute significantly in discrete steps of virus replication and pathogenesis. Transcription of genomic segments produces 10 ssRNA which is further translated by host cell ribosomes yielding the seven SPs (VP1 to VP7) and five NSPs (NS1-NS5) (Mertens and Sangar, 1985; Ratinier *et al.*, 2011). SPs help mainly in virus morphogenesis while the NSPs interact with host proteins, although they work in coordination. NS1 enhances protein synthesis by interacting with the ACUUAC hexanucleotides at the 3' untranslated region of ssRNA transcripts and increase the viral titer (Boyce *et al.*, 2004). NS2 constitutes viral inclusion bodies (VIBs) by recruiting viral ssRNAs and protein required for packaging and assembly (Kar *et al.*, 2007). NS3/NS3a is a glycosylated protein performs a central role in intracellular trafficking and egress of the mature virions. NS4 plays a role in viral counteraction to the host interferon response (Ratinier *et al.*, 2011). The distinctive role of NS5 is not established yet. These viral proteins are associated in one or another way to establish infection within the target host cells.

However, precise information about the function of these viral proteins in the virus life cycle and their interaction with host factors is not available. The viral proteins abrogate the host mechanisms through various pathways and modulate host immune response too. An understanding regarding the virus interaction with host cellular machinery to produce an infection is important to develop the methods to combat the BT disease. One of the approaches towards the virus-host interaction understanding is by PPI. The eminence of PPI study has been recognized by many researchers in identifying virus-host interaction, such as vaccinia virus

(Zhang *et al.*, 2009), JEV (Wang *et al.*, 2011), Influenza virus (Wei *et al.*, 2019), RVFV (Le May *et al.*, 2005), CHIKV (Rana *et al.*, 2017) and many more. Thus, the study of the interaction between virus and host proteins is of enormous importance for a better understanding of the molecular mechanism involved in the viral protein synthesis, replication, and infection. Moreover, the study of virus-host interactions will assist to identify checkpoints for therapeutic targets and novel vaccine design in the future. Considering the significance of viral-host protein interactions, this study was designed with the objective to "Unravel the host factors interacting with the NS3 protein during bluetongue virus replication".

The current study was focused on the better understanding of BTV NS3 protein role by recognizing its host interacting factors using the Y2H system. NS3 protein is encoded by seg 10 of BTV and it occurs in two isoforms i.e. full-length NS3 with 229 aa and NS3a with 216 aa which lacks 13 aa at N-terminus resulting from an alternative translation start site (Wu *et al.*, 1992). It consists of a long N-terminal and short C-terminal cytoplasmic domains that are allied by two TM (Bansal *et al.*, 1998; Belhouchet *et al.*, 2011). The TM domains are connected with a short extracellular loop which possesses a glycosylation site at asparagine 150 and NS3 protein can exists in both nonglycosylated and glycosylated forms in vivo (Bansal *et al.*, 1998; Beaton *et al.*, 2002). It also posses two late polybasic motifs PSAP and PPRY (92-97 aa and 114-121 aa position) upstream to the TM1 and are common in all orbiviruses. These polybasic motifs are involved in the endosomal sorting complex required for trafficking (ESCRT) of the vacuolar protein sorting pathway, a cellular budding network which forms multivesicular body (MVB) (Wirblich *et al.*, 2006; Hurley, 2008; Celma and Roy, 2009). NS3 protein localizes in the endoplasmic reticulum (ER) and Golgi apparatus before reaching the plasma membrane and form a homo-oligomer. It affects cellular functions, protein trafficking, and membrane permeability. It forms pores through lipid bilayers and promotes virus release, thus function as viroporin (Han and Harty, 2004). Studies have reported the importance of NS3 protein in BTV core assembly and its interaction with outer capsid proteins together for maturation and release of the virion (Bhattacharya *et al.*, 2007; Bhattacharya and Roy, 2008; Celma and Roy, 2011). It also produces cytotoxicity upon expression in mammalian or insect cells (van Staden *et al.*, 1995) suggesting its substantial implications for the pathogenicity of BTV infection.

Various robust techniques are available to study the interaction between virus and host proteins, including affinity purification-mass Spectrometry, fluorescent imaging-based techniques, siRNA screens, and Y2H, etc. (Meng *et al.*, 2005; Sardiu and Washburn, 2011). However, the Y2H system is the most throughput technique to study PPI recommended by many researchers (Brückner *et al.*, 2009; Rao *et al.*, 2014; Podobnik *et al.*, 2016; Gillen and Nita-Lazar, 2019). It is an in-vivo genetic approach based on the reconstitution of a functional transcription factor when two proteins or polypeptides of interest interact. It can detect the minimal interacting domains and has the capacity to demonstrate even weak protein interactions (Fields and Song, 1989) and has been used to study several virus-host interactions (Gladue *et al.*, 2012; Ohta *et al.*, 2018; Wang *et al.*, 2018; Paiano *et al.*, 2019). The current study uses the Y2H system as the primary assay for preliminary identification of BTV NS3 interacting host proteins which are further confirmed by using β- galactosidase-based assays, GST pull-down, and M2H studies.

5.1 Construction and characterization of cDNA libraries

A complex, diverse, and high-quality cDNA library is indispensable for performing Y2H based screening. Many researchers in past have described the construction of Y2H compatible libraries derived from various sources for screening host factors interacting with several viruses (Zhao *et al.*, 2004; Cao and Yan, 2013; Mahajan *et al.*, 2015; Wang *et al.*, 2018; Xu *et al.*, 2020). For BTV also Beaton *et al*. (2002) had used a cDNA library derived from human cell line and Pourcelot *et al*. (2019) had used libraries derived from bovine and culicoides cell lines for studying the host factors interacting with BTV. Albeit, as of now no one has employed the cDNA library derived from the susceptible host (i.e. sheep) or BHK-21 cell line for screening the BTV-host PPI. In the present study, two cDNA libraries derived from sheep lung tissue and naïve BHK-21 cell line were constructed. Sheep is the most severely affected host for BTV and the virus has a very strong affinity for endothelial cells (ECs), macrophages, and conventional dendritic cells (cDC). BTV replicates primarily in lymph nodes, spleen, and lungs by damaging the microvascular ECs. Besides, the lung remains the most severely affected tissue where the virus tends to localize in greater concentrations as it is rich in ECs (Uren and Squire, 1982; Wang *et al.*, 1988; DeMaula *et al.*, 2002). On the other side

BHK-21 cell line is highly susceptible to BTV infection producing higher yields of the virus with a low multiplicity of infection and supports all the serotypes of BTV (McPhee *et al.*, 1982; Wechsler and McHolland, 1988; Sekar et al., 2009). Hence, the lung tissue and BHK-21 cells were chosen to construct the cDNA libraries. The cDNA libraries were constructed using the SMART technique that employs template switching and reverse transcription properties to enhance the length of cloned templates and the quality cDNA library. Less than 1 µg of the corresponding high-quality mRNAs were used for first-strand cDNA synthesis. The ds cDNAs were amplified by LD-PCR with 22 cycles, found to be optimal for both the cDNAs to reduce the PCR induced mutations and the selection bias for smaller size cDNA. The ds cDNAs were further purified using CHROMA-SPIN TE-400 columns to exclude the incorporation of the small inserts (low molecular weight ds cDNA, adapters, and unincorporated nucleotides). The purified ds cDNAs were co-transformed with the pGADT7-rec vector into the competent yeast strain Y187. The colonies grown were collected in a freezing medium and preserved as libraries. The integration of template switching and directional cloning ensures the construction of cDNA libraries with a high yield of representatives. Various quality parameters were estimated for the generated libraries and all were found to be equivalent or above the baseline value indicating the worth of the libraries (Table 4.1). The libraries were having more than one million independent clones reflecting their complexity. Also, a high transformation efficiency and recombination rate was achieved for both the libraries (Table 4.1). Pre-freezing library titer and the cell densities were calculated to evaluate the quality of the constructed libraries, were found to be well above the standard, and were maintained post storage at -80 °C (Table 4.1). All these attributes corroborate that the constructed cDNA libraries are of high quality and appropriate for the study of BTV-host PPI. A similar type of results have been reported by Gao *et al*. (2004), Cao and Yan (2013), and Wang *et al*. (2018) for high-quality Y2H libraries. Approximately 500 ml of each library was produced while only 1 ml of the library is needed to perform one large-scale mating between prey and bait. Hence, libraries generated in this study can be used to screen host proteins interacting with various proteins of BTV as well as to screen host interactions of other viruses that affect small ruminants and can grow in BHK-21 cell line. Due to time and resource limitations only one cDNA library opted for further nucleotide sequencing and as the prey for screening. Sheep being the natural host for BTV and considering

the novelty of library from natural susceptible host origin as described earlier, sheep lung cDNA library was selected. Nucleotide sequencing and BLAST search analysis of sequenced ESTs of sheep lung cDNA library evidenced 89.8 % of the random sequences were homologous to the characterized or putative proteins or the mRNAs of the sheep (Table 4.2). The GO analysis revealed the diversity of transcripts found in the library suggesting that the constructed library is unbiased and very much suitable for preliminary screening of virus-host interactions. We have employed this library as prey to screen host proteins interacting with the NS3 protein of BTV.

5.2 Forward Library screening and confirmation by β-galactosidase assays

The Y2H assay was used for preliminary screening of host proteins interacting with BTV NS3 protein. The greatest advantage of Y2H screening is that a cloned genome prey library derived from target cell type could rapidly screen a large number of potential prey interactions that might have functional significance (Brückner *et al.*, 2009; White and Howley, 2013). The same approach was applied in this study and a genome library constructed from the BTV susceptible host was screened to determine the host proteins interacting with the NS3 protein of BTV. The Y2H screening was carried out using Matchmaker Gold Y2H System where Gal4, a yeast transcription factor is split into BD and AD. Prey proteins are expressed as a fusion to AD in the pGADT7 vector while a bait protein is expressed as a fusion to BD in the pGBKT7 vector. Only the co-expression of the bait and an interacting prey reconstitute the full transcription factor that stimulates transcription and activation of reporter genes. AUR1-C, ADE2, HIS3, and MEL1 are the four independent reporter genes that are transcribed upon the interaction between bait and prey proteins. Many studies have employed the same system to identify the virus-host interactions (Beaton *et al.*, 2002; Celma and Roy, 2011; Silva *et al.*, 2013; Vidalain *et al.*, 2015; Wang *et al.*, 2018). Large-scale virus-host PPI studies have been conducted with EBV (Calderwood *et al.*, 2007) and HCV (de Chassey *et al.*, 2008). A similar approach has been employed to identify the interacting host proteins of CHIKV, HBV, DENV, HCMV, and influenza virus (Bai *et al.*, 2005; Limjindaporn *et al.*, 2007; Dudha and Gupta, 2016; Wang *et al.*, 2016; Ohta *et al.*, 2018). Very recently, Mahajan

et al. (2021) have reported the interaction between FMDV and MARCH7, an E3 ubiquitin ligase using the fetal bovine kidney cell line (LFBK) cDNA library.

The characteristic cell rounding, cell rupture, and detachment were observed in BHK-21 cells after 72 h of infection with BTV-10 (Clavijo *et al.*, 2000). The dsRNA of BTV was extracted from the BHK-21 cells and was separated on PAGE. All the 10 segments of the virus were visible clearly by silver nitrate staining. The full-length NS3 coding region of BTV was amplified and cloned in pGBKT7-BD vector followed by the transformation in Y2H Gold cells to generate the BTV-NS3 bait. The bait was characterized for its properties before screening the prey library because the Y2H system has some significant limitations, particularly to detect false-positive or false-negative interactions. Sometimes bait or prey protein may itself can activate the Y2H reporter gene in the absence of any binding partner which hinders the true interaction between bait and prey (White and Howley, 2013). Nonetheless, the Y2H system used in this study is reported to have less probability of showing false positives owing to the presence of four independent reporter genes (Serebriiskii *et al.*, 2000). To meet the requirement, full-length NS3 bait was tested for autonomous activation of the reporter genes (AUR1-C, ADE2, HIS3, and MEL1) in absence of prey protein. The full-length NS3 bait produced white colonies on SD- Trp/X plates while no colonies were observed in presence of Aureobasidin A, a definitive selectable marker for yeast. Whereas blue colonies were observed in the positive diploid control in presence of Aureobasidin A, indicates activation of the *AUR1-C* Y2H reporter by positive control. Thus it eliminates the likeliness of auto-activation by full-length NS3 bait. The toxicity level of the bait was also tested by comparing its colony size with the colonies of empty pGBKT7-BD vector and was found to be non-toxic to the yeast cells. Further, the expression of full-length NS3 bait in yeast cells was analyzed and confirmed by western blotting. The bait was found to be well expressed, adequate expression indicates that the constructed bait can interact with the corresponding host proteins. The full-length NS3 bait qualified all the parameters, confirms that the generated bait is suitable for screening the prey library.

The full-length NS3 bait strain was grown overnight, re-suspended to the cell density of $>1x10^8$ cells/ml, and mated with the ovine library for preliminary screening of BTV-NS3

interacting host proteins. The mating was found to be of high quality on basis of evaluation parameters (Table 4.1) but blue colonies were not observed on DDO/X/A plates. Repetition of the experiment also led to the same result which unequivocally indicates either there is failure of mating experiment or there is no interaction between NS3 and prey library . As there are only two main variables in Y2H i.e. bait and prey, to verify which one failed to perform, the mating was conducted using BTV NS4 bait since changing the bait is much easier than changing the prey library (Brückner *et al.*, 2009). Several blue colonies were developed on mating between NS4 bait and the ovine library assuring that the library was working well. So the problem was left with bait only. In Y2H some full-length proteins do not yield interactions despite good expression of bait or prey. Full-length proteins fail to perform might be due to incorrect folding in yeast, precluding interaction with its partner, or due to their biology (Galletta and Rusan, 2015). On thorough analysis, the presence of two TM was observed in the NS3 sequence. The position of TM domains were located from 122 to 139 aa and 166 to 182 aa using Phobius (Käll *et al.*, 2004) and TMPred software (Hofmann and Stoffel, 1993). Previously, it has been reported that the presence of TM domains limit the BTV full-length NS3 expression in the prokaryotic system by producing toxicity (Chacko *et al.*, 2015; Mohanty *et al.*, 2016). NS3 protein of Ibaraki virus, a member of the orbivirus genus has also been documented to produce cytotoxic effect owing to the presence of membrane association and glycosylation (Urata *et al.*, 2016). NS3 protein of African horse sickness virus (AHSV), another orbivirus produces a cytotoxic effect on the protein expression in the baculovirus system (van Staden *et al.*, 1995; van Niekerk *et al.*, 2001). Literature suggests that the structure and distribution of the TM domains within the protein control the expression of cytotoxicity it produces. Mutations altering the TM domains or deletion of these TM domains reduce the cytotoxicity of the protein (van Niekerk *et al.*, 2001; Huismans *et al.*, 2004; Mohanty *et al.*, 2016). Thus, it was hypothesized that the TM domains in the NS3 protein may produce steric hindrances in its activity and might be applicable during interaction with other proteins in the Y2H system. Besides, the use of sub-fragments was found to be outperforming in Y2H than the full-length one (Galletta and Rusan, 2015)and an earlier Y2H study for BTV NS3 has also mentioned the use of the truncated NS3 only (Beaton *et al.*,

2002). Thus, the experiment was redesigned to generate truncated NS3 ($NS3_t$) bait to mate with the ovine library.

C-terminus truncated NS3 ($NS3_t$ aa 1-115) was cloned in pGBKT7-BD vector and was transformed in Y2H gold cells to generate the pGBKT7-$NS3_t$ bait. The newly constructed bait was again characterized for all the parameters in a similar manner done for full-length NS3 bait and was found to qualify all the parameters. The mating of $NS3_t$ bait with the ovine library resulted in blue colonies on DDO/X/A plates and all the mating evaluation parameters show that the mating was of high quality and reliability. In preliminary screening, a total of 65 blue colonies were developed DDO/X/A agar plates. These colonies were further patched onto the QDO/X/A agar plates for higher stringency screening and the number reduced to 37. This number includes colonies that might contain smaller inserts or more than one prey insert. To confirm this, 37 colonies were subjected to PCR and sequencing. After BLAST search and comprehensive sequence analysis, eight individual interacting preys were identified by excluding the repetitive clones or clones with small insert sizes. As the library constructed in this study was not normalized like commercial libraries hence it was obvious to get some redundant prey proteins after mating. Wang *et al.* (2018) have reported a similar type of results for Tembusu virus interaction with cDNA library from duck embryo fibroblasts origin where 133 blue colonies were identified in preliminary screening on DDO/X/A and QDO/X/A and the final number reduced to seven after sequence analysis. They also found redundancy in mating where multiple clones were noted for individual host proteins. Xu *et al.* (2020) have reported the identification of 36 clones using *the Cymbidium faberi* flower cDNA library. Also, Mahajan *et al.* (2021) have very recently identified 21 novel interactors for FMD 2C protein using LFBK cDNA library, where they also got many redundant clones on preliminary screening. Multiple colonies observed for selected eight preys were segregated by repeated streaking on DDO/X/A and patching on QDO/X/A agar plates. The eight unique plasmids were rescued from these isolated colonies using a zymolyase-based yeast isolation kit. The isolated plasmids were subjected to sequencing and analyzed by BLAST search. The sequences of the isolated plasmids were cross-checked with the sequences obtained from colony PCR products, to abolish any cross-contamination or mislabeling during the streaking and patching of a large set of colonies.

The eight host proteins determined as the interacting partners for BTV $NS3_t$ protein were transformed in yeast cells and small-scale mating of individual prey protein was done with the bait to verify their interaction. The development of blue colonies on QDO/X/A plates eliminated the doubt regarding the interaction of isolated plasmids. Further, the interactions results of prey proteins with NS3t protein were strengthened by β-Gal assays exploiting the LacZ reporter gene. Colony lift filter assay reaffirmed the interaction among the respective bait and prey proteins while ONPG assay determined the strength of their interaction. As a turnout of these yeast-based assays we could conclude that the interaction observed for prey protein DCTN2 and BTV $NS3_t$ bait protein in large-scale mating was the false-positive or a weak transient interaction. Finally, the combined results confirmed that seven out of eight proteins were interacting with the BTV $NS3_t$ protein in yeast-based system.

5.3 Confirmation of interactions by alternative assays

Based on the results from yeast-based assays NAP1L1, Tsg101, PDIA4, UBE2D4, GOLGA2, EID1 and ACTN1 proteins have shown strong interaction with BTV $NS3_t$ protein. Among the seven interactions we obtained, Tsg101 interaction with NS3 protein of BTV has already been reported by Wirblich and colleagues (2006) although, not by using the Y2H system. They have demonstrated that orbivirus NS3 protein recruits Tsg101, a cellular protein to facilitate the virus release in mammalian as well as insect cells. They also reported the interaction with Tsg101 occurs through the PASP motif of NS3 protein and mutation of this motif obliterates NS3 binding Tsg101. The calpactin light chain (p11) of the cellular annexin II complex has been reported by Beaton and colleagues (2002) as a BTV NS3 interactor using the Y2H system. They reveal that the first 13 aa of NS3 interact with high specificity and interaction with p11 helps in active cellular exocytosis. Kundlacz and colleagues (2019) have identified the BRAF as a new cellular interactor of BTV NS3. Their study has demonstrated that BRAF silencing, significantly decreases the MAPK/ERK activation and suggests that the BTV manipulates MAPK/ERK pathway for its own replication. As per our knowledge, the interacting prey proteins identified in this study except Tsg101 are not reported earlier. On the basis of yeast-based assays results and thorough analysis of the sequences and functional significance of the identified preys, NAP1L1 was selected for further validation of its interaction with BTV $NS3_t$ protein.

Nucleosome assembly protein 1-like 1 (NAP1L1) is a member of the nucleosome assembly protein family. It is a ubiquitously expressed protein and is conserved from in all eukaryotes right from yeast to humans (Ishimi and Kikuchi, 1991). It is a multifunctional protein engaged in chromatin assembly and remodeling, cell-cycle regulation, transcription regulation, and apoptosis (Park and Luger, 2006; Zlatanova *et al.*, 2007). NAP1L1 is a histone chaperone accountable for the incorporation of histone H2A-H2B dimers to complete the nucleosome. It is also involved in the nucleo-cytoplasmic shuttling of proteins that carry H2A-H2B dimers from the cytoplasm towards the chromatin assembly machinery in the nucleus. NAP1L1 plays an important role in cell cycle progression during the G1 phase and mitosis. Studies suggest that NAP1L1 interacts with some viruses through their transcription-activating proteins (Rehtanz *et al.*, 2004; Vardabasso *et al.*, 2008). Using the Y2H system only, human NAP1L1 (hNAP-1) has been identified as an interaction partner for E2 protein of BPV1 for transcription activation. hNAP1L1 is alone able to stimulate the p53 gene thus E2 protein interaction with NAP1L1 may play a significant role in BPV1 pathogenesis (Rehtanz *et al.*, 2004). hNAP-1 also binds with HIV-1 Tat protein both in vitro and in vivo and takes part in the regulation of Tat-mediated activation of viral gene expression (Vardabasso *et al.*, 2008). NAP1L1 has been reported as the primary candidate to promote CHIKV replication in vertebrates but mosquito homolog for this has not been found (Meshram *et al.*, 2018). As CHIKV is an arthropod transmitted virus-like that of BTV, the above finding may be correlated with BTV also. NAP1L1 has been confirmed as a binding partner for HCV NS5A and NS3 protein. Besides, HCV NS5A subvert the host innate response by targeting NAP1L1 leading to downmodulation of NF-kB, explains NAP1L1 role as antiviral effectors (Çevik *et al.*, 2017; Yin *et al.*, 2018). BTV NS3 has also been considered to have a role in host proteins shut down and is reported as an interferon (INF) antagonist (Chauveau *et al.*, 2013), provides an explanation for a possible role of NAP1L1 in BTV mediated immune modulation. By targeting NAP1L1, it is possible for viruses to control a subset of host genes, including cardinal components of the antiviral innate sensing mechanism. Therefore, investigation of the transcriptional level mechanism of action of NAP1L1 during viral infection is important (Çevik *et al.*, 2017). The NAP1L1 interaction with $NS3_t$ protein of BTV was validated by using GST pull-down and Mammalian two-hybrid assays. The SEAP activity of NAP1L1/NS3 interaction was manifold higher than

the negative control and was comparable to that of the positive control, confirming true interaction between NAP1L1 and BTV NS3 protein.

In this study, two high-quality Y2H compatible cDNA libraries from ovine tissue and BHK-21 cell line were constructed using the SMART technique to be used as prey in large-scale Y2H screens. The ovine library was subjected to sequencing and used for further downstream PPI studies. Sequence analysis of randomly picked clones from the ovine library showed 89.7% of ESTs were identical with the characterized or putative proteins or the mRNAs of the sheep while 4% ESTs showed homology with the goat proteins. The GO analysis of the sequenced ESTs demonstrated very less redundancy and much diversity of the transcripts was present in the ovine library. Preliminary Y2H screening between ovine cDNA library and BTV full-length NS3 protein, no interactions were found. On troubleshooting, the presence of TM domains within the NS3 sequence was found to hinder NS3 interaction with prey proteins. So, the Y2H screening was done once again using truncated NS3 which did not contain TM domains and eight putative prey proteins were identified. The identified prey proteins were confirmed by applying the β-Gal assays. Seven prey proteins were found to pass β-Gal assays and one failed to prove its interaction. These seven proteins were analyzed critically for their functional relevance in the host and NAP1L1 was selected for further validation. GST pull-down and mammalian cell assay proved NAP1L1 interaction with BTV NS3t protein, suggesting NAP1L1 as a potential interacting partner for BTV $NS3_t$. Still to claim NAP1L1 as a bonafide interacting candidate for BTV NS3 other validation and functional studies under wild type, over-expressed and knock-down conditions are need to perform in the future. Further studies are also needed to be carried out to unravel the mechanisms and pathways involved in the interaction between BTV NS3t and the remaining identified host proteins. This study will pave the way as the preliminary source for investigating the BTV-host interface. This was for the first time library scale screening of host-virus PPI of Indian variant of BTV NS3 protein was carried out. The PPI study can reveal the difference in replication strategies with the mammalian and insect host and identify the new checkpoints for novel vaccine and drug targets.

Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

BT is an infectious and non-contagious disease of ruminants transmitted by midges of the *Culicoides* genus. BTV can replicate in many species of ruminants including sheep, goats, cattle, camels, wild or zoo ruminants, and farmed cervids. Clinical symptoms are more explicit in the sheep and North American white-tailed deer while others are often asymptomatically affected. Cases range in severity from mild to rapidly fatal, and animals that survive may be debilitated. BTV is very diverse as there are 27 serotypes probably more, can reassort to form new variants. Besides, the existence of multiple serotypes complicates control strategy, as immunity to one serotype may not provide cross-protection against the other. The rapid spread of disease over a large area causes substantial constraints to the international trade of animals and animal products. Spread via vectors makes the disease more challenging to control, except by vaccination. BTV leads to different pathogenesis in mammalian and insect hosts where distinct pathology can be observed in mammalian hosts and insects mostly remain persistently affected. The difference can be associated with the involvement of host cellular factors which help the virus to make capital out of host machinery. The virus may have diversity in virus replication, packaging, and cell egress depending on the host. Precise information on host-virus interaction during virus trafficking between mammalian and insect vector cells is not available. Thus, the studies on a deeper understanding of the genetic elements in the virus life cycle, viral proteins, and the host factors involved in the whole infectious process may help to define the molecular determinants of BTV virulence and factors responsible for the subjugation of host machinery. PPI study has been acknowledged as a critical high-throughput tool to study virus-host interface.

The present study was designed for a better understanding of the NS3 protein of BTV using the Y2H system to shed light on the virus-host interaction mechanism. The Y2H compatible cDNA libraries were constructed, characterized and the best performing cDNA library was used as the prey in the Y2H system to preliminary screen the host proteins interacting with the NS3 protein of BTV. The putative interacting host proteins were identified and the interactions were further corroborated using different approaches. NS3 is 229 aa protein, encoded by the seg 10 of BTV. NS3 is the only glycoprotein of BTV and performs multiple functions including virus assembly, maturation, and intracellular trafficking. It alters membrane permeability, acts as viroporin, and promotes virus release, also act as an important virulent factor having a role in host protein shutdown.

In the present study, two cDNA libraries derived from sheep lung tissue and naïve BHK-21 cell line were constructed. The total RNAs were extracted from the sheep lung tissue and uninfected BHK-21 cells. The poly(A) enriched mRNAs were extracted from the total RNAs and corresponding first-strand cDNAs were constructed using the SMART technique. The ss cDNAs were amplified to ds cDNAs by 22 cycles of LD-PCR and the resultant ds cDNAs were purified using CHROMA-SPIN TE-400 to exclude the predominance of the small size inserts. The purified ds cDNAs from the respective sources were directly co-transformed with the pGADT7-rec vector into the competent prey yeast strain Y187. The quality parameters for the constructed cDNA libraries were measured. The sheep lung library and naïve BHK-21 library were found to contain $>1.68\times 10^6$ and 1.2×10^6 independent clones, and a recombination rate of 98% and 92%, respectively. The libraries were having cell density and titer greater than 10^7 cells/ml and 1×10^7 cfu/ml which did not decline much upon freeze-thaw. A range of inserts observed in the libraries indicating the high quality and diversity of constructed cDNA libraries. Both the libraries were found suitable for use as the prey in the Y2H system; however, considering the significance of infection in the natural host, the ovine cDNA library was selected for identifying BTV-interacting host partners. Both the libraries were aliquoted and stored in -80 ^oC for future use.

The nucleotide sequencing of the randomly selected 50 clones from the ovine cDNA library was carried out. The sequences were assembled and subjected to a BLAST search

against the nonredundant (nr) GenBank database to compare them with the currently available data. The sequences were analyzed with the homologous top hits proteins and submitted to GenBank using BankIt. 89.7% of ESTs were showing homology with the characterized or putative proteins or the mRNAs of the sheep while 4% ESTs were identical with the goat proteins. The GO analysis of the sequenced ESTs demonstrated minimum redundancy and diversity of the transcripts was observed in the ovine library. The constructed cDNA library would be one of the useful biological products to study the BTV-host interactions as well as for studying host-virus protein interactions for other important small-ruminants pathogens.

The Y2H assay was used for the preliminary screening of the cellular proteins interacting with the BTV NS3 protein. The Y2H screening used in this study was based on the activation of four independent reporter genes (AUR1-C, ADE2, HIS3, and MEL1) which are transcribed in case of positive interaction between bait and prey proteins only. Initially, the full-length NS3 coding region of BTV 10 was amplified and cloned in the pGBKT7-BD vector. The recombinant pGBKT7-NS3 plasmid was then transformed in the competent Y2H Gold cells to generate BTV-NS3 bait. Auto-activation and toxicity analysis for the constructed bait was carried out, also the expression was checked. The BTV-NS3 bait was proven to be non-autoactivating, non-toxic, and was well expressed in yeast cells. The well-characterized BTV-NS3 bait and the constructed ovine prey library were mated for screening the NS3 interacting host proteins. The forward-library screening between the BTV NS3 and prey library was of good quality but could not yield blue colonies on DDO/X/A agar plates. On repetition of an experiment by changing the bait confirmed that the BTV full-length NS3 bait was not able to interact with the host proteins. The detailed study left with the conclusion that the presence of TM domains within the NS3 proteins, impedes its interaction with prey proteins. Thus, the C-terminus truncated NS3 bait ($NS3_t$) excluding the TM domains was constructed and characterized. The mating between the BTV NS3t bait and ovine cDNA library was found to be of high grade and reliability as 3.14 % of the mating efficiency has resulted. The preliminary screening yielded 65 blue colonies on DDO/X/A agar plates which were reduced to 37 on patching onto the higher stringency QDO/X/A agar plates. These 37 colonies were PCR amplified and subjected to sequencing. On BLAST search analysis, only eight prey proteins were selected

as probable interactors by eliminating the redundant ones and the inserts with smaller sizes. Plasmids were rescued from these eight prey clones and their interactions with BTV NS3t were further confirmed by yeast-based confirmation assays like co-transformation and β-Gal assays (colony-lift filter and ONPG liquid culture assays). Considering the combined results of mating and β-Gal assays, seven prey proteins were confirmed as the interacting partners for BTV protein in a yeast-based system.

Among the seven interacting prey proteins confirmed in the yeast-based system, NAP1L1 evidenced the strong interaction with BTV $NS3_t$ protein. NAP1L1, a member of the nucleosome assembly protein family act as histone chaperon, also engaged in chromatin remodeling, cell-cycle regulation, and transcription regulation So, NAP1L1-BTV $NS3_t$ interaction was corroborated by GST pull-down assay, and the interaction was detected on immunoblot using anti-GST, anti-c-myc, and anti-NS3 antibodies. Furthermore, the NAP1L1-BTV $NS3_t$ interaction was again using an M2H system that supports post-translational modifications.

In this study, two high-quality Y2H compatible cDNA libraries from sheep lung and BHK-21 cells origin were constructed for use as prey in large-scale Y2H screens. This is the first study to report the construction of cDNA libraries derived from the BTV susceptible hosts as well as a cell line. For both, the libraries quality parameters were well above the thresholds indicating their applicability in screening the host-virus interaction. The GO analysis of the sequenced ESTs of the ovine library revealed a wide diversity of the transcripts without much redundancy emphasizing its suitability as prey in PPI screens. Initially, Y2H screening between ovine cDNA library and full-length NS3 protein of BTV could not reveal any interaction whereas changing the NS3 bait from full-length to truncated one by excluding the TM domains resulted in interactions. On preliminary screening, 65 interactions were observed, which reduced to 37 on higher stringency screening. This number was again reduced to eight upon PCR confirmation and sequence analysis. Finally, the selected eight unique prey plasmids were rescued and their interactions were confirmed on rigorous screening by co-transformation and β-Gal assays. By eliminating false positives, seven preys out of eight were confirmed positive. Based on results from yeast-based assays NAP1L1 was found to be strongly interacting with

BTV $NS3_t$. NAP1L1-BTV $NS3_t$ interaction was further confirmed by GST pull-down and M2H assays. Thus, our study confirms NAP1L1 as an interacting partner for BTV $NS3_t$ protein. However, more studies needed to be carried out to unravel the mechanisms and pathways involved in the interaction between the BTV NS3 and each of the identified host proteins.

Mini Abstract

7 MINI ABSTRACT

Bluetongue is an infectious and devastating viral disease of domestic and wild ruminants. The disease poses significant losses to livestock health and the agricultural economy. It is primarily an arthropod-transmitted disease which makes its control challenging and has become endemic in several parts of the world, including the Indian subcontinent. Thus, the present study was undertaken to better understand the role of BTV NS3 protein in the virus-host interface. For that purpose, two Y2H compatible cDNA libraries from the sheep lung and BHK-21 cells were constructed and characterized. Both the libraries were found to be diverse and of high quality for their application in the Y2H system. Sheep lung library was further sequenced and used for screening the host proteins interacting with BTV NS3 protein. Initial screening with full-length NS3 bait did not result in any interaction with the prey library. However, the modified truncated NS3 bait ($NS3_t$) without transmembrane domains resulted in multiple hits. High-quality and reliable mating was observed between the ovine library and $NS3_t$ bait with the mating efficiency of 3.14%. Preliminary screening resulted in 65 interacting blue colonies on DDO/X/A agar plates which were reduced to 37 on stringent QDO/X/A media selection. To eliminate redundant clones, all 37 colonies were PCR amplified and sequenced. The sequences were subjected to BLAST analysis and eight unique prey proteins were selected as interacting partners for BTV $NS3_t$. Plasmids of these eight prey proteins were rescued and their interaction with BTV $NS3_t$ was again confirmed by co-transformation and β-galactosidase assays. Finally, seven prey proteins were confirmed as the interacting partners for BTV $NS3_t$ protein in a yeast-based system. NAP1L1 prey protein was found to show strong interaction with BTV $NS3_t$ in a yeast-based system hence was selected for further validation. GST pull-down assay confirmed the interaction between NAP1L1-BTV $NS3_t$. Also, their interaction was again corroborated in mammalian cells using a mammalian two-hybrid assay. Thus, the results from the GST pull-down and mammalian two-hybrid assay assure true interaction between NAP1L1 and BTV $NS3_t$ protein. This is the first study to report the construction and characterization of cDNA libraries from BTV susceptible host and cell line. The constructed libraries are suitable for application in Y2H screens and NAP1L1 protein, identified by Y2H screening interacts genuinely with BTV $NS3_t$ protein. However, detailed functional studies are needed to carry out to reveal the subtle mechanism of this host protein in BTV replication and pathogenesis.

लघु सारांश

8 लघु सारांश

नीलर्सना, घरेलू और जंगली जुगाली करने वाले जानवरों का एक संक्रामक और विनाशकारी रोग है। यह रोग पशुधन स्वास्थ्य और कृषि अर्थव्यवस्था को महत्वपूर्ण नुकसान पहुचाता है। यह मुख्य रूप से सन्धिपाद संक्रमित रोग है, जिस वजह से इसका नियंत्रण चुनोतीपूर्ण है। यह रोग भारतीय उपमहाद्वीप सहित दुनिया के कई हिस्सों में स्थानिक हो गया है। इसलिये वर्तमान अध्ययन में वायरस होस्ट परस्पर क्रिया में BTV NS3 प्रोटीन की भूमिका को बेहतर ढंग से समझने के लिये प्रयत्न किया गया है। इस प्रयोजन के लिये भेड़ के फेफड़े और BHK-21 कोशिकाओं से दो Y2H संगत cDNA लाइब्रेरीज का निर्माण किया गया और उनका मुल्यांकन भी किया गया। Y2H प्रणाली में उनके अनुप्रयोग के लिये दोनों लाइब्रेरीज उच्च गुणवत्ता की पायी गई हैं। भेड़ फेफड़े की लाइब्रेरी को आगे अनुक्रमित किया गया और BTV NS3 प्रोटीन के साथ इंटरेक्ट होने वाले होस्ट प्रोटीन की जांच करने के लिये इस्तेमाल किया गया। प्रारंभिक जांच में पुरे NS3 प्रोटीन के साथ कोई इंटरैक्शन नहीं पाया गया। हालांकि, ट्रांसमेम्ब्रेन डोमेन के बिना संशोधित NS3 ($NS3_t$) के साथ कई इंटरैक्टींग प्रोटीन पाये गये। $NS3_t$ प्रोटीन के साथ उच्च गुणवत्ता और विश्वसनीय इंटरैक्शन देखा गया, जिसमें 3.14% मेटिंग इफिशिंयसी पायी गई। प्रारंभिक जांच के परिणामस्वरूप DDO/X/A की प्लेस्ट पर 65 इंटरैक्टिंग मिली कौलोनिया मिली जो QDO/X/A के चयन में 37 तक कम हो गई। दोहराये गये क्लोनों को कम करने के लिये सभी 37 कौलोनियों को पीसीआर प्रवर्धित और अनुक्रमित किया गया। अनुक्रमों का BLAST विश्लेषण किया गया और आठ होस्ट प्रोटीनों को BTV $NS3_t$ के भागीदारों के रूप में चुना गया। इन आठ होस्ट प्रोटीनों के प्लास्मिड को निकाला गया और BTV NS3t के साथ उनके इंटरेक्शन की फिरसे सह–परिवर्तन और β-galactosidase से पुष्टि की गयी। अंत में सात प्रोटीन का BTV $NS3_t$ के साथ मजबूत इंटरेक्शन पाया गया। इसलिये इसे आगे सत्यापन के लिये चुना गया। GST पुल डाऊन परख में NAP1L1-BTV $NS3_t$ के बीच के इंटरेक्शन की पुष्टि की गयी। इस अध्ययन से यह निष्कर्ष निकाला जा सकता है कि विकसित की गई लाइब्रेरीज का प्रयोग इंटरेक्शन जानने में अत्यंत उपयुक्त होगा। NAP1L1 प्रोटीन BTV विषाणु के $NS3_t$ प्रोटीन से इंटरैक्ट करता है। यद्यपि अन्य प्रयोगों से ही इस बात की पूर्ण पुष्टि होगी।

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Appendix

APPENDIX-I

1. 1.1xTE/LiAc

10X TE buffer (Clontech, USA)	1.1 ml
1 M LiAc (10X; Clontech, USA)	1.1 ml

Make final volume up to 10 ml with sterile, deionized water

2. PEG/LiAc

50% PEG 3350 (Clontech, USA)	8 ml
10X TE Buffer (Clontech, USA)	1 ml
1 M LiAc (10X)	1 ml

3. Freezing medium

YPDA medium (sterile; Clontech, USA)	100 ml
75 % glycerol (sterile)	50 ml

4. TSS

2X LB broth	10 ml
Mg^{2+} (2 M)	200 µl
Dimethyl sulfoxide (Merck, Germany)	1 ml
30% PEG-8000	7 ml
Autoclaved DW	1.8 ml

Mixed and sterilized by filtration through 0.2 µm membrane filter

5. Ampicillin Stock solution

Ampicillin (Calbiochem, Merck)	100 mg
DW	1 ml

Sterilized by filtration through 0.2 µm membrane filter & stored at -20°C in aliquots

6. Kanamycin Stock solution

Kanamycin (Calbiochem, Merck)	50 mg
DW	1 ml

Sterilized by filtration through 0.2 µm membrane filter & stored at -20°C in aliquots

7. Chloramphenicol Stock solution

Chloramphenicol (VWR, USA)	34 mg
Ethanol	1 ml

Sterilized by filtration through 0.2 µm membrane filter & stored at -20°C in aliquots

8. Aureobasidin A Stock Solution

Aureobasidin A (Clontech, USA)	1 mg
Ethanol	2 ml
Store at 4°C	

9. X-α Gal Stock Solution

X-α Gal (Clontech, USA)	100 mg
Dimethylformamide	5 ml
Store at -20°C	

10. YPDA-Kan medium

YPDA medium (Clontech, USA)	100 ml
Kanamycin stock	100 µl

11. 0.5X YPDA-Kan medium

YPDA medium (Clontech, USA)	50 ml
Autoclaved DW	50 ml
Kanamycin stock	100 µl

12. LB-Ampicillin agar plates

LB agar (autoclaved; HiMedia, India)	100 ml
Ampicillin stock	100 µl

13. LB-Ampicillin agar plates

LB agar (autoclaved; HiMedia, India)	100 ml
Kanamycin stock	100 µl

14. LB /Amp/Chl agar plates

LB agar (autoclaved; HiMedia, India)	100 ml
Ampicillin stock	100 µl
Chloramphenicol stock	100 µl

15. SD/-Trp/X agar plates

SD/-Trp agar (Clontech, USA)	100 ml
X-α Gal stock	200 µl

16. SD/-Trp/X/A agar plates

SD/-Trp agar (Clontech, USA)	100 ml
X-α Gal stock	200 µl
Aureobasidin A stock	25 µl

17. DDO/X agar plates

SD/-Leu/-Trp agar (Clontech, USA)	100 ml
X-α Gal stock	200 µl

18. DDO/X/A agar plates

SD/-Leu/-Trp agar (Clontech, USA)	100 ml
X-α Gal stock	200 µl
Aureobasidin A stock	25 µl

19. QDO/X agar plates

SD/–Ade/–His/–Leu/–Trp agar (Clontech, USA)	100 ml
X-α Gal stock	200 µl

20. QDO/X/A agar plates

SD/–Ade/–His/–Leu/–Trp agar (Clontech, USA)	100 ml
X-α Gal stock	200 µl
Aureobasidin A stock	25 µl

21. 100X PMSF

PMSF (Sigma-Aldrich, USA)	0.1742 g
Isopropanol	10 ml

Wrap the tube and store at room temperature

22. 20% w/v TCA

Trichloroacetic acid (Merck, Germany)	2 gm
Distilled water	10 ml

Store the solution at 4^{o}C

23. TCA buffer

Tris-HCl (pH 8, 1 M stock solution)	200 µl
Ammonium acetate (7.5 M stock solution)	66.6 µl
EDTA (0.5 M stock solution)	40 µl
Deionized H2O	9.7 ml
Protease inhibitor solution (50 µl/ml)	500µl, prechilled
PMSF (100X stock solution)	100 µl

Add the protease inhibitor solution and PMSF immediately prior to use

24. Tris/EDTA solution

Tris-base (1 M stock solution, not pH-adjusted)	2.0 ml
EDTA (0.5 M stock solution)	0.4 ml
Deionized H_2O	7.6 ml

25. SDS/glycerol stock solution

SDS (25% stock solution)	3.5 ml
Glycerol (100%)	3.5 ml
Tris-base (1 M stock solution, not pH-adjusted)	1.0 ml
Bromophenol blue	Spatula tip-full
Deionized H_2O	Make final volume of 12 ml

26. TCA-Laemmli loading buffer

SDS/glycerol stock solution	480 µl
Tris/EDTA solution	400 µl
β-mercaptoethanol	50 µl
PMSF	20 µl PMSF stock solution (100X)
Protease inhibitor solution	20 µl
Deionized H_2O	30 µl

27. Z buffer

$Na_2HPO_4.7H_2O$	1.61 g
$NaH_2PO_4.H_2O$	550 mg
KCl	750 mg
$MgSO_4.7H_2O$	246 mg

Final volume up to 100 ml with distilled water, adjust pH to 7.0 and autoclave

28. Z buffer/X-gal solution

Z-buffer	10 ml
β-ME	27 µl
X-gal (20 mg/ml)	167 µl

29. ONPG-Z buffer

Z-buffer	10 ml
ONPG	40 mg

Adjust pH to 7.4

30. 10X TAE buffer

Tris base	48.4 g
Glacial acetic acid (Merck, Germany)	11.4 ml
EDTA disodium salt (Merck, Germany)	3.7 g
DW	up to 1000 ml

Dilute 100 mL of 10X stock to 1 L with distilled water to prepare working solution

31. Trypsin-Versene

Trypsin	1.7 g
EDTA/Versene (disodium dihydrate)	1.4 g
1X PBS	up to 1000 ml

Sterilized by filtration through 0.2 µm membrane filter & stored at 4°C

APPENDIX-II

Preparation of competent cells by calcium chloride method

1. Inoculate 50 µl of *E. coli* DH5α cells in 5 ml LB broth (1:100 ratio) and incubate overnight at 37 °C with 180 rpm shaking.

2. Add 500 µl of overnight culture in 50 ml LB broth (1:100 ratio) and incubate at 37 °C with shaking at 180 rpm till OD_{600} reaches 0.3-0.4 (2-3 h).

3. Then, transfer the culture to a sterile 50 ml tube and centrifuge at 3500 rpm for 15 min at 4 °C in a refrigerated centrifuge (Eppendorf, Germany).

4. Discard the supernatant and resuspend the bacterial pellet in 1/5th volume of 100 mM $CaCl_2$ (i.e. 10 ml) and keep at ice for 1 h.

5. Centrifuge the resuspend culture at 3500 rpm for 10 min at 4 °C in a refrigerated centrifuge.

6. Discard the supernatant and resuspend the bacterial pellet in 1/5th volume of $CaCl_2$ and $MgCl_2$ mixture prepared with the ratio of 80 mM:20 mM respectively (i.e. 10 ml) and keep at ice for 1 h.

7. Centrifuge again at 3500 rpm for 10 min at 4 °C in a refrigerated centrifuge and discard the supernatant.

8. Finally, resuspend the pellet in 1/5th volume of 100 mM $CaCl_2$ with 20% glycerol (i.e. 5 ml), mix it homogenously and prepare the aliquots of 100 µl each.

9. Keep it overnight at -20°C and then store at -80 °C.

APPENDIX-III

A. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis of the expressed proteins was done to check the level of expression and apparent molecular weight of the expressed proteins.

Gel casting

1. The glass plates were firstly cleaned with extran followed by wiping with absolute ethanol.
2. Both the plates were fixed in casting assembly.
3. 12.5% resolving gel was poured to form 1mm thickness gel between the glass plates.
4. A layer of deionised water was loaded on to the top and the gel was allowed to polymerize at RT.
5. After polymerization of the gel, water overlay was discarded and the gel was washed with deionised water to remove unpolymerized gel.
6. The comb was secured in between the plates and the 4.5% stacking gel was poured over the resolving gel. Care was taken to avoid trapping of air bubbles between the comb and the gel.
7. The casting assembly containing polymerized gels was kept into a vertical electrophoresis apparatus.
8. Tank was filled with running buffer and the wells were flushed clean using a 22 gauge needle and syringe.

Preparation of the samples and loading

1. The bacterial pellet kept at -20ºC was thawed and treated with sample treatment buffer (STB).
2. The samples were boiled in a water bath for 10 mins followed by the centrifugation at 12000 rpm for 5 mins to pellet the cell debris.
3. The samples (20 µl) were loaded in wells alongside PageRuler^{TM} pre-stained protein ladder for reference. The un-induced and un-transformed cell lysates were also loaded to serve as the negative controls.

4. The electrodes were placed in a buffer tank and the electrophoresis was done at 90 V for 20 mins followed by 110 V till the running dye came out of gel.

5. After the run, gel was carefully removed and washed with deionised water several times. The stacking portion of the gel was torn off and the gel was kept in a tray for staining.

Staining and destaining of gel

1. The gel was immersed in a freshly prepared coomassie brilliant blue staining solution for overnight at RT.

2. After overnight staining, gel was submerged in the destaining solution.

3. The stained protein profile was visualized and compared with the reference ladder and negative controls.

B. Western Blot analysis

1. The SDS-PAGE gel was run in a similar manner as described above.

2. The gel was carefully removed, trimmed and placed in a tray containing transfer buffer.

3. The Western Blot apparatus was assembled.

4. A 0.45 μm nitrocellulose membrane (NCM) and absorbent pads were cut as per the dimensions of the gel.

5. Four pieces of absorbent pads soaked in transfer buffer were stacked one over the other on the lower electrode.

6. The NCM was wetted with transfer buffer and placed over the absorbent pads.

7. The gel was then carefully layered over the membrane avoiding trapping of air bubbles between the gel and the NCM.

8. Another set of four absorbent pads soaked in the transfer buffer were then piled one above another on the gel.

9. By rolling a glass pipette any trapped air bubbles were removed.

10. The upper electrode was placed on to the assembly and pressed tightly to provide complete contact between the electrodes, the gel and the membrane.

11. The electrodes were connected to a power supply and the blotting was performed at an applied current of 1.5mA/cm^2 for 1 h.

12. The apparatus was disassembled and both the gel and the membrane were carefully removed.

13. The NCM was placed in a tray and washed twice with TBS-Tween 20 (TBST).

14. The blotted NCM was then immersed in 3% BSA blocking buffer and incubated at 4ºC for overnight.

15. The membrane was washed twice with the TBST at an interval of 10 mins between each wash followed by a 10-min hold wash with TBS.

16. The blot was treated with primary antibody at a fixed dilution.

17. The membrane was again washed thrice in a similar manner as described in step 15.

18. The blot was treated with secondary antibody at a fixed dilution.

19. The membrane was again washed thrice in a similar manner as described in step 15.

20. The blot was developed by submerging the NCM in the substrate solution until the bands became visible.

21. Soon after the appearance of bands, substrate solution was drained and the reaction was stopped by adding distilled water.

Solutions used in SDS-PAGE and western blotting

A. SDS PAGE

1. Stock Solution A (30% acrylamide stock solution)

Acrylamide (SRL, India)	29.2 g
Bis acrylamide (N'N' methlenebisacrylamide) (GenDEPOT)	0.8 g
DW	up to 100 ml

Mixed and stored at cool and dark place

2. Stock solution B (1.5 M Tris HCl buffer, pH 8.8)

Tris (Tris hydroxymethyl aminomenthane) (Sigma- Aldrich, USA)	18.2 g
SDS (Sigma- Aldrich, USA)	0.4 g
HCl (Merck, Germany)	to adjust pH to 8.8
DW	up to 100 ml

Mixed and stored in a cool and dark place

3. Stock Solution C (0.5 M Tris HCl buffer, pH 6.8)

Tris (Sigma- Aldrich, USA)	6.1 g
SDS (Sigma- Aldrich, USA)	0.4 g
HCl (Merck, Germany)	to adjust pH to 6.8
DW	up to 100 ml

Mixed and stored in a cool and dark place

4. Solution D (10% Ammonium persulfate)

Ammonium persulfate (VWR, USA)	100 mg
DW	up to 1 ml

Solution was prepared just before use

5. 12.5% Resolving gel

Solution A	7.5 ml
Solution B	4.5 ml
Solution D	0.08 ml
TEMED (N,N,N′, N′-Tetramethylethylenediamine) (Sigma- Aldrich, USA)	0.01 ml
DW	6 ml

6. 4.5% Stacking gel

Solution A	0.9 ml
Solution C	1.5 ml
Solution D	0.02 ml
TEMED	0.01 ml
DW	3.6 ml

7. Electrophoresis buffer

Tris Base	3 g
Glycine	14.4 g
SDS	1 g
DW	up to 1000 ml

8. Sample treatment buffer

SDS	0.1 g
2-Mercaptoethanol (Merck, Germany)	0.1 ml
Solution C	1 ml
Glycerine	2 ml
Bromophenol Blue dye (Sigma- Aldrich, USA)	1 mg
DW	up to 10 ml

9. Coomassie brilliant blue solution

Coomassie Brilliant blue (Merck, Germany)	1.25 g
Glacial acetic acid (Merck, Germany)	50 ml
Methanol (Merck, Germany)	250 ml
DW	up to 500 ml

10. Distaining solution

Glacial acetic acid	7 ml
Methanol	25 ml
DW	up to 100 ml

B. Western Blotting reagents

1. Transfer buffer

Tris Base	6.36 g
Glycine	7.56 g
Methanol	100 ml
DW	up to 500 ml

2. Tris base sodium chloride solution (TBS)

Tris Base	6.06 g
Sodium chloride (Sigma-Aldrich, USA)	8.76 g
DW	up to 1000 ml

3. TBS-Tween solution (TBST)

TBS	100 ml
Tween-20 (0.05%) (Sigma- Aldrich, USA)	50 µl

4. Blocking buffer

3% Bovine serum albumin (Calbiochem, Germany)	0.3 g
TBST	10 ml

5. Substrate solution

Sodium acetate (50 mM) pH 5.0	15 ml
DAB (Sigma- Aldrich, USA)	3 mg
H_2O_2 (30%) (Merck, Germany)	4.5 µl

APPENDIX-IV

Buffers and reagents used for RNA-PAGE

1. Lysis buffer for dsRNA extraction

100 mM Tris EDTA buffer	200 µl
10% SDS	1ml
3 M Sodium acetate	1ml
Autoclaved DW	17.8 ml

2. Tris-EDTA buffer

Tris-HCl (100 mM)	1 ml (of 1M stock)
EDTA 10mM	200 µl (of 0.5 M stock)
Autoclaved DW	8.8 ml

3. Running buffer (10X)

Tris Base (Sigma- Aldrich, USA)	30 g
Glycine (Merck, Germany)	144 g
DW	up to 1000 ml

4. Fixing and Stopping solution for silver staining of ds RNA

Glacial acetic acid (Merck, Germany)	50 ml
DW	up to 500 ml

5. Staining solution for silver staining of ds RNA

Silver nitrate ($AgNO_3$)	100 mg
DW	up to 100 ml
Prepare fresh	
Formaldehyde (add just before use)	150 µl

6. Developing solution for silver staining of ds RNA

Sodium carbonate ($NaCO_3$)	3 gm
DW	up to 100 ml
Prepare fresh and chilled it on ice	
Formaldehyde (add just before use)	150 µl
Sodium thiosulfate (add just before use)	20 µl

7. Sodium thiosulfate solution (10 mg/ml) for silver staining of ds RNA

Sodium thiosulfate	10 mg
DW	Up to 10 ml

Store at 4°C

Gel casting

a. Resolving gel 10%

30% Acrylamide/Bis-acrylamide solution	3.3 ml
Resolving buffer (1.5 M, pH 8.8)	2.5 ml
10% APS	100 µl
TEMED	4 µl
DW	4 ml

b. Stacking gel 5%

30% Acrylamide/Bis-acrylamide solution	670 µl
Stacking buffer (0.5 M, pH 6.8)	500 µl
10% APS	40 µl
TEMED	4 µl
DW	2.7 ml

VITAE

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