Molecular characterization of Japanese encephalitis virus (JEV) strain SA14-14-2 and the development of recombinant Newcastle disease virus-based immunogen against JEV

A Thesis

submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

by

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Dedicated to my parents

For their unwavering support, encouragement and faith in me

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DECLARATION

I hereby declare that the research embodied in this thesis entitled **"Molecular characterization of Japanese encephalitis virus (JEV) strain SA14-14-2 and the development of recombinant Newcastle disease virus-based immunogen against JEV"** is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India under the supervision of Dr. Sachin Kumar.

In keeping with the general practice of reporting scientific observations, due acknowledgments have been made wherever the work of other investigators are referred to.

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CERTIFICATE

It is certified that the work described in this thesis entitled **"Molecular characterization of Japanese encephalitis virus (JEV) strain SA14-14-2 and the development of recombinant Newcastle disease virus-based immunogen against JEV"** by Barnali Nath (Roll No. 146106006), submitted to Indian Institute of Technology Guwahati, India for the award of the degree of Doctor of Philosophy, is an authentic record of results obtained from the research work carried out under my supervision at the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India. This thesis or any part thereof has not been submitted elsewhere for the award of any degree or diploma.

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Abbreviations

µm	Micrometer
µl	Microliter
3D	Three-dimensional
Å	Angstrom
AES	Acute encephalitis syndrome
ANOVA	Analysis of variance
APA	American Psychological Association
APMV-1	Avian paramyxovirus-1
ASA	Average solvent accessibility
BHK-21	Baby hamster kidney cells
bp	Base pairs
cDNA	Complementary DNA
CDS	Coding sequences
CEF	Chicken embryo fibroblasts
CSF	Cerebrospinal fluid
DI	Domain I of the Japanese encephalitis virus envelop protein
DII	Domain II of the Japanese encephalitis virus envelop protein
DIII	Domain III of the Japanese encephalitis virus envelop protein
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dpi	Days post-immunization
E	Envelope protein of Japanese encephalitis virus
ELISA	Enzyme-linked immunosorbent assay
F	Fusion protein of Newcastle disease virus
FBS	Fetal bovine serum
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HA	Hemagglutination assay
HDV	Hepatitis delta virus
HEp-2	Human epithelial type 2 cells
HI	Hemagglutination inhibition
HLA	Human leukocyte antigen
HN	Hemagglutinin-neuraminidase protein of Newcastle disease virus
ICPI	Intracerebral pathogenicity index
IEDB	Immune Epitope Database and Analysis Resource
IFN	Interferon
IL	Interleukin
IP	Immunoperoxidase
JE	Japanese encephalitis
JEV	Japanese encephalitis virus

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kb	Kilobases
L	RNA-dependent RNA polymerase of Newcastle disease virus
M	Matrix protein of Newcastle disease virus
MDT	Mean death time
MOI	Multiplicity of infection
MVEV	Murray Valley encephalitis virus
N	Nucleoprotein of Newcastle disease virus
NCBI	National Center for Biotechnology Information
ND	Newcastle disease
NDV	Newcastle disease virus
nm	Nanometre
NS1	Non-structural protein 1 of Japanese encephalitis virus
OIE	World Organization for Animal Health
ORF	Open reading frame
P	Phosphoprotein of Newcastle disease virus
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
pg	Picogram
PHK	Primary hamster kidney cells
pNDV	rNDV full-length expression plasmid
pNDV-Ejev	Plasmid bearing the E gene of JEV
pNDV-NS1jev	Plasmid bearing the NS1 genes of JEV
PVS	Protein Variability Server
RNA	Ribonucleic acid
rNDV	Recombinant NDV
rNDV-Ejev	rNDV expressing the E protein of JEV
rNDV-NS1jev	rNDV expressing the NS1 protein of JEV
RNP	Ribonucleoprotein complex
RT	Reverse transcription
RT-PCR	Reverse transcription PCR
SD	Standard deviation
SLEV	Saint Louis encephalitis virus
sNS1	Secreted hexameric form of NS1
SPF	Specific pathogen-free
$TCID_{50}$	50% endpoint tissue culture infectious dose
Th	T helper cells
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
WHO	World Health Organization
WNV	West Nile virus

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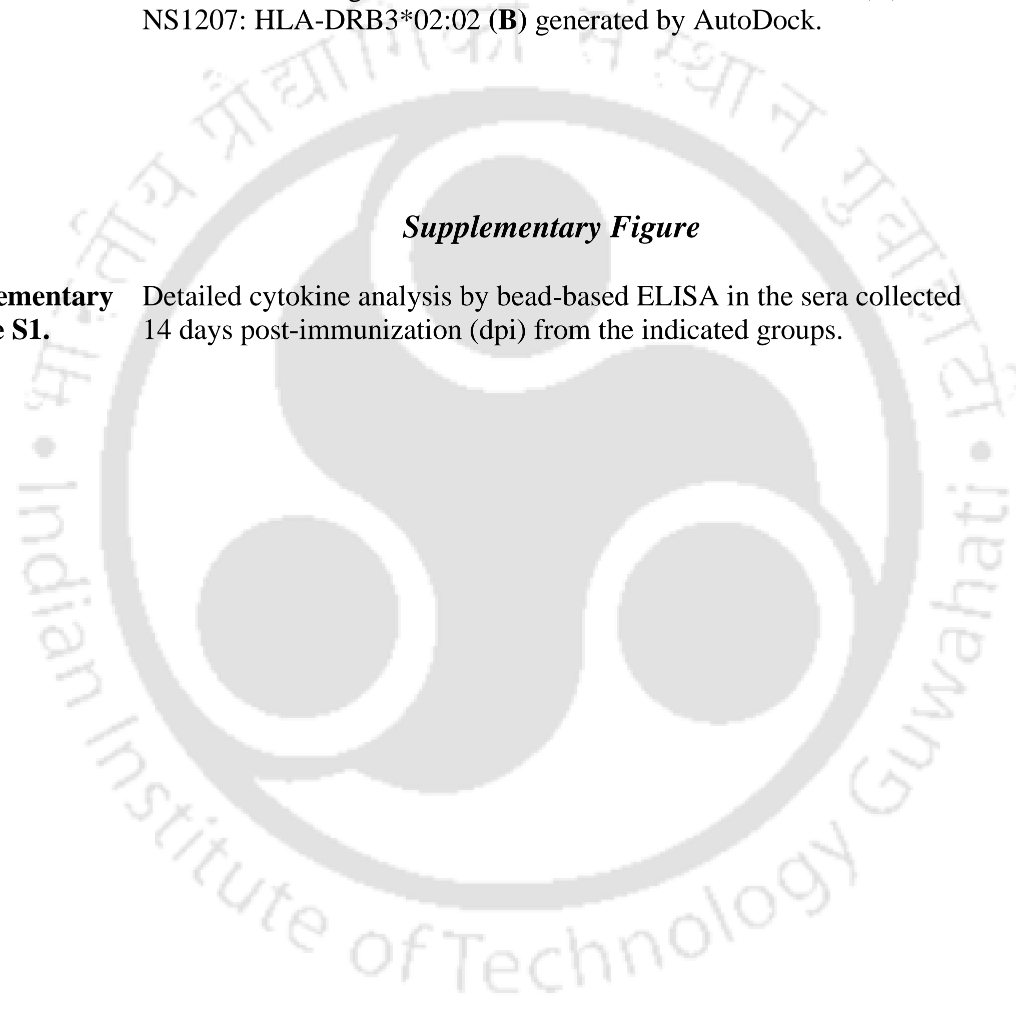

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Abstract

Japanese encephalitis (JE) – the mosquito-borne zoonosis, is a global public health concern. It is the predominant cause of acute encephalitis syndrome (AES) in Asia, the Western Pacific, and Australia. It is caused by the Japanese encephalitis virus (JEV), which belongs to the genus *Flavivirus* and is the prototype member of the JE serocomplex. The envelope protein (E) of JEV is a major antigenic determinant and responsible for immunogenic responses as well as receptor binding, membrane fusion and virion assembly. The non-structural protein 1 (NS1) of JEV helps in viral replication, generates an immune response and serves as a diagnostic marker for JEV infections.

Recently, JE/AES cases are reported to occur in areas with an established JE vaccination program that uses the JEV live attenuated vaccine strain SA14-14-2. We characterized the SA14-14-2 strain in baby hamster kidney (BHK-21) cells and observed an enhanced replication following its passage in BHK-21 cells. On sequence analysis and docking studies, the cell-culture adapted vaccine strain showed a relevant point mutation identical to its wild-type parent strain SA14, which suggests the possibility of reversion of SA14-14-2 following its adaptation in permissive host cells.

Newcastle disease virus (NDV) is a notable virus of the poultry industry, but it does not affect humans due to its natural host-range restriction. NDV is used as a broad-spectrum vaccine vector to express several human and animal immunogenic proteins. We generated recombinant NDVs (rNDVs) individually expressing the E and NS1 proteins of JEV (rNDV-Ejev and rNDV-NS1jev). The rNDVs induced immunity against JEV upon intranasal immunization in BALB/c mice. They produced sufficient neutralization antibody titers against both NDV and JEV and also T helper cells (Th1 and Th2) mediated immune responses. On

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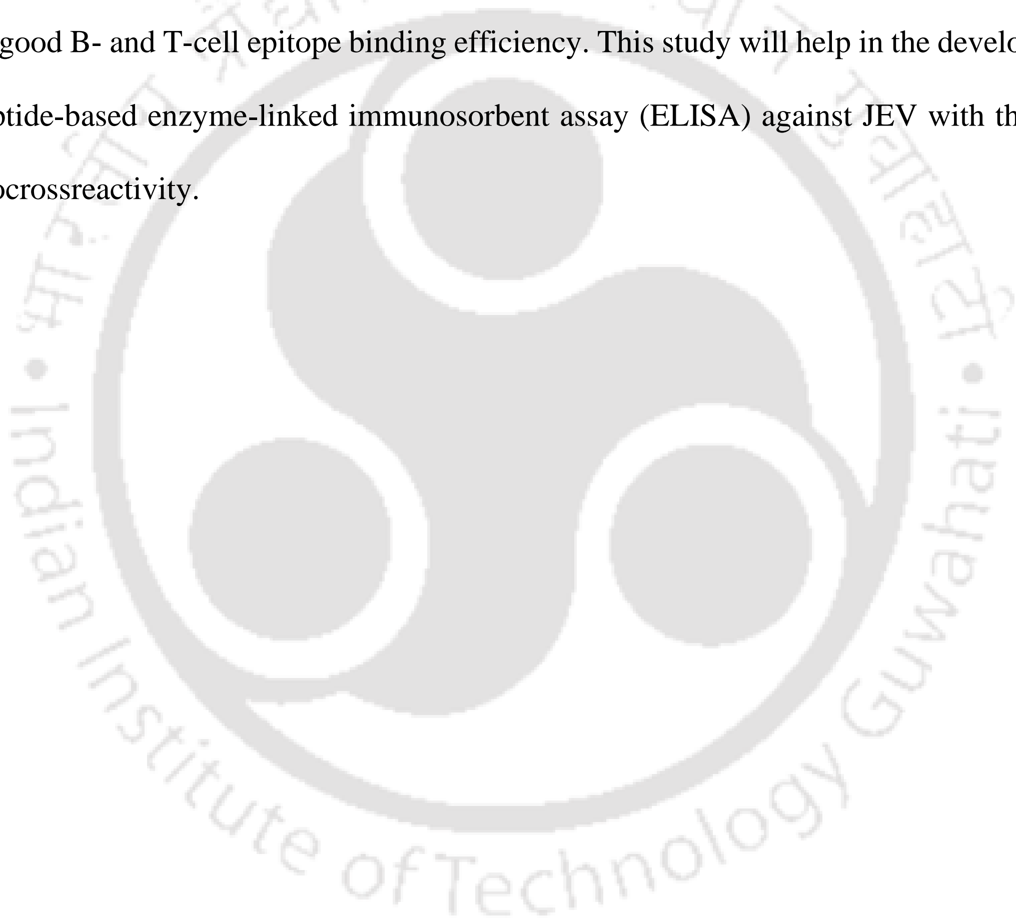
comparison with rNDVs expressing NS1, and a combination of both E and NS1, the results suggested rNDV-Ejev can be a promising live viral-vectored vaccine against JEV.

The differential diagnosis of JEV with the existing diagnostic kits is inefficient because of the immunocrossreactivity between the envelope proteins of JEV and other antigenically similar and co-circulating flaviviruses such as West Nile virus (WNV) and Dengue virus. Based on *in-silico* analysis, we identified four epitope-based peptides on the JEV E and NS1 proteins, which are conserved, surface-exposed, solvent-accessible, and predicted to have good B- and T-cell epitope binding efficiency. This study will help in the development of a peptide-based enzyme-linked immunosorbent assay (ELISA) against JEV with the least immunocrossreactivity.



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Chapter 1
Introduction and Review of Literature

1.1. Introduction

Flaviviruses are arthropod-borne, primarily ticks and mosquitoes, and can infect humans. The mosquito-transmitted *flaviviruses* include Yellow fever, Dengue, Japanese encephalitis, West Nile and Zika viruses that cause widespread morbidity and mortality globally. Nine genetically and antigenically related *flaviviruses* constitute the JE serocomplex (Cook and Holmes 2006). Out of these, four *flaviviruses* have led to epidemic outbreaks of encephalitis in humans: JEV, WNV, Murray Valley encephalitis virus (MVEV) and Saint Louis encephalitis virus (SLEV) (Solomon 2004).

1.1.1. Current Distribution of JE

JEV is the most important member of the JE serocomplex. Out of the members of JE serocomplex, JEV has the highest incidence globally, with an estimated 68,000 clinical cases per year, of which 75% cases occur in children aged 0–14 years (Khan, Borah et al. 2015). Although JE is mostly asymptomatic, the case-fatality rate among symptomatic cases can be as high as 30%. Around 30%–50% of patients with encephalitis suffer from permanent neurologic or psychiatric sequelae (World Health Organization 2019). JE cases are presently seen in Asian countries like India, Bangladesh, Burma, China, Indonesia, Japan, Malaysia, Nepal, North and South Korea, Pakistan, Philippines, Russia, Singapore, Sri Lanka, Taiwan, Thailand (Hills, Griggs et al. 2010). Recently, JE has spread to newer territories, including Russia, Papua New Guinea and Northern Australia (Halstead 1992, Mackenzie, Gubler et al. 2004, Campbell, Hills et al. 2011) (Figure 1.1).

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In India, on average, 5,97,542,000 people live in JE-endemic regions. Around 1,500 to 4,000 cases are reported every year from the 104 JE endemic districts spread across 11 states of India (Kabilan 2004, Rustagi, Basu et al. 2019). Of all JE endemic states of India, Assam is the worst affected state for the last five years (Staff 2019). Recently, adults have been seen to exceed the pediatric age group in the number of JE cases in Assam, India (Borah, Dutta et al. 2011).

The strains of JEV are divided into five genotypes: G1-G5 (Solomon, Ni et al. 2003). Mostly, G3 is found to be circulating in Southeast Asian countries (Mackenzie, Gubler et al. 2004). However, in recent years, G1 has replaced G3 as the significant circulating genotype in Asia and G3 has spread to Europe and Africa. The current JE vaccine is derived from G3, and it can protect against G1-G4 JEV genotypes. However, the current vaccines have low protective efficacy against the emerging G5 genotype (Cao, Fu et al. 2016).

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Figure 1.1. Recent global distribution of areas at risk of Japanese Encephalitis (JE) by the Centers for Disease Control and Prevention (CDC). <https://wwwnc.cdc.gov/travel/yellowbook/2020/travel-related-infectious-diseases/japanese-encephalitis>

Note: The boundaries and names of countries used in this map do not imply the expression of any opinion on the part of the CDC/IITG concerning the legal status of any country or territory.

1.1.2. Virus

JEV is an icosahedral, enveloped and small *Flavivirus* of approximately 50 nanometers (nm) diameter. It has a positive-sense, single-stranded RNA genome of approximately 11 kilobases (kb) in length and the genome is capped at the 5′ end and un-polyadenylated at the 3′ end. The genome has a single, long, open reading frame (ORF) that encodes for a polyprotein flanked by 5′ and 3′ non-translated regions (NTRs). The JEV polyprotein is cleaved by various host and viral proteases into three structural (capsid [C], membrane [M], and envelope [E]), and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (Sumiyoshi, Mori et al. 1987, Chambers, Hahn et al. 1990, Unni, Ruzek et al. 2011) (Figure 1.2). E and NS1

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are the immunogenic proteins capable of inducing protective immunity against JEV infection (Chen, Pan et al. 1999, Luca, AbiMansour et al. 2012, Rastogi, Sharma et al. 2016).

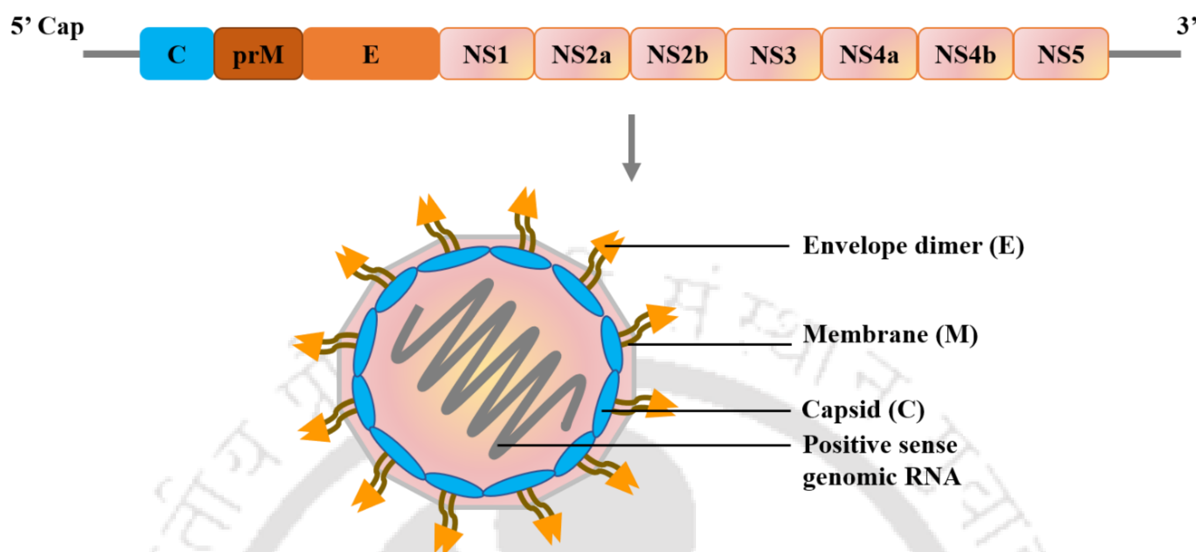

Figure 1.2. The schematic representation of the Japanese encephalitis virus. JEV has a positive-sense, single-stranded RNA genome of approximately 11 kilobases, which is capped at the 5′ end and un-polyadenylated at the 3′ end. The JEV polyprotein (encoded by a single, long, open reading frame) is cleaved by various host and viral proteases into three structural (capsid [C], membrane [M], and envelope [E]), and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. These proteins assemble to form the icosahedral, enveloped structure of JEV.

1.1.3. Transmission

The complex ecosystem of JEV transmission includes migrating ardeid birds, *Culex* spp. mosquitoes, local pig populations, and accidental dead-end hosts such as humans and horses. The migrating ardeid birds and the domestic and feral pig populations are the reservoir and amplification hosts for JEV, respectively (Monath and Heinz 1996, Pearce, Learoyd et al. 2018) (Figure 1.3). Humans are accidental dead-end hosts, because they usually do not develop high enough concentrations of JE virus in their bloodstreams to infect feeding mosquitoes. Apart from humans, JEV cases have been reported from cattle, sheep, goats, dogs, cats, poultry, wild mammals, reptiles and amphibians as all of them are susceptible hosts for mosquito vector (Oliveira, Cohnstaedt et al. 2017). JEV transmission occurs mainly in rural agricultural areas

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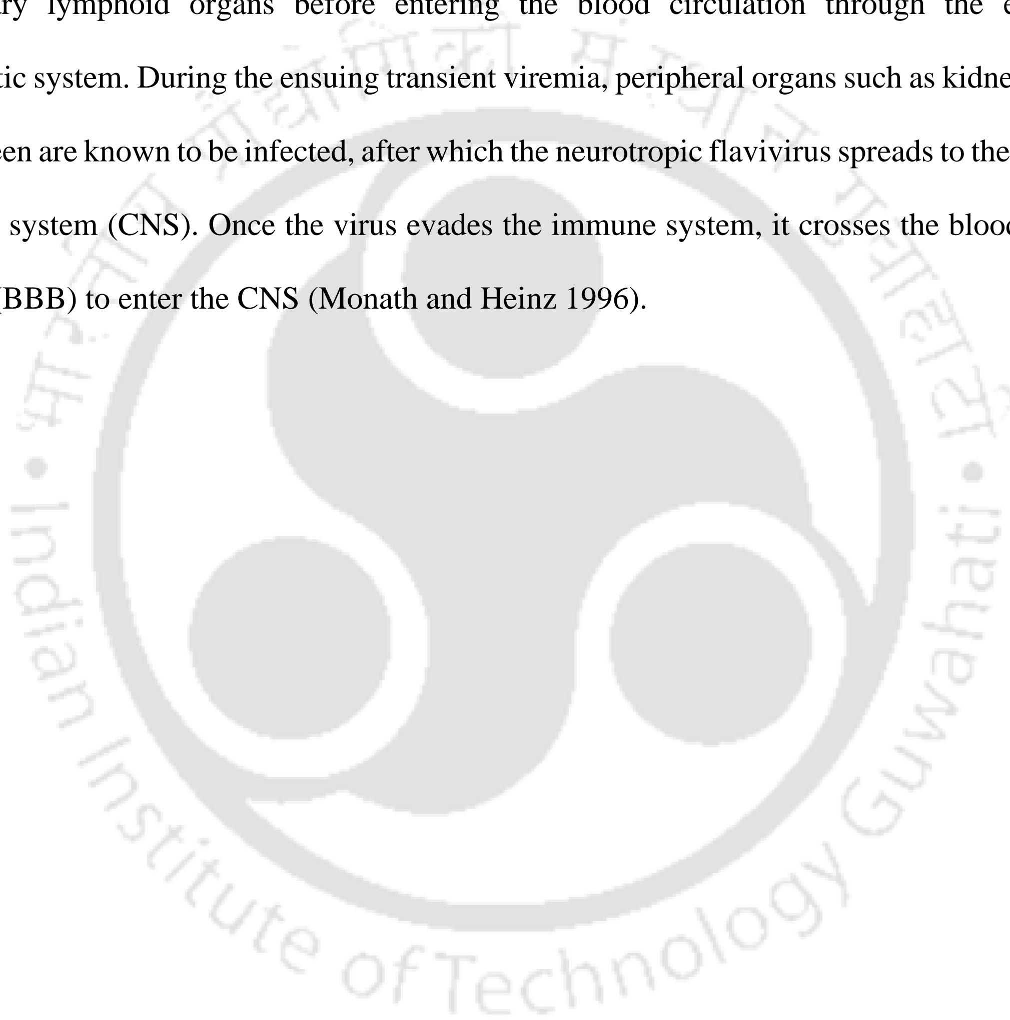
associated with rice paddy fields and flooding irrigation practices during the rainy season (Centers for Disease Control and Prevention 2019).

Upon entry through mosquito bite, the virus infects Langerhan's dendritic cells in the skin and is carried to nearest draining lymph nodes, thereby initiating a round of early immune response. Unfortunately for the host, this is not sufficient to counter the virus and it spreads to secondary lymphoid organs before entering the blood circulation through the efferent lymphatic system. During the ensuing transient viremia, peripheral organs such as kidney, liver and spleen are known to be infected, after which the neurotropic flavivirus spreads to the central nervous system (CNS). Once the virus evades the immune system, it crosses the blood–brain barrier (BBB) to enter the CNS (Monath and Heinz 1996).



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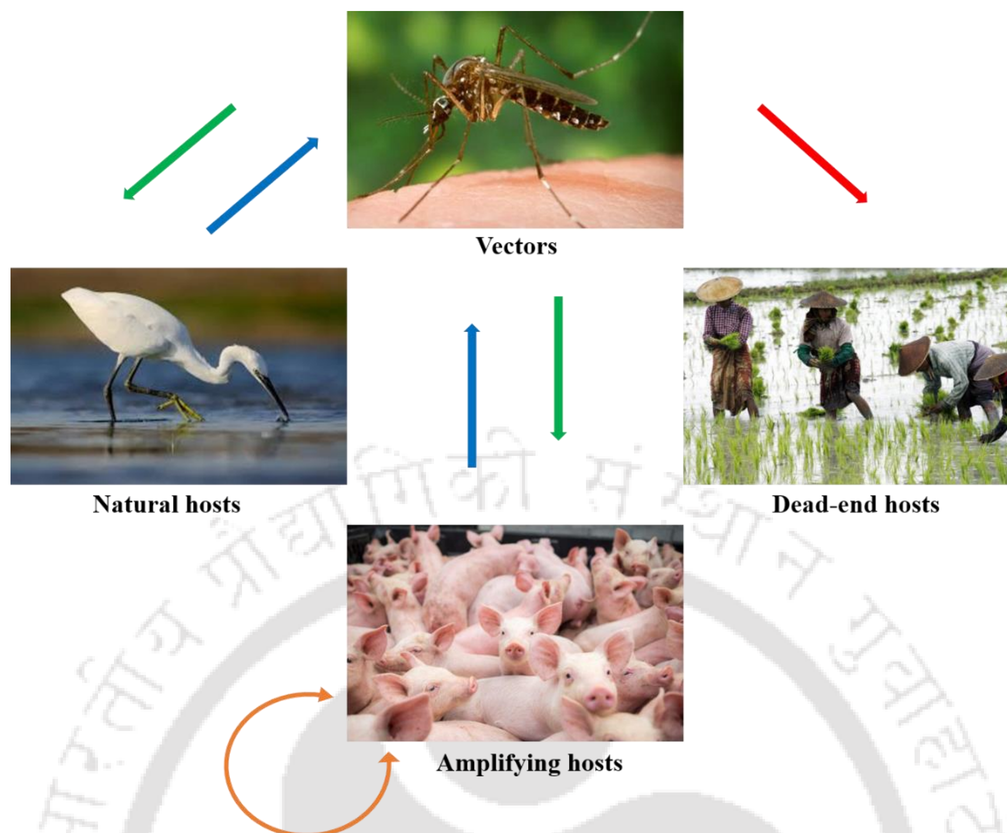

Figure 1.3. The transmission cycle of the Japanese encephalitis virus (JEV) (Filgueira and Lannes 2019). Wading and water birds are the natural hosts and carriers of JEV. *Culex* spp. mosquitoes transmit JEV between birds and to other species; domestic and feral pigs are the carriers and amplifiers of JEV. Vector-free transmission of JEV occurs in the pig population. Humans and horses are generally dead-end hosts due to low viremia.

1.1.4. Symptoms and Treatment

The incubation period (time from infection to developing symptoms) of JE is between 5-15 days. Clinical consequences of JEV infection ranges from a non-specific febrile illness to severe meningoencephalitis. Initial symptoms include fever with headache and vomiting. Over the next few days, patients suffer from a reduced consciousness level, seizures (particularly in children), parkinsonian movement disorders (like tremors, slowing and stiffening movements), and acute flaccid paralysis (involving weakness of the muscles of respiration and swallowing). Pathological and imaging studies have shown that the anatomical sites of damage include the basal ganglia and the anterior horns of the spinal cord.

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Specific antiviral treatment for JE is not available. The treatment is mostly symptomatic and supportive (Centers for Disease Control and Prevention 2019).

1.1.5. Diagnosis

For the confirmation of *Flavivirus* infection, the WHO prescribes the following diagnostic tests:

i. Serological detection through IgM antibody capture ELISA (MAC-ELISA)
ii. Plaque reduction neutralization test (PRNT)
iii. Virus isolation and viral genomic detection by reverse transcription PCR (RT-PCR)

1.1.6. Vaccines

Antiviral therapy against JEV is still not well established, and human immunization is the current method of choice to attain protection against JEV (Yun and Lee 2014). Currently, four different types of JE vaccine for humans are licensed in various areas of the world:

i. Mouse brain-derived killed-inactivated vaccine JE-VAX (BIKEN). However, due to severe drawbacks and limitations, the production of JE-VAX was halted in 2006, and all the remaining stock expired in 2011 (Fischer, Lindsey et al. 2010, Halstead and Thomas 2011)
ii. Cell culture-derived live attenuated vaccine SA14-14-2 (Chengdu Institute of Biological Products)
iii. Cell culture-derived killed-inactivated vaccines viz. JEBIK V (BIKEN); ENCEVAC, KD-287, or JEIMMUGEN INJ (Kaketsuken), IC51, IXIARO, JESPECT, or JEEV (Intercell AG)

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iv. Cell culture-derived live attenuated chimeric vaccines viz. ChimeriVax-JE, IMOJEV, JE-CV, or THAIJEV (Sanofi-Aventis) (Beasley, Lewthwaite et al. 2008, Wilder-Smith and Halstead 2010, Halstead and Thomas 2011)

Also, the new experimental JE vaccines can be categorized into:

i. recombinant protein-based vaccines (Mason, Dalrymple et al. 1989, Seif, Morita et al. 1995, Seif, Morita et al. 1996, Verma, Kumar et al. 2009)
ii. plasmid DNA-based vaccines (Kaur and Vrati 2003)
iii. poxvirus-based vaccines (Konishi, Pincus et al. 1991, Mason, Pincus et al. 1991, Jan, Yang et al. 1993)

1.1.7. Pig vaccination

A potential strategy to control JE is the vaccination of pigs, the amplifying hosts. Four attenuated strains, 's-,' 'm,' 'at,' and ML-17, have been developed and used as live virus vaccines for the prevention of swine stillbirth in the field due to JE (Fujisaki 1975). According to a study conducted in JE endemic areas, the vaccination of 50% of the total pig population each year resulted in an 82% reduction in the annual incidence of JE in pigs (without taking into consideration pig-to-pig direct transmission of JE) (Diallo, Chevalier et al. 2018). However, it is not widely implemented due to the following reasons: 1) pig populations have high turnover rates due to short gestation period (114 days) and large litter size. So, new-born pigs have to be vaccinated annually, which is very costly. 2) Young pigs have maternal antibodies, and this reduces the effectiveness of live attenuated vaccines (Tobias, Svenja et al. 2009).

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1.2. Review of Literature

1.2.1. History

The clinical recognition of JE dates back to the 19th century when it appeared as recurring 'summer encephalitis' (Mansfield, Hernández-Triana et al. 2017). It is hypothesized that JEV originated in the Indonesia-Malaysia region of Southeast Asia and evolved after that into four genotypes (G1-G4). These four genotypes then spread across entire Asia (Solomon, Ni et al. 2003). The first clinical case of JE was recorded in Japan in the year 1871. After a span of half a century, again, a massive JE outbreak occurred in Japan, where more than 6000 cases were documented. A series of outbreaks occurred in 1927, 1934 and 1935 in Japan. In 1924, a viral agent was isolated from the brain tissue of an infected patient in Japan. After about 10 years, this viral agent was recognized to be JEV by transfection into monkey brains (Tobias, Svenja et al. 2009). Since the 1870s, it has become the preeminent cause of epidemic encephalitis in Asia and the Western Pacific (Hills, Griggs et al. 2010).

In India, the first clinical case of JE was recorded at Vellore, Tamil Nadu, in 1955 (Reuben and Gajanana 1997), and within a decade, a total of 65 confirmed cases were reported from South India. In 1973, the first significant outbreak of JE was reported from the districts of Bankura and Burdwan in West Bengal (Banerjee, Sengupta et al. 1976). A total of 700 reported cases and 300 deaths occurred in that outbreak. Again in 1976, another outbreak occurred in West Bengal, with 307 reported cases and 126 deaths. In 2005, the most prolonged epidemic of JE was reported from Gorakhpur district, Uttar Pradesh, India (Kulkarni, Sapkal et al. 2018). Recently, in 2012 and 2016, two large JE outbreaks were reported from Malkangiri and Manipur, respectively (Dwibedi, Mohapatra et al. 2015, Kulkarni, Sapkal et al. 2018). In 35 years, 107,552 reported cases and 34,461 deaths were recorded due to JE from various parts of India (Dutta, Rangarajan et al. 2010, Taraphdar and Chatterjee 2015). Now JE is declared as

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a notifiable disease in India due to its expanding geographical distribution (Solomon, Ni et al. 2003).

1.2.2. JE Emergence and control strategies

The boundless emergence of JE in Southeast Asian countries is due to unprecedented growth in population, increase in rice farming (both in cropping area and density), pig rearing, and a lack of proper surveillance systems and vaccination programs in underdeveloped/developing areas. However, countries like Japan and South Korea have developed successful JE control programs. The JE incidence rates in those countries have remained stable for more than two decades. Their key control strategies involve: 1) large-scale immunization and surveillance programs for humans, 2) urbanization and hygienic housing facilities 3) better agricultural practices with a decrease of irrigated land 4) separation of pig rearing from human settlements and pig immunization (Tobias, Svenja et al. 2009).

1.2.3. Life-cycle of JE

JEV infects multiple cell types with the help of several key receptor molecules (Table 1.1). It has the unique capacity to use multiple endocytic pathways interchangeably for entry into different cell lines and establish an infection (Nain, Abdin et al. 2016). JEV binds to the host cell surface receptors/attachment factors with the help of its E protein. It is followed by receptor-mediated endocytosis. Based on cell-type, JEV can enter the cell through three different endocytic pathways: 1) clathrin-mediated endocytosis, 2) caveolae-mediated endocytosis, or 3) clathrin-independent route (in case of neuronal cells) (Nain, Abdin et al. 2016). The viral RNA is then released in the cytoplasm by a low pH-dependent uncoating of the virus envelope in the acidic endosome (Sanchez-San Martin, Liu et al. 2009). The virus then hijacks the host machinery and membranes for its genome replication (Westaway, Mackenzie et al. 1997, Uchil and Satchidanandam 2003).

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Cell line	Receptors/Attachment factors	References
BHK-21 (Baby hamster kidney)	Heparin sulfate, Vimentin (Type III intermediate filament protein), Heat-shock protein HSP90β	(Chen, Lin et al. 1996, Su, Liao et al. 2001, Hung, Tsai et al. 2011, Liang, Yu et al. 2011)
Vero (African green monkey kidney)	Mr 74000 molecule, α5β3 integrin (vitronectin receptor)	(Kimura 1994, Chu and Ng 2004)
Neuro2a (Mouse neuroblastoma)	Heat-shock protein HSP70	(Ren, Ding et al. 2007, Das, Laxminarayana et al. 2009)
Microglial (Mouse glial)	Laminin receptor, CD4	(Thongtan, Wikan et al. 2012)
C6/36 (Mosquito larvae)	Mr 53000 molecule	(Boonsanay and Smith 2007)
CHO (hamster ovary)	Heparin sulfate	(Su, Liao et al. 2001)

Table 1.1. Japanese encephalitis virus receptors/attachment factors in different cell lines.

1.2.4. Immunogenic proteins of JEV

It has been demonstrated that the passive administration of monoclonal antibodies against the E protein protects mice against JEV infection (Kimura-Kiroda and Yasui 1988). Furthermore, sub-viral particles consisting of only the E protein and plasmid vectors expressing different forms of E protein were effective in generating a protective immune response in mice against JEV infection (Konishi, Pincus et al. 1992, Ashok and Rangarajan 1999, Chang, Hunt et al. 2000). However, the NS1 based subunit and DNA vaccines were reported to be partially protective against JEV infections (Srivastava, Putnak et al. 1995, Lin, Chen et al. 1998,

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Ishikawa, Wang et al. 2011). Hence, these two immunogenic proteins can be used to develop rNDV-based vaccine candidates against JEV.

1.2.4.1. Envelope protein

It plays a pivotal role in virus infection by helping in receptor binding, membrane fusion and virion assembly (Monath 1990, McMinn 1997). Ninety homodimers of E protein form the major component of the mature JEV virion (Monath 1990, McMinn 1997, Chen, Pan et al. 1999). The E protein of JEV exists as a monomer in solution and assembles as an anti-parallel dimer in its crystal lattice. JEV E protein has a three-domain organization: an ectodomain, a stem region, and a transmembrane domain, and it exhibits disulfide connectivity like the E proteins of other flaviviruses (Luca, AbiMansour et al. 2012, Liu, Zhao et al. 2019). The ectodomain has domain I (DI), which is the central domain located between the extended domain II (DII) and the globular domain III (DIII). DI is composed of a nine-stranded β-barrel. DI interacts with DII and forms the DI-II hinge region (Luca, AbiMansour et al. 2012). DII is formed out of two extended loops that protrude from DI. The larger loop of DII contains the conserved fusion peptide at its tip. DIII contains an immunoglobulin-like fold and is connected to DI by a short peptide linker. It has been reported that many of the effective neutralizing monoclonal antibodies recognize the lateral ridge of DIII of the *Flavivirus* E protein (Nybakken, Oliphant et al. 2005, Sánchez, Pierson et al. 2005, Oliphant, Nybakken et al. 2006, Sultana, Foellmer et al. 2009, Brien, Austin et al. 2010). Also, epitope mapping of neutralizing antibodies on the JEV E protein shows that the critical residues for neutralization also fall into the DI–DII hinge, DI lateral ridge, and the buried fusion loop (Luca, AbiMansour et al. 2012).

1.2.4.2. NS1

The NS1 is a highly conserved protein amongst flaviviruses and is present in diverse forms. The intracellular monomeric form plays a primary role in viral replication, whereas, the

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membrane-bound dimeric form (mNS1) and the secreted hexameric form (sNS1) play essential roles in generating an immune response (Rastogi, Sharma et al. 2016). sNS1 is released transiently during JEV replication and serves as a diagnostic marker for JEV infections. (Li, Counor et al. 2012, Rastogi, Sharma et al. 2016). NS1 can activate the Toll-like receptors (TLRs) and inhibit the complement system (Young, Hilditch et al. 2000, Macdonald, Tonry et al. 2005). NS1 protein has three domains: the β-roll (amino acid residues 1 to 29), wing (38 to 151), and β-ladder (181 to 352) domains (Akey, Brown et al. 2014, Brown, Akey et al. 2016, Xu, Song et al. 2016).

1.2.5. Reverse Genetics

Reverse genetics is a method to study the *unknown function* of a known gene. This approach is opposite to the traditional forward genetics, where an *unknown gene* is studied for a known function. Reverse genetics became feasible after the introduction of recombinant DNA technology.

Reverse genetics: Known gene/protein--mutate the gene--explore resulting mutant phenotype

Forward genetics: Known mutant phenotype--screen mutants--identify the gene(s) causing the phenotype

1.2.5.1 Reverse genetics in the context of virology

It starts with the engineering of mutations in known viral genes (DNA or cDNA) and the subsequent recovery of infectious viral particles to explore the unknown function of the viral genes or the resultant phenotype.

The first reverse genetics system for an RNA virus was established for the Poliovirus, a positive-sense RNA virus (Racaniello and Baltimore 1981). For a negative-sense RNA virus, the minimum infectious unit is not an RNA molecule, but a core structure called

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ribonucleoprotein complex (RNP). In functional RNPs, the genomic RNAs have to be encapsulated with the nucleoprotein (N) and form a complex with the polymerase (L) and phosphoprotein (P). Due to technical difficulties in reconstituting biologically active RNPs, genetic manipulation of negative-sense RNA viruses has lagged than that of the positive-sense RNA viruses. In 1994, Schell, Mebatsion and Conzelmann first reported the recovery of Rabies virus, a non-segmented, negative-sense, RNA virus belonging to the family of *Rhabdoviridae*, entirely from cDNA. Reverse genetics of negative-sense RNA viruses progressed rapidly in the next years, as documented by the generation not only of non-segmented negative-sense RNA viruses (Conzelmann 1998, Roberts and Rose 1999) but also of segmented negative-sense RNA viruses, including Bunyamwera virus (Bridgen and Elliott 1996) and Influenza viruses (Fodor, Devenish et al. 1999, Hoffmann, Neumann et al. 2000, Neumann, Whitt et al. 2002).

The use of vaccines is one of the most effective and inexpensive ways of combating diseases. Reverse genetics is widely used to develop safe and effective live vaccines, broad-spectrum vaccine vectors, genetically tagged recombinant viruses--they facilitate the serological differentiation of vaccinated animals from infected animals (DIVA approach). Reverse genetics is also used to investigate the structure and function of viral genes and their proteins, study the interaction of viral proteins with host receptors and develop gene therapy tools (Mebatsion 2005).

1.2.5.2. NDV as a vaccine vector

NDV is an attractive vaccine vector candidate for both human and animal uses (Samal 2011). It presents a promising candidate for the rational design of live attenuated vaccine and vaccine vectors because of its modular nature of transcription, minimum recombination frequency and an absence of DNA phase during replication. The genome of NDV is quite easy to manipulate

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using a reverse genetics system (Huang, Elankumaran et al. 2003, Bukreyev, Huang et al. 2005). NDV-based live attenuated vaccines and bivalent (dual) vaccines are economically very popular for the poultry industry. NDV acts as a potential vaccine vector for humans because of its attenuation due to natural host range restriction (Samal 2011). In terms of safety, efficacy and cost-effectiveness, it presents an outstanding vaccine candidate. The rNDV expressing foreign protein is explored as a viral vector by many scientists around the globe. The following properties of NDV can be attributed for its credibility as a viral vector:

i. It grows with high titers in embryonated chicken eggs, cell culture and respiratory tract of avian and non-avian species.

ii. It infects naturally via the respiratory tract and is thus useful to deliver protective antigens derived from respiratory pathogens. Besides, it induces both local and systemic immune responses.

iii. It elicits both humoral and cellular immune responses.

iv. It has a modular genome with only six essential and two accessory genes that are easy to manipulate.

v. It does not integrate with the host genome as it replicates in the host cytoplasm and shows the least genetic recombination.

vi. rNDV expressing the foreign antigen shows quite a high and stable expression of foreign protein after many passages both *in vitro* and *in vivo* (Huang, Elankumaran et al. 2003)

vii. It can be attenuated using reverse genetics for the development of stable vaccine and vaccine vector.

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1.2.5.3. rNDV-based vaccines for humans

NDV offers a vital vaccine candidate for humans in terms of safety, efficacy and cost-effectiveness. rNDV confers immunogenic response against different antigenic challenges: human influenza A virus (IAV) HA protein (Ge, Deng et al. 2007, DiNapoli, Nayak et al. 2009), human and simian immunodeficiency viruses (HIV and SIV) Gag protein (Nakaya, Cros et al. 2001, Nakaya, Nakaya et al. 2004, Lawrence, Wanjalla et al. 2013), HIV glycoproteins (Khattar, Samal et al. 2011, Khattar, Samal et al. 2013), human respiratory syncytial virus (RSV) F glycoprotein (Martinez-Sobrido, Gitiban et al. 2006), human parainfluenza virus 3 HN protein (Bukreyev, Huang et al. 2005), severe acute respiratory syndrome-related coronavirus (SARS-CoV) spike glycoprotein (DiNapoli, Kotelkin et al. 2007), ebola virus GP envelope protein (DiNapoli, Yang et al. 2010), rift valley fever virus (RVFV) structural glycoproteins Gn and Gc (Kortekaas, de Boer et al. 2010). In 2016, Wang et al. developed an rNDV-based multivalent vaccine candidate against the *Flavivirus* WNV by expressing the pre-membrane/envelope (PrM/E) proteins. It showed potential immunogenicity in mice, horses, chickens, ducks and geese (Wang, Yang et al. 2016).

1.2.5.4. Recovery of rNDV

To initiate an infectious cycle of negative-strand RNA viruses (i.e., recovery of the virus), the intracellular reconstitution of the RNP entirely from cloned cDNA is required. Most of the systems for recovery of rNDV are based on co-transfection of the cell (Hep-2 or BSRT7/5) with a plasmid expressing the full-length antigenomic RNA with three other support plasmids encoding viral N, P and L proteins all under the control of bacteriophage T7 RNA polymerase promoter. The T7 RNA polymerase was provided by either a recombinant vaccinia virus expressing the T7 gene or a cell line constitutively expressing the T7 polymerase, e.g., BSRT7/5 (Samal 2011) (Figure 1.4). For the construction of the vaccine vector, the foreign

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gene (not more than 3.8 kb) must be flanked by NDV specific gene-start and gene-end sequences at the intergenic sequence without perturbing the rule of six (Bukreyev, Skiadopoulos et al. 2006). The level of foreign gene expression is found to be more in the case of its insertion at the 3′ end of the genome (Huang, Krishnamurthy et al. 2001, Carnero, Li et al. 2009).

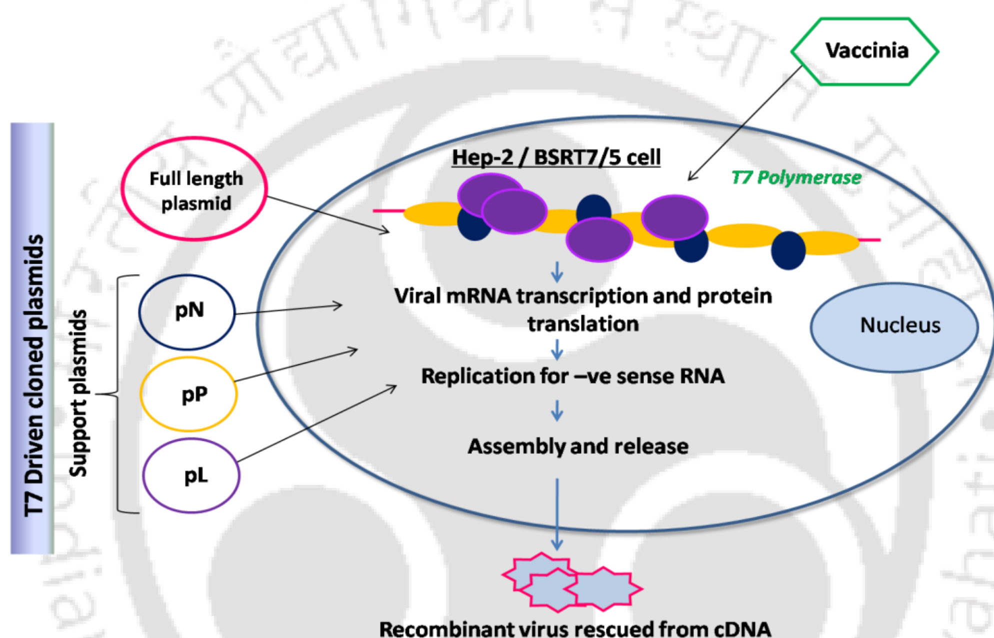

Figure 1.4. The schematic representation of the recovery of recombinant Newcastle disease virus developed using reverse genetics.

1.2.6. Overview of the viral vaccine development process

This process has various phases, from exploration to clinical development. Candidate vaccines first undergo a thorough investigation of host-virus interactions, including viral replication and anti-viral infectivity assay, cell-viability assay etc. It is followed by the identification of potential viral antigens by library screening and epitope mapping. The comprehensive screening and profiling of the immune response (by neutralization and T-cell monitoring) is one of the most critical steps in viral vaccine development. After thorough characterization, the

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safest and most effective lead candidates are chosen for further downstream processing (harvesting, filtration, concentration and purification), clinical development and final production.

1.3. Rationale for the study

JEV is a global public health issue and remains a challenge for clinicians (Calvert, Dixon et al. 2014). It is well understood that RNA viruses replicate at very high mutation rates and exist as quasispecies (Sole, Sardanyes et al. 2006). Also, there can be a genetic drift and loss of information from a stable quasispecies if the replication threshold is crossed (Mas, Ulloa et al. 2004, Sole, Sardanyes et al. 2006). Moreover, there is an increased rate of mutation in cells grown *in vitro*, leading to the emergence of an antigenic variant (Loeb, Essigmann et al. 1999, Crotty, Cameron et al. 2001, Sole, Sardanyes et al. 2006). Although the JEV has been explored to a greater extent, there may be some newer circulating strains that behave differently than our present understanding of its molecular biology.

The cell-culture derived live attenuated vaccine SA14-14-2, first licensed for commercial application in China, is the most widely used vaccine in the JE endemic areas (Yu 2010). However, there is always a risk for reversion of the live attenuated SA14-14-2 strain to high virulence (Monath 2002, WHO 2005, Beasley, Lewthwaite et al. 2008, Nath, Gupta et al. 2017). In JE endemic areas, vaccinated individuals are continuously exposed to natural JEV infection, and hence, the durability of protective immunity conferred by the vaccine merits further investigation (Yun and Lee 2014). The National Vector Borne Disease Control Programme (NVBDCP) of India reported that till August 2013, a total of 209 confirmed cases and 18 deaths occurred due to JE in West Bengal, India, even after the implementation of JE vaccination programs (Directorate of National Vector Borne Disease Control Programme 2013).

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Also, additional quality control issues need to be addressed about the presence of adventitious agents in the uncharacterized primary hamster kidney (PHK) cells used for vaccine production of SA14-14-2 (Tsai 2000, WHO 2007). On the other hand, in the case of inactivated vaccines, there is always a risk of improper inactivation of virulent JEV (Ferguson, Kurane et al. 2007, Kim and Samal 2016, Chanthavanich, Limkittikul et al. 2018). Multiple doses at specified intervals is another limitation associated with the inactivated vaccine. The recently developed India's first indigenous vaccine against JEV is JENVAC. It is an inactivated Vero cell-derived vaccine prepared from an Indian strain of JEV. Even though it is highly effective against all known strains of JEV, it requires booster doses and is quite expensive for the rural population (Singh, Mitra et al. 2015).

Thus, in JE endemic Asian countries, the need of the hour is a safer and cheaper JE vaccine, which is efficacious and provides adequate protection with a single dose. In such a situation, NDV-based viral-vectored vaccines offer an alternate live vaccine approach with a host of advantages mentioned in section 1.2.5.2.

Another crucial aspect is that the members of the JE sero-complex have similar antigenicity, and their co-circulation poses challenges in the effective differential diagnosis of JEV. Co-infection of JEV, WNV, and other flaviviruses have been reported in India (Khan, Dutta et al. 2011). The present JEV ELISA kit in use throughout the country is found to cross-react with closely related flaviviruses *viz.,* Dengue and WNV (Johnson, Goodman et al. 2016). This immunocrossreactivity can be eliminated by using only conserved and immunodominant regions of JEV proteins with high solvent accessibility as an immunogen.

1.4. Objectives

i. Development of recombinant NDVs expressing E and NS1 proteins of JEV

ii. Evaluation of the immunogenicity of recombinant NDVs expressing E and NS1 proteins of JEV in mice

iii. *In-silico* approach in designing epitope-based peptides against JEV

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Chapter 2

Adaptation of the Japanese encephalitis virus (JEV) strain SA14-14-2 in Baby hamster kidney cells

2.1. Abstract

A large number of JE/AES cases are reported to occur in areas with established or developing JE vaccination programs. Partial vaccine coverage and the emergence of new variants of JEV may be playing an important role. In the present study, we have characterized the widely used JEV live attenuated vaccine strain SA14-14-2 in BHK-21 cells. The vaccine strain showed enhanced replication following its serial passage in BHK-21 cells. Nucleotide and amino acid sequence analysis of the E protein of the cell-culture adapted vaccine strain showed a significant point mutation identical to its wild-type parent strain SA14. This study suggests the possibility of reversion mutation and exaltation of vaccine strains following their adaptation to individual host cells.

2.2. Introduction

Around 81% of JE cases occur in areas with well-established or developing JE vaccination programs, while approximately 19% of cases occur in areas with minimal or no such programs (Campbell, Hills et al. 2011). In India, there has been an exponential rise in the number of JE cases since 2011 (Borah, Dutta et al. 2011). Currently, the JE endemic states in India, along with other developing Southeast Asian countries, are hit by an unrestrained JE toll because of the JEV vaccine crunch and its failure (Bora 2016, Banerjee, Sen Gupta et al. 2017).

The live attenuated vaccine SA14-14-2 developed in China is of considerable interest in JEV endemic countries. It is known to replicate well in PHK cells, C6/36 mosquito cells, continuous African green monkey kidney (Vero) cells, Rhesus monkey kidney (LLC-MK2)

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cells and BHK21 cells (Yu 2010). In the present study, we showed the adaptation of JEV vaccine strain SA14-14-2 in kidney cells and its probable association with the mutation in amino acid sequence.

2.3. Materials and Methods

2.3.1. Cells and Viruses

BHK-21 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY) at 37°C in 5% CO_2. The live attenuated JEV vaccine strain SA14-14-2 was procured from the Department of Health and Family Welfare, Government of Assam, India. The sequence of the vaccine strain was extracted from GenBank (Accession number JN604986). SA14-14-2 was passaged >20 times in 80% confluent BHK-21 cells at a multiplicity of infection (MOI) of 0.01. The JEV titers in 50% endpoint tissue culture infectious dose ($TCID_{50}$) units/µl were determined on monolayers of BHK-21 cells using the Reed and Muench method (Reed 1938). The BHK-21 cells were adsorbed for 1 hour with 100 $TCID_{50}$/ml of the JEV inoculum and further maintained with DMEM with 2% FBS till 72 hr. The infected BHK-21 cells were freeze-thawed for three cycles, centrifuged at 3000xg, and the supernatant was collected and stored at −80°C.

2.3.2. Immunoperoxidase staining

The presence of JEV antigens in the BHK-21 cells was visualized by immunoperoxidase (IP) staining using JEV specific primary antibody (Thermo Scientific, USA), which binds to the E protein. The secondary antibody is joined to the enzyme, peroxidase, that catalyses a reaction in which the JEV antigen is specifically stained brown.

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2.3.3. Real Time PCR

For real-time PCR, total RNA was extracted from uninfected and infected BHK-21 cells using RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. The RNA was treated with DNase I (NEB, USA) for 30 min at 37°C to remove genomic DNA contamination. RNA was quantified at 260 nm absorbance, and purity was accessed by the A260/A280 ratio (>1.8). The cDNA was synthesized by the PrimeScript first-strand cDNA synthesis kit (TaKaRa, Japan). For real time PCR, JEV E gene specific primers were used (forward primer – 5'GGGAAGGGAAGCATTGACAC3' and reverse primer - 5'GCCCCAACTTGCGCTGAATA3'. Cycling parameters were fixed according to the manufacturer's protocol, and JEV gene expression from the cDNA was quantified using GAPDH as an internal control. Data were analyzed using SDS v2.0.6 software, and the results were statistically expressed as mean ±SD of at least three independent experiments (Applied Biosystems, USA).

2.3.4. RT-PCR, Sequencing and Alignment

Furthermore, the JEV genomic RNA was extracted from the supernatant of infected cells (1st, 13th and 20th passage) using TRIzol (Invitrogen, USA). The cDNA was synthesized using NS1 and E gene-specific reverse primers (NS1 Rev- 5'CGGAATTCCTAAGCAGCGACTAGCAC3' and E Rev– 5'CCCAAGCTTCTAAGCATGCACATTGGTCGC3') followed by PCR using gene-specific forward and reverse primers (NS1For- 5'CTAGCTAGCATGGACACTGGATGTGCCATTG 3', and E For – 5'CGGGATCCATGGCGTTTAATTGTCTGGGAATGGGCAATC3'). The PCR amplified E and NS1 gene products were sequenced using a Big Dye terminator v 3.1 matrix standard kit and 3130xl Genetic Analyzer data collection software v3.0 (Applied Biosystems Inc). The sequencing was repeated three times from three independent RNA

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preparations from each of passage 1, 13 and 20 to ensure a consensus sequence. Amino acid sequence comparison of the viral E protein was performed by ClustalW multiple alignment algorithm of the MegAlign program of the DNASTAR Lasergene 7 software package. JEV wild-type strain SA14/China/1954 (U14163.1) and four recently reported strains (KC526869, KC526870, KC526871 and KC526872) were used to compare the sequences of passaged SA14-14-2.

2.3.5. Three-dimensional structure prediction

The three-dimensional (3D) structures of the E protein of JEV strain SA14-14-2 before and after passage were predicted by I-TASSER (Zhang 2007, Roy, Kucukural et al. 2010). The predicted structures were analyzed in PyMOL software (DeLano 2002). The structure of heparan sulfate was taken from the National Center for Biotechnology Information (NCBI) Pub-Chem compound database. The heparan sulfate was downloaded in Structure Data File (SDF) format and converted to Protein Data Bank (PDB) coordinates using Open Babel converter.

2.3.6. Docking

Receptor and ligand were prepared for docking using AutoDock (v1.5.2) by adding polar hydrogen atoms to receptor proteins and merging its non-polar hydrogen atoms. All bonds of ligands were set to be rotatable. These modified molecules are saved in PDBQT format, as shown earlier (Sanner 1999, Morris, Huey et al. 2009). Grid box was generated to receptor protein covering the whole of protein binding sites. The distance between the two connecting grid points was taken as 1 Angstrom (Å). The center of the grid was kept at (x, y, z) = (92.548, 83.081, 92.538), and the number of grid points in x, y, and z-axes was set to 80×118×66 as shown previously (Konc, Konc et al. 2011). Finally, the docking of heparan sulfate to viral E protein was performed using AutoDock Vina (Morris, Huey et al. 2009, Trott and Olson 2009).

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2.4. Results

2.4.1. Cells and Viruses

The infection of JEV strain SA14-14-2 showed an increased cytopathic effect in BHK-21 cells up to 20 passages (Figure 2.1 A). Further passage of SA14-14-2 in BHK-21 cells showed no enhancement in its cytopathic effect, and hence, we performed experiments using the 20th passage of the JEV strain. The cytopathic effects include the formation of multinucleated giant cells initially and proceeded further to cell detachment. The JEV vaccine strain showed an increased titer of $10^{3.5}$, 10^{5}, $10^{5.5}$, $10^{6.5}$, $10^{6.5}$, $10^{6.5}$, $10^{5.5}$, $10^{5.5}$ $TCID_{50}$/mL at 16, 24, 32, 40, 48, 56, 64, 72 hr post-infection, respectively after twenty passages as compared to the initial passage (Figure 2.1 C).

2.4.2. Immunoperoxidase staining

The JEV specific antigens were further confirmed by positive IP staining using its specific antibody (Figure 2.1 B).

2.4.3. Real Time PCR

The mRNA expression level of the viral E protein increased by approximately 1.303-fold in BHK-21 cells following passage 20 (Figure 2.1 D). This fold change can be related to the increase of E protein level and the consequent increase of cytopathic effect following passage 20.

2.4.4. RT-PCR, Sequencing and Alignment

The replication of JEV after each passage was confirmed by RT-PCR analysis using E and NS1 gene-specific primers (Figure 2.1 E). The E and NS1 gene sequences of the passaged JEV were submitted to GenBank (Accession number KY683775). The E and NS1 gene sequences of the

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passaged JEV were analyzed for their putative amino acid sequences. The nucleotide sequence of the E and NS1 genes of the BHK-21 adapted JEV strain SA14-14-2 showed substitution mutation at G768C, T836A (both in E gene) and T2323C (in NS1 gene) nucleotide positions. A comparison of the amino acid sequences between the E protein of JEV strain SA14-14-2 (passage 1 and 20), parental wild-type strain SA14, and four recently reported sequences showed differences in several positions (Figure 2.2). Interestingly, the putative amino acid sequence of the E protein of the BHK-21 adapted JEV strain SA14-14-2 showed a point mutation of M279K reverting it to the parental wild type strain (SA14/China/1954) (Figure 2.2).

2.4.5. Three-dimensional structure prediction and Docking

Docking studies between the JEV E protein and cell surface-expressed heparan sulfate gave a population of possible conformations and orientations for the ligand at the binding site. The best conformation was chosen to have the lowest energy. First passage JEV has the lowest energy of -6.4kcal/mol with D10, F11, H319, G413, A424, and W425. Passage 20th JEV has the lowest energy -7.3kcal/mol with N154, N151, S152, A362, T147, T146, G163, and K369 (Figure 2.3).

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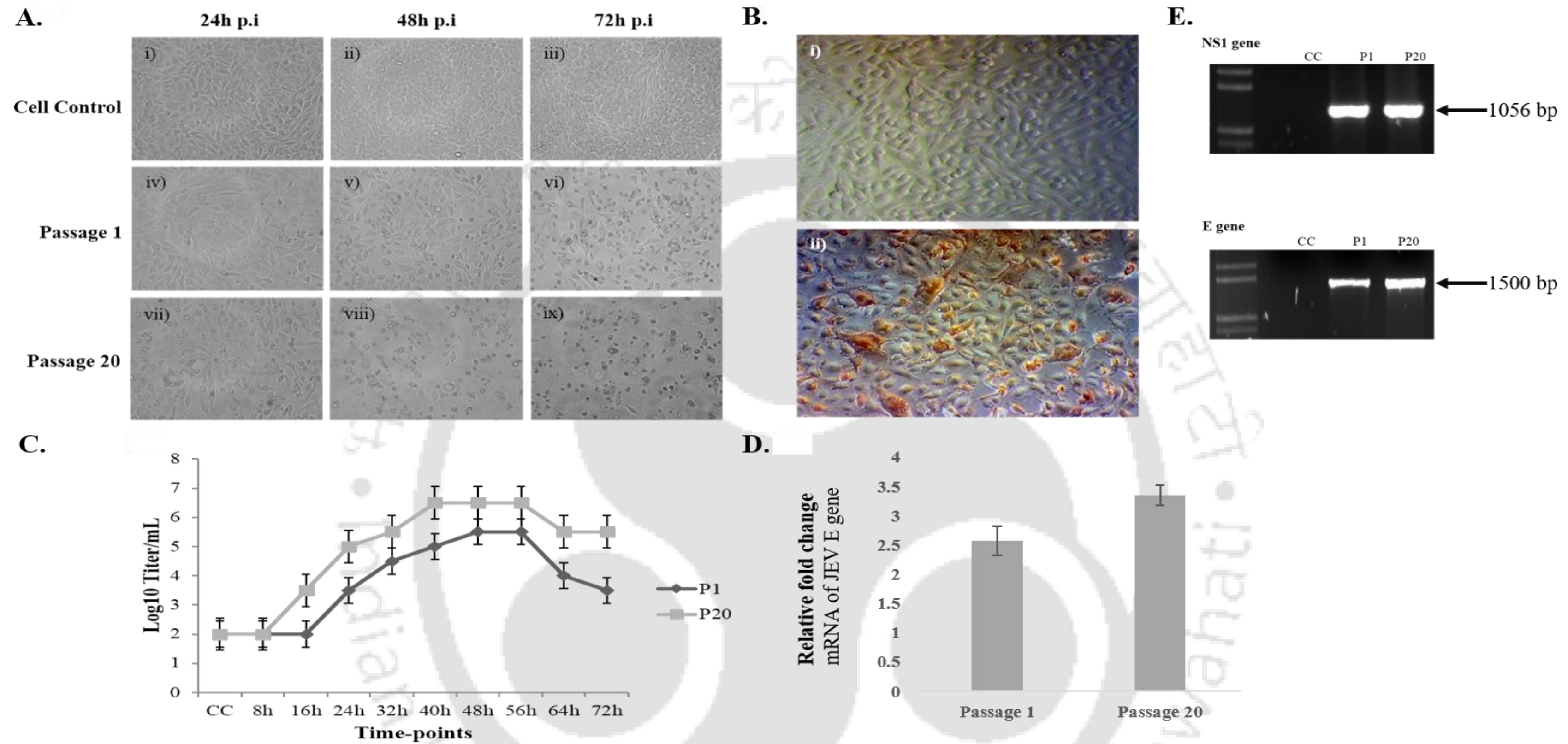

Figure 2.1. Infectivity of Japanese encephalitis virus (JEV) vaccine strain SA14-14-2 in BHK-21 cells at 24- (i, iv, vii), 48- (ii, v, viii), 72- (iii, vi, ix) hours post-infection (h.p.i) **(A)**. Immunoperoxidase staining of BHK-21 cells following its infection with JEV. No peroxidase staining was observed in uninfected cells **(B)**. Infectivity of JEV in BHK-21 cells showing $TCID_{50}$/ml titer of JEV following its passage **(C)**. Real-time expression analysis of the JEV E gene in BHK-21 cells following its passage **(D)**. PCR amplification of E (1500 bp) and NS1 (1056 bp) genes of JEV **(E)**.

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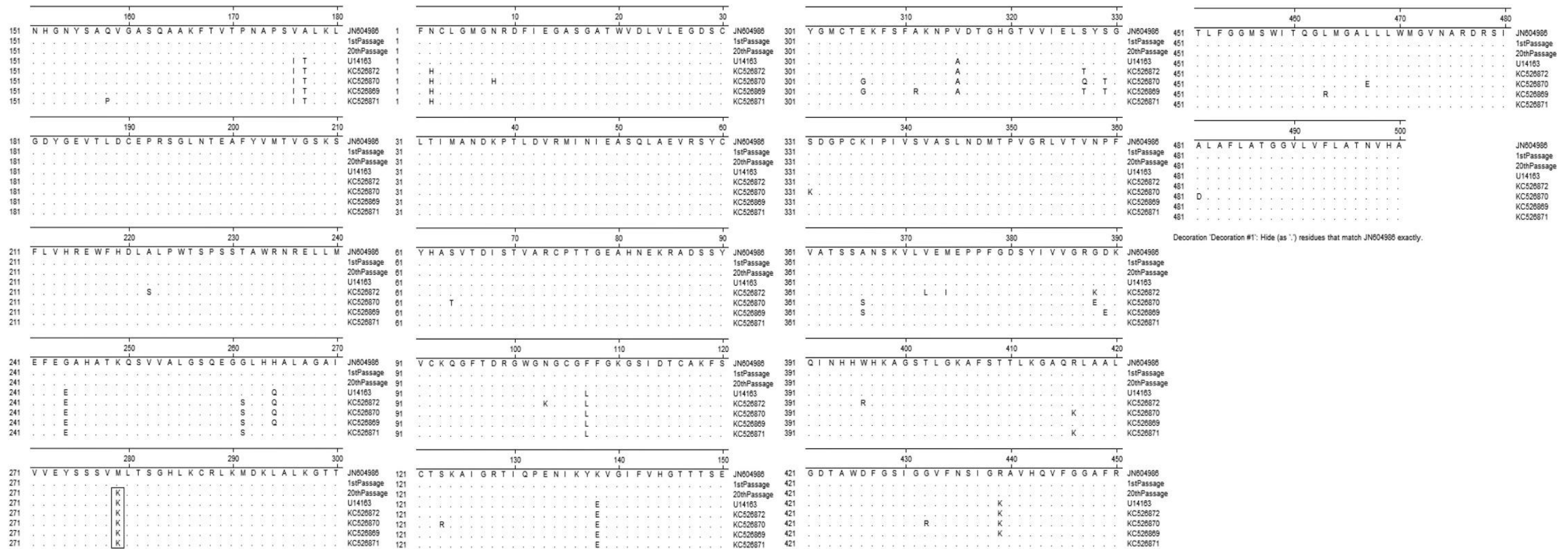

Figure 2.2. Amino acid sequence comparison of the E protein of Japanese encephalitis virus (JEV). Amino acid sequence alignment was performed by the ClustalW multiple alignment algorithm of the MegAlign program of the DNASTAR Lasergene 7 software package. GenBank accession number JN604986 has been taken as the reference sequence for JEV strain SA14-14-2 and aligned with the 1st and 20th passages. Other sequences used in the study were from JEV strains SA14/China/1954 (U14163), JEV/IND/11/WB/JEV45/Midnapore/India/2011(KC526872), JEV/IND/11/WB/JEV47/Hoogly/India/2011(KC526870), JEV/IND/11/WB/JEV46/Midnapore/India/2011(KC526869), JEV/IND/12/WB/JEV50/Malda/India/2012 (KC526871).

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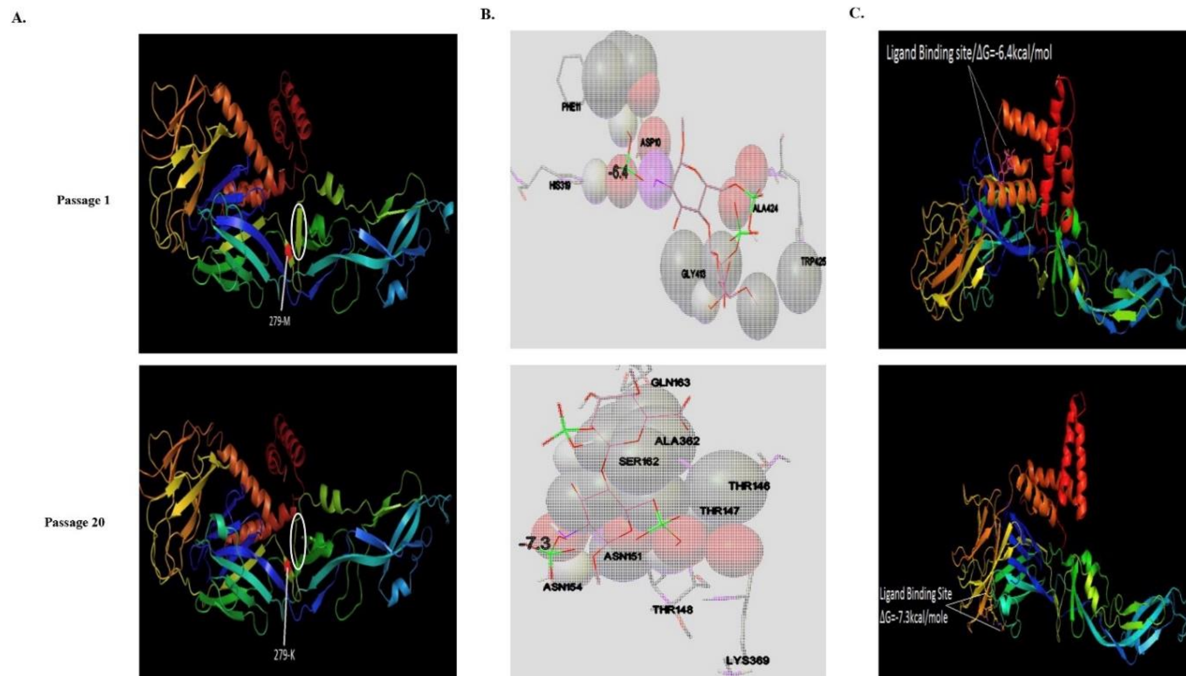

Figure 2.3. 3D structure of the E protein of the 1st and 20th passage of Japanese encephalitis virus (JEV) vaccine strain SA14-14-2 as predicted by I-TASSER (threading) **(A)**. The mutation M279K leads to a structural difference in one beta-strand **(B)**. Docking of E protein of the 1st and 20th passage and heparan sulfate. Receptor-ligand interaction occurs at different amino-acid positions in passage 1 and passage 20 **(C)**. The lowest free energy of receptor-ligand interaction and its position in the case of passage 1 and 20. The binding is more robust in the case of the mutated protein (passage 20).

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2.5. Discussion

The JEV vaccine strain SA14-14-2 went through a mutation on the serial passage *in vitro*, which had a corresponding effect on its replication and cytopathic effect. Our result corresponds with the earlier reports of enhanced cytopathic effect of *Flavivirus* and *Pestivirus* (belonging to the family *Flaviviridae*) owing to an increase in the binding affinity for cells following its passage in permissive cell lines (Mandl, Kroschewski et al. 2001, Monath, Arroyo et al. 2002, Kumar, Barman et al. 2015). Flaviviruses are known to experience selective mutational pressure during replication in different host systems (Monath, Arroyo et al. 2002). Moreover, structural proteins mutate faster than other viral proteins in order to selectively escape the host immune system (Wensvoort, Boonstra et al. 1990, Kwang, Littledike et al. 1992). Our results showed a point mutation M279K in the E protein of the BHK-21 adapted JEV vaccine strain SA14-14-2. It has been reported that amino acid substitutions in the E protein play a key role in determining the neurovirulence of JEV (McMinn 1997). A single mutation in the E protein hinge region of flaviviruses led to an increase in neurovirulence in mice and monkeys (Monath, Arroyo et al. 2002). The JEV vaccine strain SA14-14-2 was derived from the wild-type JEV strain SA14 isolated from a pool of *Culex pipiens* mosquito larvae.

Molecular characterization of SA14-14-2 showed that there are eight critical amino acid substitutions in the E protein responsible for its attenuation. Out of these eight, mutation of Lysine (K) to Methionine (M) (nucleotide position 1813, amino acid position 279) in the E protein of SA14 was one of the critical substitutions. Studies on the neuroattenuation stability of the vaccine strain SA14-14-2 demonstrated that its genetic characteristics are highly stable even after multiple *in vitro* and *in vivo* passages (Yu 2010). The critical reversion mutation M279K in the E protein of the vaccine strain is located within the beta-strand of the secondary structure of E protein in the DI-II hinge region (Monath, Arroyo et al. 2002). Lysine is a

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positively charged, basic amino acid thatplays an essential role in binding sites of proteins and actively participates in hydrogen bonding (Zandarashvili, Li et al. 2011). The mutation M279K led to an increase in the net positive charge and hydrophilicity of the E protein. As a result, there was a probable increase in the binding affinity of the E protein for BHK-21 cells by altering the electrostatic protein-protein interaction and increasing fusogenic activity under low pH conditions during viral infection (Hasegawa, Yoshida et al. 1992, Arroyo, Guirakhoo et al. 2001, Mandl, Kroschewski et al. 2001). Sulfated form of glycosaminoglycans (GAGs), including heparan sulfate on cell surfaces, are highly negatively charged biopolymers and play a determining role in the early stage of JEV infection *in vitro* (Su, Liao et al. 2001). The E protein of JEV binds to cell surface-expressed heparan sulfate, one possible receptor of JEV (Su, Liao et al. 2001, Chien, Chen et al. 2008). During protein-ligand interaction, a better binding affinity equals lower free-energy (Mobley and Dill 2009). Our prediction by software showed that heparan sulfate binds more tightly with the E protein of BHK-21 adapted virus (passage 20) with a binding energy of -7.3kcal/mol as compared to its initial passage with a binding energy of -6.4kcal/mol. We could conclude that because of the reversion mutation M279K, there was a structural change in the E protein of SA14-14-2, which most likely accounted for the change in binding position and an increase in binding affinity of E protein for heparan sulfate receptor. However, the estimation of the increase in E protein level by quantitative western blot and ELISA assays are not performed here and remains for future studies

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Chapter 3

Development of recombinant Newcastle disease viruses (rNDVs) expressing E and NS1 proteins of JEV

3.1. Abstract

The NDV isolates recovered from different cases in chicken flocks in Assam, India during 2014 –15 were genotypically and pathotypically characterized. After that, we performed the complete genome sequencing of the NDV strain isolated from Pandu, Assam, India. Clinicopathological and genetic analysis showed the virulent nature of the NDV strain Pandu. On molecular phylogenetic and evolutionary distance analyses, the NDV strain Pandu formed a distinct clade within the genotype XIII of class II NDV, suggesting a new sub-genotype XIIIc.

It was followed by the development of an rNDV individually expressing the E and NS1 proteins of JEV (rNDV-Ejev and rNDV-NS1jev). The recovered rNDV-Ejev and rNDV-NS1jev were characterized in 9-days-old specific pathogen-free (SPF) embryonated chicken eggs and cell culture.

3.2. Introduction

Newcastle Disease (ND) is a viral disease of the wild and domestic avian species. ND is caused by the NDV, a well-characterized avian paramyxovirus-1 (APMV-1) under the family *Paramyxoviridae* (Lamb, Collins et al. 2005, Alexander and Senne 2008). NDV is an enveloped virus containing a non-segmented, negative-sense, single-stranded RNA genome, which falls into three different genome size categories of 15,186 nt, 15,192 nt and 15,198 nt in length (Krishnamurthy and Samal 1998, de Leeuw and Peeters 1999). Its strains encode six essential proteins, namely nucleoprotein [N], phosphoprotein [P], matrix [M], fusion [F],

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hemagglutinin-neuraminidase [HN] and RNA-dependent RNA polymerase [L] (Lamb, Collins et al. 2005, Alexander and Senne 2008).

The F glycoprotein is an integral membrane protein and a major protective antigen (Samal 2011). Based on disease signs and lesions produced, NDV isolates have been historically classified into three major clinicopathologic groups, namely, lentogenic (low virulent), mesogenic (moderately virulent) and velogenic (highly virulent) (Hanson and Brandly 1955, Samal 2011). The activation of NDV infectivity depends upon cleavage of the F protein, and hence, the amino acid sequence at the F protein cleavage site is established as the molecular determinant of NDV virulence (Nagai, Klenk et al. 1976, Toyoda, Sakaguchi et al. 1987). The mean death time (MDT) in embryonated chicken eggs and intracerebral pathogenicity index (ICPI) in day-old chicks are the internationally accepted tests to determine the pathotype of NDV isolates (Alexander 2009, Samal 2011). According to the World Organization for Animal Health (OIE), the virus should have an ICPI of ≥ 0.7 or at least three arginine or lysine residues between residue number 113 and 116 and phenylalanine at residue 117 to be considered as virulent (OIE 2012). Phylogenetically, strains of NDV are divided into two classes (class I and class II), and further classified into twenty-one genotypes (I–XXI) based on genetic differences (Dimitrov, Ramey et al. 2016, Dimitrov, Abolnik et al. 2019). The present study will be useful to understand the biology of circulating strains of NDV in India.

NDV has been developed as a viral vaccine vector using reverse genetics, with several advantages over other viral vaccine vectors for human usage (Bukreyev and Collins 2008, Samal 2011). NDV can infect humans, but is non-pathogenic to humans due to its natural host range restriction. There is no pre-existing immunity to NDV in the human population (Huang, Elankumaran et al. 2003, Bukreyev and Collins 2008). Unlike pox, adeno and herpes viral vectors, its genome encodes only six essential proteins and hence, it is easy to modulate using advanced molecular biology tools. This translates to a lesser competition between vector

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proteins and the expressed foreign proteins for immune responses (Kim and Samal 2016). NDV does not establish persistent infection or integrate with the host genome as it replicates in the cytoplasm of host cells (Samal 2011). Owing to these salient features, we have generated and characterized rNDVs individually expressing the E and NS1 genes of JEV.

3.3. Materials and Methods

3.3.1. Molecular characterization of Newcastle disease virus strains isolated from different cases in Northeast India during 2014-15

Three different NDV outbreaks were reported during the period June 2014 to February 2015 from Northeast India (Figure 3.1). The details of the outbreak have been summarized in Table 3.1. The collected serum samples from the ailing birds were tested for the presence of NDV specific antibody by hemagglutination inhibition (HI) assay and commercial ELISA [IDEXX, USA]. Infected tissue samples such as the brain, bursa, lungs, liver, kidney, spleen and intestine were collected from ailing and dead birds. Collected tissues were fixed in 10% neutral buffered formalin for approximately 48 hrs. Formalin-fixed tissue samples were routinely processed for hematoxylin and eosin staining after paraffin embedding and sectioning (3 micrometers) following standard procedure (Wakamatsu, King et al. 2006).

Tissue samples containing viruses were inoculated in the allantoic cavities of 9-days-old SPF embryonated chicken eggs, and infected allantoic fluids were collected 48 hr post-inoculation. The presence of the virus in collected allantoic fluids was confirmed by hemagglutination assay (HA) using 1% chicken RBC. Moreover, the isolated virus strains were plaque purified using chicken embryo fibroblasts (CEF) following the standard protocol (Alexander and Senne 2008). The virulence of the virus strains was determined by the clinicopathological assays: MDT and ICPI following the standard protocol (OIE 2012).

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The viral genomic RNA was extracted from the homogenized tissue samples and allantoic fluids using TRIzol® Reagent (Invitrogen, Grand Island, NY, USA). Reverse transcription (RT) was performed following the manufacturer's protocol using a SuperScript™III RT enzyme (Invitrogen, Grand Island, NY, USA) by NDV specific forward primer (NDV 4163 forward: 5′ AGC CTG CTA TCC YAT AGC AAA TGC 3′). The cDNA synthesized by the RT reaction was further amplified by PCR using NDV F and HN gene-specific primer pairs (F 4544 forward: 5′ GCT GCT AGC ATG GGC TCC AGA CCT TC 3′ and F 6205 reverse: 5′ CGT GGT ACC TCA CAT TTT TGT AGT GGC 3′; HN 6412 forward: 5′ CGG GGT ACC ATG GAC MGC GCM GTT AG 3′ and HN 8147 reverse: 5′ CGG GAT CCC TAR CCA GAC CTG GCT TCT C 3′ where M stands for A/C, R stands for A/G). The number in the primer indicates the position of the nucleotide with respect to the complete genome sequence of NDV. Degenerate consensus primers were designed using available GenBank sequences of different strains of NDV (GenBank accession numbers: NC_002617, FJ986192, AY562988, JF950510). The amplified 1661 and 1735 base pairs (bp) of F and HN genes, respectively, were purified and cloned into the pGEM®-T vector (Promega, Madison, WI, USA). The cloned gene products were sequenced by the Sanger sequencing method and analyzed by DNA Star software.

3.3.2. Complete genome sequencing of NDV/Chicken/Pandu/01/15

The complete genome sequencing of NDV strain Pandu was performed, and the genome editing and final assembly of the nucleotide sequence were performed using the Lasergene sequence analysis software package (Lasergene, version 5.07; DNASTAR, Inc., Madison, WI). Phylogenetic and evolutionary analysis of NDV strain Pandu was performed using MEGA6 software (Tamura and Kumar 2002, Tamura, Stecher et al. 2013). The F gene sequence of NDV strain Pandu was used to construct a phylogenetic tree to infer its evolutionary relationship with other representative strains belonging to genotype I-XVIII. Besides, the F gene sequence

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was used to determine the phylogenetic relationships of NDV strain Pandu with other representative genotype XIII strains. The evolutionary distances between NDV strain Pandu and representative strains belonging to genotype II, IV, VII, XIIIa and XIIIb was computed since these are the commonly circulating genotypes in Southeast Asia. To further test sub-genotypes or clades within the genotype, evolutionary distances were computed between sub-genotype XIIIa, XIIIb and the representative genotype XIII strain in India – NDV isolate Cockatoo/India/7847/1982. For the assignment of sub-genotypes and genotypes, the recently proposed nomenclature was followed (Diel, da Silva et al. 2012).

3.3.3. Cells and Viruses

The rNDV strain, derived from the parent strain (GenBank accession number AY845400) available in the laboratory, was amplified in 9-days-old SPF embryonated chicken eggs (Kumar, Kumar et al. 2018, Kumar, Kumar et al. 2019). The parent strain was procured from a veterinary store in Guwahati, Assam. The infected allantoic fluid was harvested 96 hr post-inoculation and clarified. The growth of rNDV was confirmed by HA and RT-PCR. BHK-21, chicken embryo fibroblast (DF-1) and human epithelial type 2 (HEp-2) cells were grown and maintained in DMEM (Sigma, USA) containing 10% FBS (Invitrogen, Grand Island, NY). All the cells were maintained at 37ºC in 5% CO_2.

The JEV vaccine strain SA14-14-2 (GenBank accession number JN604986) was obtained from the Department of Health and Family Welfare, Government of Assam, India. The BHK-21 cells were infected with JEV at an MOI of 0.01 and harvested 72 hr post-infection to prepare the virus stock. The recombinant vaccinia virus strain Ankara expressing T7 RNA polymerase - MVA/T7 (a generous gift of Dr. Bernard Moss, NIH), was amplified in primary chicken embryo fibroblast cells and stored at -80 °C.

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3.3.4. Construction of rNDV plasmids expressing E and NS1 genes of JEV

The rNDV full-length expression plasmid (pNDV), available in the laboratory and reported earlier (Kumar, Kumar et al. 2018, Kumar, Kumar et al. 2019), was used to clone the E and NS1 genes of JEV. The complete antigenome of NDV was segmented into five fragments and cloned sequentially into the linker in the pUC19 vector. A unique restriction site of *Asc*I (3236nt to 3243nt) was created in the noncoding region between the P and M genes of NDV by site-directed mutagenesis. The pNDV sequence was flanked on both sides with a T7 promoter and Hepatitis delta virus (HDV) ribozyme sequence with a T7 termination sequence.

Total RNA was extracted from BHK-21 cells infected with JEV vaccine strain (SA14-14-2) 72 hr post-infection using RNeasy Mini Kit (QIAGEN) following the manufacturer's protocol. The cDNA was synthesized by the PrimeScript first-strand cDNA synthesis kit (TaKaRa, Japan). The cDNA was synthesized using JEV E gene-specific reverse primer (E Rev– 5´CCCAAGCTTCTAAGCATGCACATTGGTCGC3´). The JEV E complete gene (978-2477, positions corresponding to JEV complete genome sequence) was designed to contain the gene start and gene end sequences of the M gene of NDV. The primers containing *Asc*I sites, NDV gene end, gene start transcriptional signals, and an intergenic sequence was used to amplify the E gene cassette. The extra ATG and TCA sequences were added to use as a start codon and stop codon respectively, for the E gene. Ex Taq DNA polymerase was used to amplify the E gene cassette (Takara, Japan) and digested with *Asc*I (NEB, USA) for further cloning. The sequence integrity and consistency of the "rule of six" was confirmed by sequence analysis. The orientation of the cloned E gene was confirmed by PCR and restriction digestion. Similarly, the NS1 gene of the JEV was cloned into the pNDV vaccine vector. The rNDV full-

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length expression plasmids bearing the E and NS1 genes of JEV were assigned the names pNDV-Ejev and pNDV-NS1jev, respectively.

3.3.5. Recovery of rNDVs expressing E and NS1 genes of JEV

The HEp-2 cells were grown overnight to 80-90% confluence in six-well culture plates and were co-transfected with 3µg of pNDV-Ejev along with 1µg each of support plasmids pN-NDV, pP-NDV and pL-NDV (available in the laboratory) using 6 microliters (µl) of Lipofectamine 2000 reagent (Invitrogen, USA). Along with the transfection mixture, one focus forming unit per cell of MVA/T7 was added for the successful expression of T7 RNA polymerase. The media was replaced with DMEM containing 2% FBS and 10% allantoic fluid 6 hr post-transfection. The HEp-2 cells were scraped, frozen, thawed three times and centrifuged 96 hr post-transfection. The resulting supernatant (100 µl) was inoculated in the allantoic cavity of 9-days-old SPF embryonated chicken eggs. The infected allantoic fluid was harvested 96 hr post-inoculation and tested for the HA activity. The presence of the rNDVs in the allantoic fluid was further confirmed by RT-PCR, followed by nucleotide sequencing of the amplified products. The recovered virus was passaged ten times in 9-days-old SPF embryonated chicken eggs and DF-1 cells. The integrity of the E gene of JEV in the recombinant virus was checked by RT-PCR and nucleotide sequencing of the amplified product after every passage. Similarly, pNDV-NS1jev was also recovered. The successfully recovered infectious recombinant viruses were named, rNDV-Ejev expressing E and rNDV-NS1jev expressing NS1 genes of JEV.

3.3.6. Characterization of rNDVs expressing E and NS1 genes of JEV

The passaged rNDVs were purified using the standard protocol for sucrose density gradient purification (Duesberg and Robinson 1965). The expression of E and NS1 proteins of JEV by the rNDVs was examined by western blot analysis. Briefly, BHK-21 cells were infected with

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rNDV, rNDV-Ejev, and rNDV-NS1jev, at an MOI of 0.01. The cell lysates were harvested 48 hr post-infection and analyzed using a monoclonal antibody against the HN protein of NDV (a kind gift of Dr. R. Iorio, University of Massachusetts, USA), anti-E JEV antibody (GeneTex, USA) and anti-NS1-JEV antibody (GeneTex, USA). Also, the western blot analysis was carried out using partially purified rNDV-Ejev and rNDV-NS1jev from the allantoic fluid.

The pathogenicity index of the rNDVs (1st, 5th and 10th passage) was determined by MDT in 9-days-old SPF embryonated chicken eggs as per the standard protocol (Kumar, Nayak et al. 2011). MDT is the meantime calculated in hrs for the minimum lethal dose of virus to kill all the inoculated 9-days-old SPF embryonated chicken eggs. The MDT values of less than 60 hrs for velogenic and more than 90 hrs for lentogenic strains of NDV have been well established (Alexander and Allan 1974). Briefly, a series of 10-fold (10^{-6} to 10^{-9}) dilutions of fresh infective allantoic fluid in sterile phosphate-buffered saline (PBS) was made, and 100 µl of each dilution was inoculated into the allantoic cavities of five replicates of 9-days-old SPF embryonated chicken eggs. The eggs were incubated at 37°C, examined every 8 hrs for 7 days, and the time-points of the embryo deaths were recorded.

The multicycle growth kinetics of rNDV, rNDV-Ejev and rNDV-NS1 were determined in BHK-21 cells. BHK-21 cells were infected separately with the 10th passage of rNDVs at an MOI of 0.01. The cell culture supernatants were collected and replaced with an equal volume (200 µl) of fresh medium at 12-hr intervals until 96 hr post-infection. The titers of virus in the samples were quantified by $TCID_{50}$ assay in BHK-21 cells (Reed and Muench 1938).

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3.4. Results

3.4.1. Molecular characterization of Newcastle disease virus strains isolated from different cases in Northeast India during 2014-15

All three isolates were named according to the place and year of isolation. The clinical signs observed among infected birds are summarized in Table 3.1. Microscopically, the liver showed severe hemorrhages along with multiple areas of focal aggregation of mononuclear infiltrating cells and focal areas of coagulative necrosis and heterophils in some areas (Figure 3.2 A). Spleen showed the depletion of lymphocytes from its nodules and congestion of the blood vessels (Figure 3.2 B). Also, extensive proliferation of fibrous connective tissue was noticed in isolated areas around spleen nodules. Lung showed extensive hemorrhages and congestion of the blood vessels (Figure 3.2 C). Proventriculus showed mild hemorrhages with hypertrophy of the proventricular gland (Figure 3.2 D). The MDT and ICPI values of NDV/Chicken/Hajo/01/14, NDV/Chicken/Polashbari/01/14 and NDV/Chicken/Pandu/01/15 suggested the velogenic nature of NDV strains (Table 3.1).

3.4.2. Complete genome sequencing of NDV/Chicken/Pandu/01/15

The complete genome of NDV strain isolated from Pandu, Assam was sequenced and characterized. The genome was found to be 15,192 nt long (GenBank accession number KY774445). The features of all the six genes, along with their deduced proteins, are summarized in Figure 3.3. The genetic characterization of NDV/Chicken/Pandu/01/15 showed virulent cleavage site ^{112}-RRQKRF-^{117} in its F protein. The nucleotide and deduced amino acid sequence of the F and HN protein of NDV/Chicken/Pandu/01/15 showed a low sequence identity with the vaccine strain LaSota. The F and HN gene of NDV/Chicken/Pandu/01/15 showed 83.5% and 81.7% nucleotide sequence identity with LaSota, respectively. Similarly, the F and HN protein of NDV/Chicken/Pandu/01/15 NDV showed 88.1% and 87.8% amino

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acid sequence identity with LaSota, respectively. On molecular phylogenetic analysis, NDV/Chicken/Pandu/01/15 clustered with the strains of the genotype XIII (Figure 3.4 A). The NDV/Chicken/Pandu/01/15 appeared to branch out as a topologically distinct clade within genotype XIII, along with two other isolates with a confident bootstrap value of 100% (Figure 3.4 B). On evolutionary distance analysis, NDV/Chicken/Pandu/01/15 showed a minimum distance of 9.2% and 11.2% with sub-genotype XIIIa and XIIIb viruses, respectively (Supplementary Table S1). In the present study, the data from phylogenetic and evolutionary analyses of NDV/Chicken/Pandu/01/15 suggests its independent evolution among genotype XIII viruses. NDV/Chicken/Pandu/01/15 formed a topologically distinct clade along with two other geographically distant strains upon phylogenetic analysis (Figure 3.4 A). Moreover, NDV/Chicken/Pandu/01/15 showed high bootstrap support at their defining nodes with sub-genotypes XIIIa and XIIIb (Figure 3.4 B). Also, the new clade was separated by an inter-populational mean evolutionary distance of 7.0% and 8.0% from sub-genotype XIIIa and XIIIb, respectively. However, the isolated new clade showed a close relationship of 6.4% with the evolutionarily parental strain NDV/Cockatoo/India/7847/1982 (Supplementary Table S2).

3.4.3. Construction and recovery of rNDVs expressing E and NS1 genes of JEV

The pNDV vaccine vector was modified by the successful insertion of the E and NS1 genes derived from the JEV vaccine strain (SA14-14-2). Each gene ORF was flanked by the gene start and gene end sequences of the M gene of NDV. The E and NS1 transcriptional cassettes were inserted into the unique *Asc*I site created between the P and M genes in the pNDV backbone to yield plasmids pNDV-Ejev and pNDV-NS1jev (Figure 3.5 A). The insertion of E and NS1 genes in pNDV-Ejev and pNDV-NS1jev was confirmed by the release of 1500 bp and 1056 bp fragments, respectively, by digestion with *Asc*I restriction enzyme (Figure 3.5 B). The clones were found to be in the correct orientation and intact upon nucleotide sequencing

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and restriction digestion. rNDV-Ejev and rNDV-NS1jev were successfully recovered following the transfection of pNDV-Ejev and pNDV-NS1jev, respectively, in the HEp-2 cells.

3.4.4. Characterization of the rNDVs expressing E and NS1 genes of JEV

The expression of E and NS1 proteins of JEV by the rNDVs was analyzed by western blot of the partially purified rNDV-Ejev and rNDV-NS1jev from the allantoic fluid (Figure 3.5 C). The NDV HN specific monoclonal antibody showed a band of ~74 kDa corresponding to HN protein in rNDV, rNDV-Ejev, and rNDV-NS1jev. In parallel, anti-E and anti-NS1 antibodies of JEV showed corresponding bands of ~55 kDa and ~40 kDa in rNDV-Ejev and rNDV-NS1jev lanes respectively. Similar results were observed in western blot analysis from the BHK-21 cells infected with rNDV-Ejev and rNDV-NS1jev (Figure 3.5 C). The multicycle growth kinetics of rNDV, rNDV-Ejev, and rNDV-NS1jev was determined in BHK-21 cells (Figure 3.5 D). The MDT in 9-days-old SPF embryonated chicken eggs for both rNDV-Ejev and rNDV-NS1jev was more than 120 hrs (Table 3.2).

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Figure 3.1. The geographical locations from where Newcastle disease cases were investigated during 2014–15 in Northeast India.

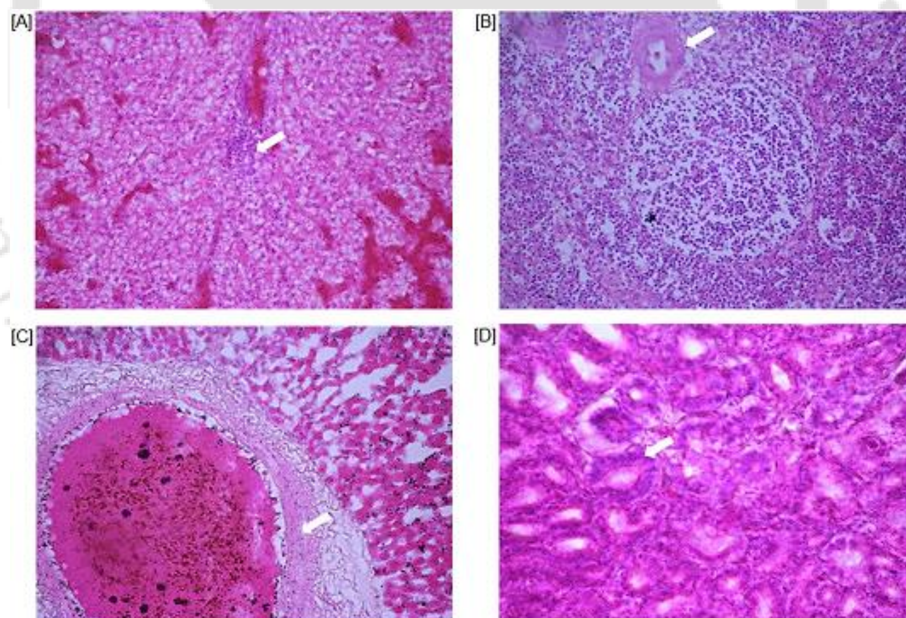

Figure 3.2. Histopathological examination of Newcastle disease virus-infected tissue samples isolated from the liver **(A)**, spleen **(B)**, lung **(C)**, and proventriculus **(D)**. The liver showed severe hemorrhages along with multiple areas of heterophil aggregation. Spleen showed congestion of the blood vessels. Lung showed extensive hemorrhages and congestion of the blood vessels. Proventriculus showed mild hemorrhages and hypertrophy of the proventricular gland. Lesions are marked with a white arrow.

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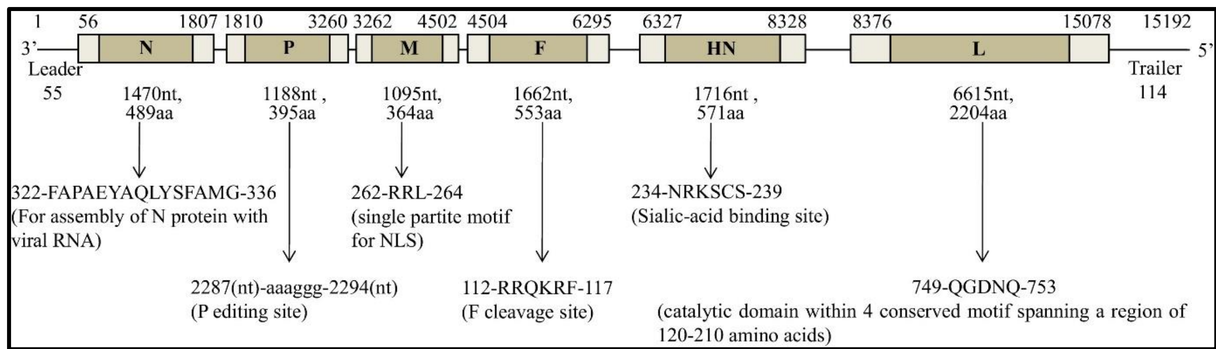

Figure 3.3. Genetic characteristics, deduced protein characteristics, and distinctive features of the Newcastle disease virus isolate NDV/Chicken/Pandu/01/15.

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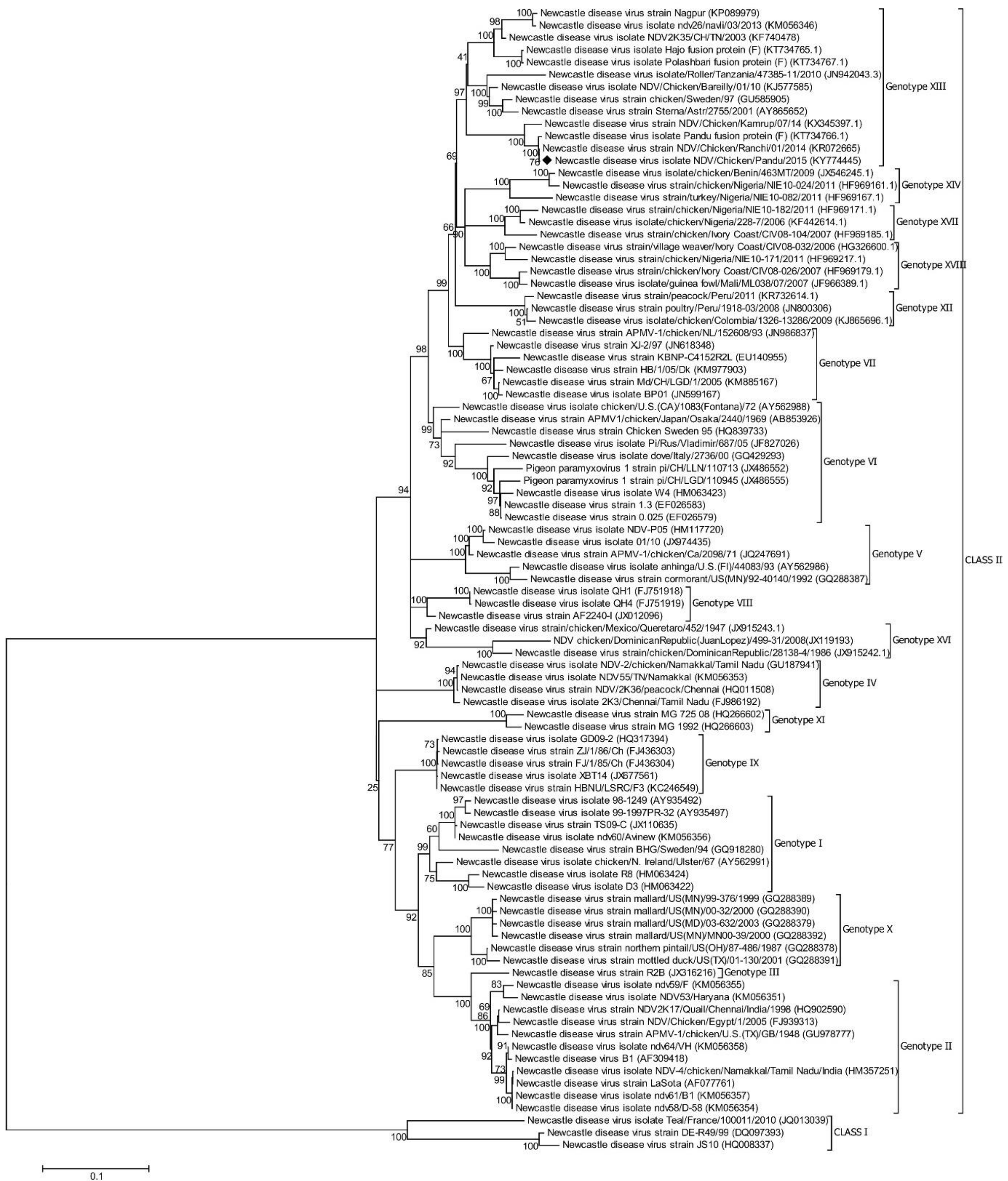
A.



B.

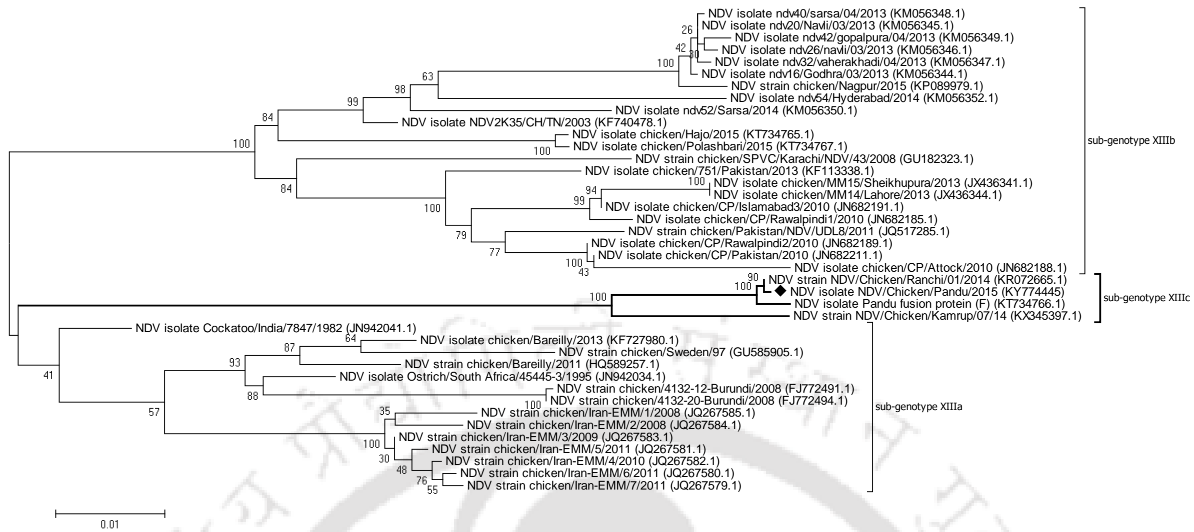


Figure 3.4. Molecular phylogenetic analysis of Newcastle disease virus isolate NDV/Chicken/Pandu/2015 based on the complete fusion (F) gene **(A)**. The evolutionary history of NDV/Chicken/Pandu/2015 was inferred by using the maximum likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (−21682.1080) is shown. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 37.8294% sites). A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 1.6998)]. The analysis involved 93 nucleotide sequences. There were a total of 1661 positions in the final dataset.

Molecular phylogenetic analysis of NDV isolate NDV/Chicken/Pandu/2015 based on the complete F gene of genotype XIII viruses (**B**). The evolutionary history of NDV/Chicken/Pandu/2015 was inferred by using the maximum likelihood method based on the Tamura 3-parameter model. The tree with the highest log likelihood (−6817.2884) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.4278)]. The analysis involved 40 nucleotide sequences. There were a total of 1654 positions in the final dataset.

The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6.

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Sample	Year of isolation	Location of outbreak	Clinical signs	Vaccination status	F protein cleavage site	% amino acid identity of F protein with LaSota	% amino acid identity of HN protein with LaSota	HI titer (log2)	MDT (hrs)	ICPI
NDV/Chicken/Hajo/01/14	2014	Hajo, Assam	Respiratory distress	Vaccinated with LaSota	^{112}-RRQKRF-^{117}	88.8	85.5	7	63	1.7
NDV/Chicken/Polashbari/01/14	2014	Polashbari, Assam	Sudden death	Vaccinated with LaSota	^{112}-RRQKRF-^{117}	88.6	85.8	6	58	1.7
NDV/Chicken/Pandu/01/15	2015	Pandu, Assam	Respiratory distress, tracheal hemorrhage, caecal hemorrhage, lung congestion	Unvaccinated	^{112}-RRQKRF-^{117}	88.1	87.8	7	49	1.8

Table 3.1. Details of the Newcastle disease virus cases investigated by the Viral Immunology Lab, IIT Guwahati during 2014–15 from Assam, Northeast India.

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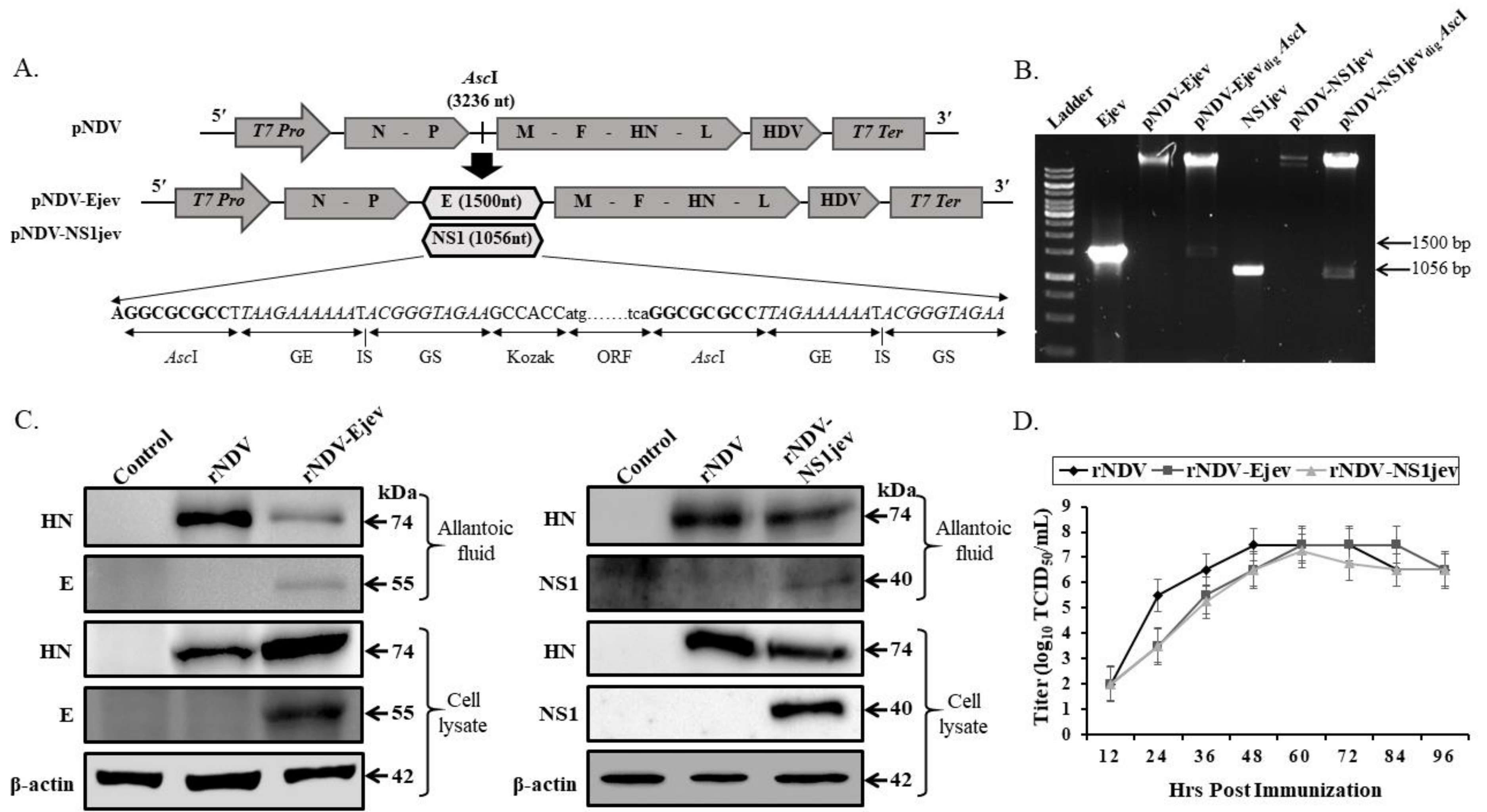

A.
pNDV
pNDV-Ejev
pNDV-NS1jev
AscI
(3236 nt)
5′
3′
T7 Pro
N - P
M - F - HN - L
HDV
T7 Ter
E (1500nt)
NS1 (1056nt)
AGGCGCGCCTTAAGAAAAAATACGGGTAGAAGCCACCatg.......tcaGGCGCGCCTTAGAAAAAATACGGGTAGAA
AscI
GE
IS
GS
Kozak
ORF
B.
Ladder
Ejev
pNDV-Ejev
pNDV-Ejev dig AscI
NS1jev
pNDV-NS1jev
pNDV-NS1jev dig AscI
1500 bp
1056 bp
C.
Control
rNDV
rNDV-Ejev
rNDV-NS1jev
HN
E
NS1
β-actin
kDa
74
55
40
42
Allantoic fluid
Cell lysate
D.
rNDV
rNDV-Ejev
rNDV-NS1jev
Titer (log10 TCID50/mL)
Hrs Post Immunization

Figure 3.5. Construction and confirmation of recombinant plasmids pNDV-Ejev and pNDV-NS1jev. A schematic representation of pNDV backbone (upper) and recombinant plasmids pNDV-Ejev and pNDV-NS1jev (lower). pNDV consists of the T7 promoter, N-nucleoprotein, P-phosphoprotein, M-matrix protein, F-fusion protein, HN-hemagglutinin-neuraminidase protein, L-large polymerase protein, HDV-hepatitis delta virus ribozyme sequence and T7 terminator. The transcription cassettes encoding JEV E and NS1 genes were cloned into the *Asc*I site at the position 3236 nucleotide in between the P and M genes of the NDV anti-genome. The E and NS1 ORFs are flanked by NDV gene end (GE), an intergenic sequence (IS) and NDV gene start (GS). Also, a Kozak sequence is placed upstream of the E and NS1 ORFs **(A)**. pNDV-Ejev$_{dig}$*Asc*I and pNDV-NS1jev$_{dig}$*Asc*I represent the digestion of recombinant plasmids pNDV-Ejev and pNDV-NS1jev with *Asc*I and the release of E (1500 bp) and NS1 (1056 bp) **(B)**. Confirmation of NDV, JEV E and NS1 specific protein expression by rNDV-Ejev and rNDV-NS1jev by western blot using the monoclonal antibody of NDV-HN, a polyclonal antibody of JEV-E and a monoclonal antibody of JEV-NS1 respectively, in allantoic fluid and cell-lysates of BHK-21 cells 48 hr post-infection **(C)**. Comparison of the multicycle growth kinetics of rNDV, rNDV-Ejev, and rNDV-NS1jev. The virus titer in cell culture supernatant samples was determined by $TCID_{50}$ assay. $TCID_{50}$ data represent the mean ± standard deviation of three independent experiments **(D)**.

Passage level	**MDT**		
	rNDV	rNDV-Ejev	rNDV-NS1jev
1st	>120 hrs.	>120 hrs.	>120 hrs.
5th	>120 hrs.	>120 hrs.	>120 hrs.
10th	>120 hrs.	>120 hrs.	>120 hrs.

Table 3.2. Mean Death Time (MDT) values in hours of the 1st, 5th and 10th passage of rNDV, rNDV-Ejev and rNDV-NS1jev.

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3.5. Discussion

The North Eastern part of India is quite vulnerable to NDV outbreaks because of its diversity and geographical factors (Nath, Barman et al. 2015, Das and Kumar 2016). These outbreaks are detrimental to the poultry industry in this part of the country (Islam, Barbaruah et al. 2005). The present report of the NDV case in Pandu from Northeast India provides additional grounds for the continued evolution of genotype XIII viruses in the country (Jakhesara, Prasad et al. 2014, Morla, Shah et al. 2016). Our result of phylogenetic and evolutionary distance analyses suggests NDV/Chicken/Pandu/01/15, similar to the recent isolates Ranchi (KR072665) and Kamrup (KX345397). A high bootstrap value supports the new clade in the tree topology. The strains belonging to the assigned clade have an average nucleotide distance of 7% and 8% with sub-genotype XIIIa and XIIIb strains, respectively.

Moreover, the three isolates were independently reported from geographically distant locations. Since the new clade fulfilled the criteria for the sub-genotype assignment, we can propose the evolution of a tentative sub-genotype XIIIc under genotype XIII, as discussed in the recent report on NDV isolate Kamrup (Diel et al., 2012; Das and Kumar, 2016). NDV genotype XIII could now be divided into three sub-genotypes XIIIa, XIIIb and XIIIc, with sub-genotype XIIIc, presently comprising recently isolated strains from the eastern and North-eastern part of India. The low amino acid sequence identity between the F and HN proteins of the outbreak and vaccine strains might be one of the reasons for the vaccine failure in several cases in different parts of India (Kumar and Kumar 2015, Nath, Barman et al. 2015). This leads to a new challenge for the development of genotype matched vaccine against NDV in India (Kim, Wanasen et al. 2013, Wang, Wang et al. 2014). The present study also emphasizes the importance of understanding the dynamic evolution of NDV from unknown reservoirs in the North Eastern part of India. It is crucial to monitor the rich avian biodiversity of Northeast

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India because a falling off in effective control and bio-security criteria will likely make this region a hub of emerging virulent NDV strains.

In this study, we have successfully rescued rNDVs expressing the foreign genes E and NS1 of JEV. We have earlier reported rNDVs expressing the foreign proteins of classical swine fever virus (CSFV), a member of family *Flaviviridae* in their native conformation (Kumar, Kumar et al. 2018). The proper folding and glycosylation pattern of proteins play an influential role in the immunogenicity of enveloped viruses (Haigwood, Nara et al. 1992, Fournillier, Wychowski et al. 2001). The introduction of the foreign genes into the rNDV particles did not increase the virulence of the rescued viruses in 9-days-old SPF embryonated chicken eggs implying that the expression of JEV immunogenic proteins by rNDV does not pose a biosafety hazard. The multicycle growth kinetics of rNDV, rNDV-Ejev, and rNDV-NS1jev suggested that the replication of rNDV-Ejev and rNDV-NS1jev was slightly retarded compared to that of rNDV. However, they achieved similar titer at 60 hr post-infection. This result corroborates with the previous reports that the introduction of foreign genes to the RNA genome of negative-sense RNA viruses reduces the replication potential of the virus (Sakai, Kiyotani et al. 1999, Wertz, Moudy et al. 2002).

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Chapter 4

Evaluation of the immunogenicity of rNDVs expressing E and NS1 proteins of JEV in mice

4.1. Abstract

rNDV-Ejev and rNDV-NS1jev were used for intranasal immunizations in female, 6-7 weeks old, BALB/c mice to evaluate the relative contributions of each of the two immunogenic proteins (E and NS1) of JEV. The vaccination of rNDV-Ejev and rNDV-NS1jev showed effective immunity against JEV upon immunization. The rNDVs vaccination produced adequate neutralization antibody titers against both NDV and JEV. The cytokine profiling of the vaccinated mice showed an effective Th1 and Th2 mediated immune response. The study also provided an insight that E, when used in combination with NS1, could reduce the efficacy of only E based immunization in mice. Our results suggested rNDV-Ejev to be a promising live viral-vectored vaccine against JEV. This study implies an alternative and economic strategy for the development of a recombinant vaccine against JEV.

4.2. Introduction

Anti-viral therapy against JEV is not well established, and immunization is an effective way to protect humans against its infection (Yun and Lee 2014). Various viral vectors have been explored to express foreign genes for recombinant vaccine production (Nascimento and Leite 2012). Replicating viral vectors, without requiring the involvement of the complete pathogen, can be an efficient way to express the foreign antigen intracellularly, and induce humoral, cellular, and mucosal immune responses successfully (Bukreyev and Collins 2008). In this study, we have used the rNDV system to evaluate the relative individual and combined

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contributions of E and NS1 proteins in the induction of neutralizing antibodies against JEV in mice.

4.3. Materials and Methods

4.3.1. Immunization study

Female, inbred, BALB/c mice, aged 6-7 weeks and weighing 25-30 grams, were purchased from disease-free small animal house at the Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana. They were housed in micro isolator cages in biosafety level-2 facility in Centre for Medical Biotechnology, Maharshi Dayanand University, Rohtak, India and fed with free access to diet and water. The experimental protocol was approved by IAEC vide no. CAH/153-165 dated 17-12-2018. All the animal research was conducted according to the guidelines approved by the Institutional Animal Care and Use Committee of the Maharshi Dayanand University, Rohtak, India.

A total of 6 groups (n=12 per group) of mice were housed separately and acclimatized for one week. Four groups of mice were anesthetized by isoflurane and inoculated intranasally with 25 µl of freshly harvested allantoic fluid (10th passage) containing 1000 $TCID_{50}$ of rNDV, rNDV-Ejev, rNDV-NS1jev, and a combination of 1000 $TCID_{50}$ each of rNDV-Ejev and rNDV-NS1jev. A group of 12 mice was mock-infected with uninfected/clear allantoic fluid. Another group of 12 mice was anesthetized by isoflurane and inoculated subcutaneously with the recommended dose of JEV vaccine strain SA14-14-2. The immunized mice were weighed and examined twice daily (every alternate day) until day 7, for clinical signs, change in activity, and behavior. Oral and cloacal swabs were collected on 1, 3, 5, and 7 days post-immunization (dpi) to measure the virus shedding. On 14, 21 and 35 dpi, blood was collected from three mice of each group by cardiac puncture, and sera samples were prepared for serology (Figure 4.1 A). NDV-specific and JEV-specific serum antibody responses were evaluated in collected sera

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samples using NDV-specific HI assays, JEV-specific ELISA, and virus neutralization tests following the standard protocol (Alexander and Chettle 1977, Gauger and Vincent 2014, Kumar, Barman et al. 2016, Kumar, Kumar et al. 2018).

4.3.2. Cytokine profiling

The collected serum samples of 14 dpi from all the six groups, as mentioned above, were used for cytokine profiling using the bead-based ELISA kit - Bio-Plex ProTM mouse cytokine group I panel 8-Plex (Bio-Rad, USA). The serum samples were screened in triplicates for 8 target cytokines, viz. IL1-β, IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN-γ, TNF-α following the manufacturer's protocol. Briefly, 6.5 micrometers (µm) magnetic beads were incubated with serum samples. The beads exhibit distinct fluorescence intensities and are coated with specific cytokine capturing antibodies. The cytokine captured beads were then incubated with biotinylated secondary antibody, followed by streptavidin-phycoerythrin conjugated reporters. Fluorescence intensities were detected using the Bio-Plex® MAGPIX™ System, and a standard curve analysis was performed using the inbuilt software. The limits of detection (LoD) were as follows: 0.6 picogram (pg)/ml for IL-2; 2.1 pg/ml for IL-4, 0.3 pg/ml for IL-5, 1 pg/ml for IL-10; 1.2 pg/ml for IFN-γ, 1.4 pg/ml for TNF-α. The individual LoD values were subtracted from the measured values of cytokine levels in the sera samples.

4.3.3. Statistical analysis

rNDV- and JEV- specific antibody responses, induction of rNDV- and JEV- neutralizing antibodies by rNDV vaccine constructs and cytokine levels were statistically analyzed from the sera samples of three mice sacrificed at individual time-points. The results were analyzed using Microsoft Excel. For multiple comparisons in cytokine profiling, one-way analysis of variance ANOVA (non-parametric or mixed) analysis in GraphPad Prism 8.0.2 was used. We have compared the mean of each group with the mean of every other group. For correction of

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multiple comparisons, we have used the Tukey hypothesis testing. The final *p*-value style was set to APA style (American Psychological Association) $*p < 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

4.4. Results

4.4.1. Immunization study

No visible clinical signs were observed in the mice immunized with the rNDVs (Figure 4.1 B). The nasal and cloacal swabs collected from the immunized mice on 1, 3, 5, and 7 dpi did not exhibit any shedding of rNDV, rNDV-Ejev or rNDV-NS1jev. All the immunized mice except the respective control groups showed seroconversion against NDV and JEV. The serological titers are expressed on a $\log_2$ scale for all data sets. All of the immunized groups except the mock and the JEV vaccine showed HI titer of more than 2 against NDV in sera collected on 14 dpi, and the titer progressively increased until 35 dpi (Figure 4.1 C). The HI titer against NDV was almost comparable in groups rNDV, rNDV-Ejev, and rNDV-NS1jev on 14, 21 and 35 dpi, except for the fact it was approximately 1-fold lower in rNDV-NS1jev group on 14 dpi (Figure 4.1 C). However, the progressive increase of HI titer against NDV from 14 to 35 dpi was less in the combination group (rNDV-Ejev + rNDV-NS1jev). A titer of 2.5 was observed on 14 dpi and a titer of 3 on 35 dpi.

JEV-specific serum antibody responses on 14, 21 and 35 dpi were determined by ELISA (Figure 4.1 D). After the JEV vaccine group, the highest antibody titers were observed in the rNDV-Ejev vaccinated group, while the lowest titer was recorded in the combination group (rNDV-Ejev + rNDV-NS1jev) (Figure 4.1 D).

The virus neutralization test assessed the ability of sera to neutralize NDV and JEV. All of the immunized groups except the mock and JEV vaccine group showed neutralizing antibody titers against NDV. A titer of 4 was observed for rNDV and rNDV-Ejev groups on 14 dpi (Figure 4.2 A). A higher titer of 5 and 6 was observed for rNDV and rNDV-Ejev groups

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on 21 and 35 dpi, respectively (Figure 4.2 A). The effective serum neutralization was highest in rNDV and rNDV-Ejev groups, followed by rNDV-NS1jev and combined (rNDV-Ejev + rNDV-NS1jev) groups.

Sera from the mock group and mice immunized with rNDV had low levels of JEV neutralizing antibodies, whereas sera from mice immunized with rNDV-Ejev or rNDV-NS1jev individually or together induced considerable levels of JEV neutralizing antibodies (Figure 4.2 B). Effective serum neutralization was the highest in the JEV vaccine group on 21 dpi. The rNDV-Ejev group closely followed it with a titer of 3, 4 on 21, 35 dpi, respectively. The JEV neutralization titers were comparable on 35 dpi in the JEV vaccine and rNDV-Ejev groups. JEV neutralization titers were lower in the rNDV-NS1jev group and the lowest in the combination group (Figure 4.2 B).

4.4.2. Cytokine profiling

The vaccination of rNDVs showed an upregulation of the Th1 mediated pro-inflammatory (IL-2, IFN-γ, TNF-α) and Th2 mediated anti-inflammatory (IL-4, IL-5, IL-10) cytokine responses in all the groups as compared to the mock-infected group (Figure 4.3). After 2 weeks, there was a significant difference in IL-2 levels between the experimental groups, $F(5, 12) = 98.20$, $p < 0.001$. Similarly, there were significant differences in IL-4, IL-5, IL-10, IFN-γ and TNF-α levels, with p-value summaries of $p = 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$ and $p = 0.004$, respectively, after multiple comparisons between experimental groups.

On multiple comparisons, the expression levels of IL-2, IL-10, and TNF-α were higher in all the rNDVs groups with respect to the mock-infected groups. However, the expression levels of IL-5 were not significant in the rNDVs groups with respect to the mock-infected groups, except for the E and NS1 combination group. The expression level of cytokines was

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represented as pg/ml (Figure 4.3). A detailed figure indicating individual comparisons between each of the experimental groups is shown in Supplementary Figure S1.

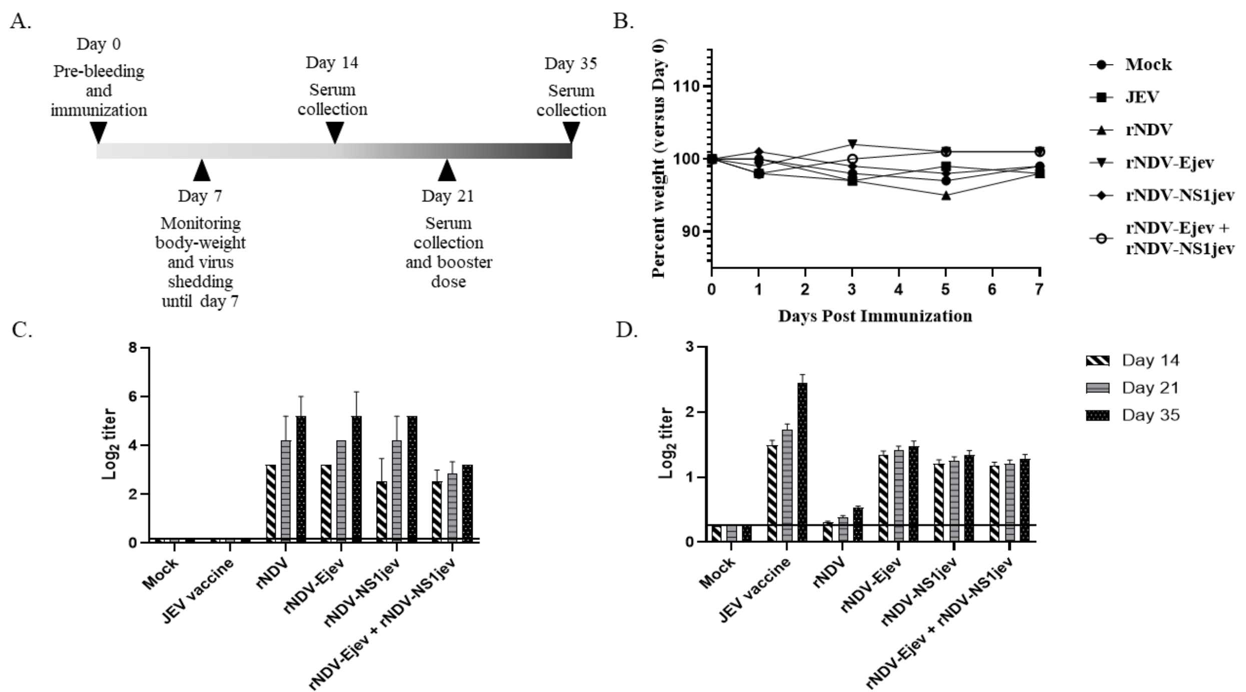

Figure 4.1. Schematic representation of the animal experiment timeline **(A)**. Bodyweight of mice was recorded on 1, 3, 5, 7 days post-immunization (dpi), and the weight loss was calculated as a percent of the weight on day 0. Data depicts the mean ± standard deviation from 12 mice per group **(B)**. Recombinant NDV (rNDV) and Japanese encephalitis virus (JEV) specific serum antibody responses in mice 14, 21 and 35 dpi in the indicated groups. Specific serum antibody responses were determined by hemagglutination inhibition (HI) assay against rNDV **(C)** and ELISA against JEV **(D)**. Titers were expressed as mean reciprocal $\log_2$ titers. Each bar represents the mean of three mice sacrificed at each time-point in all groups. Data represent the mean ± standard deviation of three independent experiments.

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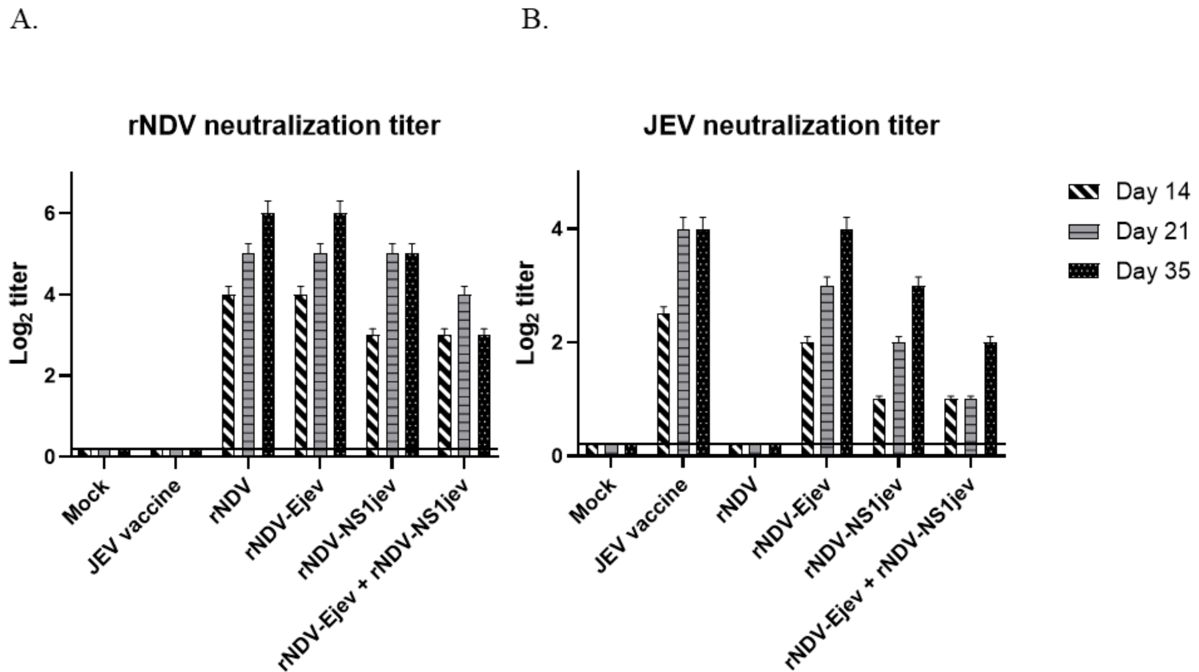

Figure 4.2. Evaluation of recombinant NDV (rNDV) and Japanese encephalitis virus (JEV) neutralizing serum antibody titers 14, 21- and 35-days post-immunization (dpi) in the indicated groups. Sera were analyzed for the ability to neutralize rNDV **(A)** and JEV **(B)**. The serum neutralizing antibody titers were expressed as mean reciprocal log_2 titers. Each bar represents the mean of three mice sacrificed at each time-point in all groups. Data represent the mean ± standard deviation of three independent experiments.

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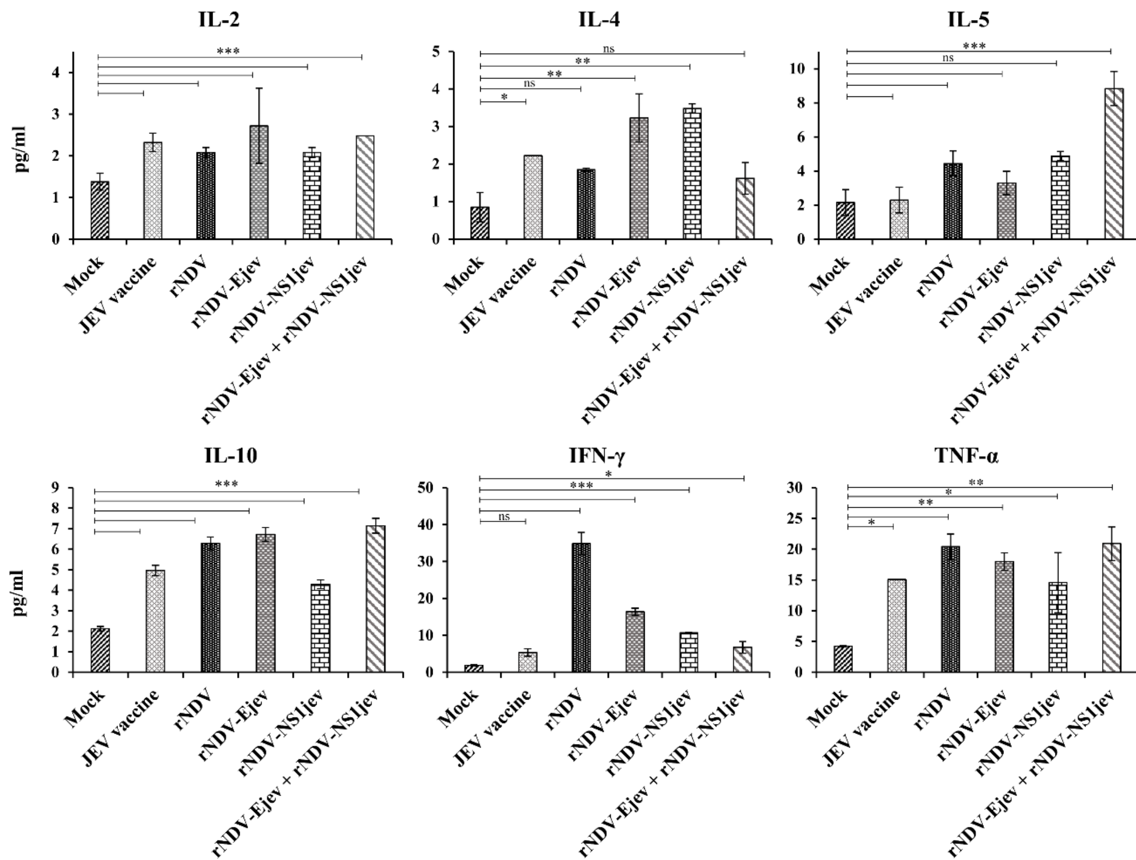

Figure 4.3. Cytokine levels quantitated by bead-based ELISA in the sera collected 14 days post-immunization (dpi) from the indicated groups. Values represent the mean of three mice sacrificed at 14 dpi, and data are expressed as picogram (pg)/ml ± standard deviation of the mean. The comparisons of the experimental groups with only the mock control group are shown in the figure. Multiple comparisons were made using the One-way ANOVA (and non-parametric or mixed) analysis using GraphPad Prism 8.0.2. There were significant differences in IL-2, IL-4, IL-5, IL-10, IFN-c and TNF-α levels, with p-value summaries of $p < 0.001$, $p = 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$ and $p = 0.004$ respectively after multiple comparisons between experimental groups.

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4.5. Discussion

NDV has been shown to replicate efficiently in mice. NDV, which belongs to APMV-1, is naturally attenuated in mice and shown to replicate in the trachea and nasal epithelium of mice. Although NDV infects mice and produces mild and transient clinical symptoms, it does not cause any apparent disease. It is quite fascinating that the seroconversion of mice post NDV vaccination makes it an ideal vector to deliver foreign proteins in mice (Khattar, Samal et al. 2013). Moreover, the ease of growing NDV in cell lines established for human vaccine development is an added advantage. Besides, NDV is well adapted to grow in embryonated chicken eggs, which facilitates its efficient bulk production (Kim and Samal 2016).

Both rNDV-Ejev and rNDV-NS1jev were used for intranasal immunizations in mice to evaluate the relative contributions of each of the two immunogenic proteins of JEV. Interestingly, the rNDVs did not show any shedding post-immunization, suggesting its inability to transmit horizontally and its safety as a vaccine vector. Our results show that both rNDV-Ejev and rNDV-NS1jev, when immunized individually or in combination, induced NDV-specific HI suggesting the efficient replication of rNDV in mice.

Also, we evaluated the immunogenicity of the combination of rNDV-Ejev and rNDV-NS1jev to address whether the two proteins behave in an additive or interdependent manner and have an added benefit over individual expressions. The replication of rNDV and rNDV-Ejev was almost similar, while, the rNDV-NS1jev and combined (rNDV-Ejev + rNDV-NS1jev) groups showed lower replication kinetics compared to rNDV and rNDV-Ejev groups in mice. NDV specific serum neutralizing antibodies were highest in rNDV and rNDV-Ejev groups and slightly reduced in rNDV-NS1jev and combined (rNDV-Ejev + rNDV-NS1jev) groups. The difference could be due to the lower replication of the virus, as reflected in the HI titer. An almost similar trend was observed in the case of JEV specific serum neutralizing

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antibodies. The highest JEV serum neutralizing titer was recorded in the JEV vaccine group followed by rNDV-Ejev, rNDV-NS1jev and combined (rNDV-Ejev + rNDV-NS1jev) group. It suggests that both rNDV-Ejev and rNDV-NS1jev are independently protective, and rNDV-Ejev alone has better protective efficacy than rNDV-NS1jev. However, when rNDV-NS1jev is combined with rNDV-Ejev, the protective effect gets compromised. This result corroborates with previous findings where it was shown that the combination of recombinant viral vaccine vectors expressing different immunogenic proteins reduces the immunogenic response (Kumar, Nayak et al. 2011).

JEV and rNDVs immunized groups exhibited independent cytokine expression profiles. Upregulation of both Th1 and Th2 mediated cytokine response was observed in mice immunized with rNDV-Ejev compared to the mice immunized only with the JEV vaccine strain. This could be due to the synergistic effect of the immunogenic E protein of JEV and the rNDV backbone proteins. The upregulation of Th1 and Th2 cytokines due to rNDV backbone could be an added advantage for its use in the vaccine formulation. Thus, we can hypothesize that in mice, the rNDV backbone acts as an effective adjuvant for a better cell-mediated immune response in comparison to the live attenuated JEV vaccine.

Our results of effective neutralization of JEV infectivity by antibodies produced by the intranasal inoculation of especially rNDV-Ejev suggested it to be a suitable live viral vaccine-vector for mice. rNDV-Ejev is the first NDV-vectored JEV vaccine candidate with the potential to induce neutralizing antibodies against JEV. Although confirmatory challenge experiments with virulent JEV strains would be required to estimate the efficacy of the proposed rNDV-vectored vaccines, we could not perform the challenge experiments owing to animal ethical limitations, and it remains for further studies. However, this can be considered as a marked step towards using rNDV-vectored vaccines against important flaviviruses.

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Chapter 5

In-silico approach to designing epitope-based peptides for viral detection systems against JEV

5.1. Abstract

Like JEV, other antigenically similar and co-circulating flaviviruses such as the WNV and Dengue virus are also associated with AES. The differential diagnosis of JEV with the existing diagnostic kits is a challenge because of the immunocrossreactivity between the envelope proteins of JEV, WNV, and Dengue virus. Using 100 sequences of the JEV E protein and 50 sequences of the JEV NS1 protein available in the NCBI GenBank database, our *in-silico* analysis with a host of web-based bioinformatics servers identified four epitope-based peptides on the JEV E and NS1 proteins. These peptides are conserved, surface-exposed, solvent-accessible, and are predicted to have good B- and T-cell epitope binding efficiency. These peptides are indicators of the eventual development of a peptide-based ELISA against JEV with the least immunocrossreactivity.

5.2. Introduction

Diagnostic kits for JE infection are commercially available. Laboratory diagnosis of JE is accomplished by the JEV-specific IgM antibody-capture ELISA (MAC-ELISA) using cerebrospinal fluid (CSF) and serum samples. However, Johnson et al. reported that the specificity of JEV MAC-ELISA is low due to cross-reactivity with other antigenically similar co-circulating flaviviruses (Johnson, Goodman et al. 2016). Several reports have confirmed that the co-infection of JEV, WNV and Dengue is associated with AES (Katyal, Bhardwaj et al. 2000, Pierson and Diamond 2008, Khan, Dutta et al. 2011). The differential diagnosis of JEV is a challenge because there is an extensive similarity between the E proteins of WNV and

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JEV (Gangwar, Shil et al. 2012). Also, NS1 is highly conserved amongst flaviviruses, which results in immunocrossreactivity (Rastogi, Sharma et al. 2016).

We aimed to eliminate the immunocrossreactivity that interferes with the specific diagnosis of JEV, which can be achieved by using only short, immunodominant and unique peptides of the JEV viral proteins in detection systems like ELISA. The peptides should be recognizable by both B-cell and T-cell receptors and should exhibit good binding affinities with the receptors for a valid diagnosis. Thus, the peptides should co-present both B-cell and T-cell epitopes (Roehrig, Johnson et al. 1992, Singh, Rothman et al. 2010). In this study, we have identified four potential B-cell and T-cell epitope-based peptides in JEV E and NS1 proteins, which can be synthesized for designing JEV specific diagnostics, as well as JEV peptide vaccine formulations.

5.3. Materials and methods

5.3.1. Identification of peptides

The sequences of JEV E (n=100) and JEV NS1 (n=50) proteins were retrieved from the NCBI GenBank. All the sequences were compared among themselves and predicted for their amino acid sequence variability using the Protein Variability server (PVS) following the Wu-Kabat variability co-efficient (http://imed.med.ucm.es/PVS/). PVS is a web server for protein sequence variability analysis designed to facilitate conserved epitope discovery (Garcia-Boronat, Diez-Rivero et al. 2008). The running average variability values for a stretch of 12 amino acids starting from position 1 of the proteins was computed, which gave a smoothed-out profile of 12-mer protein stretch variability for both E and NS1. Hereafter, the average solvent accessibility (ASA) profiles of each protein were predicted using the SABLE server (https://sable.cchmc.org/). Like the variability profile, the ASA profile was also smoothed out for both E and NS1 by taking a running average over the ASA values for 12 amino acids at a

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time. From the two above mentioned profiles, the amino acid stretches that showed the least protein variability with the highest average ASA values were shortlisted.

5.3.2. Prediction of T- and B-cell epitopes

The T-cell and B-cell epitopes of E and NS1 proteins were predicted using two web-based tools, viz., the IEDB (Immune Epitope Database and Analysis Resource) (https://www.iedb.org/) and ABCpred servers (http://crdd.osdd.net/raghava/abcpred/) (Saha and Raghava 2006, Vita, Mahajan et al. 2019). These servers are known to predict the major histocompatibility complex, MHC class I and MHC class II T-cell epitope binding, linear and discontinuous B-cell epitopes using antigen structure, and B-cell binding affinities with accuracies ranging from 60-90% (Zhang, Wang et al. 2008, Dey, Nandy et al. 2017). The IEDB server determined the MHC class I and MHC class II binding affinities for Human Leukocyte Antigens (HLA) of the JEV E and NS1 amino acid stretches, which were shortlisted by protein variability and ASA profiles. Seven-allele HLA reference set was selected to achieve broad coverage of the population. Following the IEDB-recommended procedure, the percentile ranks for the amino acid stretches were obtained. Binding affinities of the linear and conformational B-cell epitopes on JEV E and NS1 proteins were estimated by the IEDB Ellipro tool (http://tools.iedb.org/ellipro/). The protein structures of JEV E and NS1 available in Protein Data Bank (https://www.rcsb.org/) were used for the study (Berman, Westbrook et al. 2000, Burley, Berman et al. 2018). For further verification of B-cell epitope prediction, the ABCpred server was used to validate the binding affinities for B-cell epitopes.

The antigenicity for the shortlisted peptides was predicted by Kolaskar and Tongaonkar method as derivable from the IEDB platform (Kolaskar and Tongaonkar 1990). Sequence alignments of selected JEV E and NS1 peptides with E and NS1 protein sequences

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of Dengue and WNV virus reference strains were performed using Mega 6 (Tamura, Stecher et al. 2013).

5.3.3. Docking studies

The availability of crystal structures of the JEV E protein, PDB ID: 5MV1, and the C-terminal β-ladder domain of JEV NS1 protein (NS1-C), PDB ID: 5O19, helped us to locate the shortlisted epitope-based peptides on the proteins. It also allowed us to check whether the peptides are surface exposed or not, using the PyMOL molecular visualization system (https://pymol.org/2/) (Poonsiri, Wright et al. 2018, Liu, Zhao et al. 2019).

The peptides E_{156} and $NS1_{207}$ were selected for docking with HLA-DRB1*07:01 and HLA-DRB3*02:02, respectively, their best performing HLA proteins predicted from IEDB. The 3D structures of the peptides and the HLAs were predicted by I-TASSER (Zhang 2007, Roy, Kucukural et al. 2010). The predicted structures were analyzed in PyMOL software (DeLano 2002). Receptor and ligand were prepared for docking using AutoDockTools by adding polar hydrogen atoms to receptor proteins and merging its non-polar hydrogen atoms. All active bonds of E_{156} and amide bonds of $NS1_{207}$ were set to be rotatable. These modified molecules were saved in PDBQT format (Morris, Huey et al. 2009). Grid box was generated for the receptor protein, covering the whole of protein for blind docking. The distance between two connecting grid points was taken as 1 Angstrom (Å) for both dockings. For E_{156}: HLA-DRB1*07:01 docking, the center of the grid was kept at (x, y, z) = (71.935, 72.097, 69.895) and the number of grid points in x, y, and z-axes was set to 76 × 64 × 62. For $NS1_{207}$: HLA-DRB3*02:02 docking, the center of the grid was kept at (x, y, z) = (72.121, 72.071, 72.517) and the number of grid points in x, y, and z-axes was set to 46 × 82 × 60. Finally, the docking of peptides with their corresponding HLA proteins was done using AutoDock4 via Genetic

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Algorithm (GA) and Lamarckian GA (LGA). The resulting docked PDBQT files were visualized in PyMOL software.

5.4. Results

5.4.1. Identification of peptides

The details (GenBank accession number, source and genotype) of the downloaded full coding sequences (CDS) of E and NS1 proteins of JEV are enlisted in the Supplementary Tables S3 A and S3 B. The CDS sequences were downloaded from all the five genotypes of JEV and from geographically diverse regions. The amino acid stretches with the least protein variability, and the highest ASA values are compiled in Supplementary Tables S4 A and S4 B. Out of those amino acid stretches, the shortlisted peptides are highlighted by straight yellow horizontal lines in Figures 5.1 A and 5.1 B and enlisted in Table 5.1.

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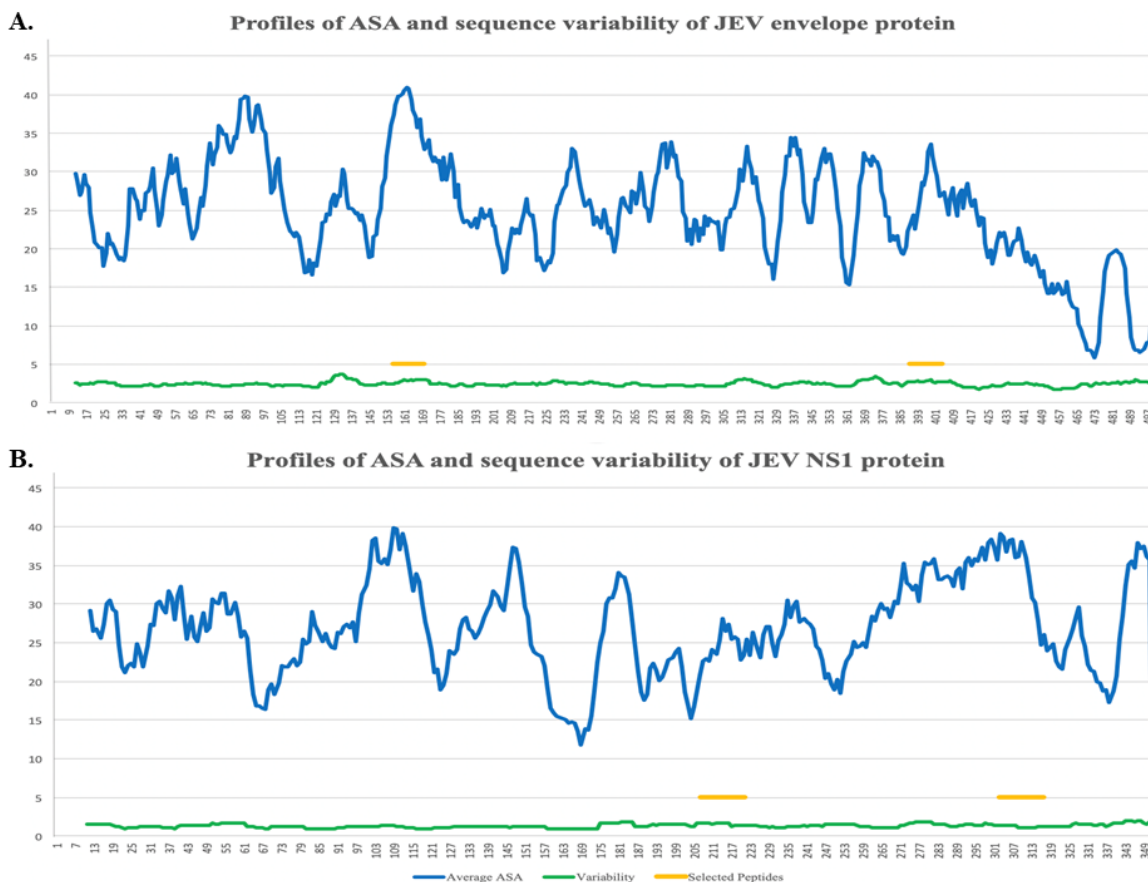

Figure 5.1. Profiles of average solvent accessibility (ASA) (blue) in percentage and amino acid sequence variability (green) in numbers of the 100 Japanese encephalitis virus (JEV) E **(A)** and 50 JEV NS1 **(B)** protein fragments plotted against amino acid numbers. The short horizontal yellow lines are the shortlisted amino acid stretches that are the most conserved and have the highest solvent accessibility.

Region	Start	End	Peptide	Name
E	156	170	SAQVGASQAAKFTIT	E_{156}
E	390	404	KQINHHWHKAGSTLG	E_{390}
NS1	207	221	NDTWKLERAVFGEVK	$NS1_{207}$
NS1	303	317	DSGKLITDWCCRSCS	$NS1_{303}$

Table 5.1. Shortlisted peptides of Japanese encephalitis virus (JEV) E and NS1 proteins with low protein variability (conserved) and high ASA.

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5.4.2. Prediction of T- and B-cell epitopes

The HLA-DRB allele binding and MHC Class II T-cell epitope prediction for JEV E and NS1 are shown in Tables 5.2 A and 5.2 B, respectively, where a low percentile rank means high binding affinity. The peptides E_{156}, E_{390}, $NS1_{207}$ and $NS1_{303}$ exhibited the highest binding affinities with the alleles HLA-DRB1*07:01, HLA-DRB1*07:01, HLA-DRB3*02:02 and HLA-DRB1*03:01 respectively. The HLA-DP/DQ allele binding and MHC Class I T-cell epitope prediction gave poor results and hence, are not shown here.

The predicted B-cell linear and discontinuous epitope potential of JEV E and NS1 by IEDB Ellipro analysis are shown in Tables 5.3 A, 5.3 B, 5.4 A and 5.4 B. The parts of the epitopes that match with our shortlisted conserved segments with high ASA are highlighted in bold. Epitopes with high scores have a high potential for antibody binding. For JEV E, IEDB predicted 13 B-cell linear epitopes, out of which the epitope with the best score contained regions from our shortlisted peptide E_{390} (Table 5.3 A). For JEV NS1, IEDB predicted 6 B-cell linear epitopes, out of which the first 2 epitopes with the best scores contained regions from our shortlisted peptides $NS1_{207}$ and $NS1_{303}$ (Table 5.3 B).

In the case of B-cell discontinuous epitopes, 5 regions overlapped with our shortlisted peptides for JEV E and 2 regions overlapped with our shortlisted peptides for JEV NS1 with scores ranging from 0.67 to 0.74 (Table 5.4 A and 5.4 B).

ABCpred server predictions further validated the excellent binding affinity of the shortlisted peptides. Here again, the parts of the epitopes that match with our shortlisted conserved segments with high ASA are highlighted in bold. In ABCpred, a high score (maximum 1) indicates good binding affinity, with a default threshold of 0.51. Our shortlisted peptides showed good scores ranging from 0.76 to 0.93, with the best score of 0.84 for E_{390} and 0.93 for $NS1_{303}$ (Table 5.5).

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The average antigenicity of the selected epitope-based peptides was found to be higher than 1, as shown in Table 5.6. The sequence alignments of the selected JEV E and NS1 peptides with the E and NS1 protein sequences of Dengue virus and WNV reference strains showed that the peptides were unique to JEV.

A.

Allele	Start	End	Length	Method Used	Peptide	Percentile Rank
HLA-DRB1*07:01	156	170	15	Consensus (comb.lib./smm/nn)	SAQVGASQAAKFTIT	20.00
HLA-DRB5*01:01	156	170	15	Consensus (smm/nn/sturniolo)	SAQVGASQAAKFTIT	29.00
HLA-DRB1*03:01	156	170	15	Consensus (smm/nn/sturniolo)	SAQVGASQAAKFTIT	34.00
HLA-DRB3*02:02	156	170	15	NetMHCIIpan	SAQVGASQAAKFTIT	37.00
HLA-DRB3*01:01	156	170	15	Consensus (comb.lib./smm/nn)	SAQVGASQAAKFTIT	40.00
HLA-DRB1*15:01	156	170	15	Consensus (smm/nn/sturniolo)	SAQVGASQAAKFTIT	59.00
HLA-DRB4*01:01	156	170	15	Consensus (comb.lib./smm/nn)	SAQVGASQAAKFTIT	69.00
Allele	**Start**	**End**	**Length**	**Method Used**	**Peptide**	**Percentile Rank**
HLA-DRB1*07:01	390	404	15	Consensus (comb.lib./smm/nn)	KQINHHWHKAGSTLG	17.00
HLA-DRB5*01:01	390	404	15	Consensus (smm/nn/sturniolo)	KQINHHWHKAGSTLG	40.00
HLA-DRB3*02:02	390	404	15	NetMHCIIpan	KQINHHWHKAGSTLG	49.00
HLA-DRB1*15:01	390	404	15	Consensus (smm/nn/sturniolo)	KQINHHWHKAGSTLG	65.00
HLA-DRB3*01:01	390	404	15	Consensus (comb.lib./smm/nn)	KQINHHWHKAGSTLG	75.00
HLA-DRB4*01:01	390	404	15	Consensus (comb.lib./smm/nn)	KQINHHWHKAGSTLG	78.00
HLA-DRB1*03:01	390	404	15	Consensus (smm/nn/sturniolo)	KQINHHWHKAGSTLG	89.00

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B.

Allele	Start	End	Length	Method Used	Peptide	Percentile Rank
HLA-DRB3*02:02	207	221	15	NetMHCIIpan	NDTWKLERAVFGEVK	18.00
HLA-DRB5*01:01	207	221	15	Consensus (smm/nn/sturniolo)	NDTWKLERAVFGEVK	21.00
HLA-DRB1*07:01	207	221	15	Consensus (comb.lib./smm/nn)	NDTWKLERAVFGEVK	35.00
HLA-DRB3*01:01	207	221	15	Consensus (comb.lib./smm/nn)	NDTWKLERAVFGEVK	37.00
HLA-DRB1*03:01	207	221	15	Consensus (smm/nn/sturniolo)	NDTWKLERAVFGEVK	60.00
HLA-DRB1*15:01	207	221	15	Consensus (smm/nn/sturniolo)	NDTWKLERAVFGEVK	60.00
HLA-DRB4*01:01	207	221	15	Consensus (comb.lib./smm/nn)	NDTWKLERAVFGEVK	61.00

Allele	Start	End	Length	Method Used	Peptide	Percentile Rank
HLA-DRB1*03:01	303	317	15	Consensus (smm/nn/sturniolo)	DSGKLITDWCCRSCS	9.50
HLA-DRB3*02:02	303	317	15	NetMHCIIpan	DSGKLITDWCCRSCS	59.00
HLA-DRB1*15:01	303	317	15	Consensus (smm/nn/sturniolo)	DSGKLITDWCCRSCS	66.00
HLA-DRB3*01:01	303	317	15	Consensus (comb.lib./smm/nn)	DSGKLITDWCCRSCS	71.00
HLA-DRB4*01:01	303	317	15	Consensus (comb.lib./smm/nn)	DSGKLITDWCCRSCS	86.00
HLA-DRB5*01:01	303	317	15	Consensus (smm/nn/sturniolo)	DSGKLITDWCCRSCS	89.00
HLA-DRB1*07:01	303	317	15	Consensus (comb.lib./smm/nn)	DSGKLITDWCCRSCS	90.00

Table 5.2. IEDB MHC-II binding predictions of JEV E **(A)** and NS1 **(B)**. Binding predictions are made for human HLA-DRB. Low percentile rank means high binding affinity.

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A.

No.	Chain	Start	End	Peptide	Number of residues	Score
1	A	375	401	EPPFGDSYIVVGRGD**KQINHHWHKAGS**	27	0.808
2	A	340	353	VSVASLNDMTPVGR	14	0.783
3	A	64	118	SVTDISTVARCPTTGEAHNEKRADSSYVCK QGFTDRGWGNGCGLFGKGSIDTCAK	55	0.78
4	A	361	369	VATSSANSK	9	0.756
5	A	13	22	EGASGATWVD	10	0.742
6	A	241	257	EFEGAHATKQSVVALGS	17	0.718
7	A	325	334	ELSYSGSDGS	10	0.703
8	A	298	317	GTTYGMCTEKFSFAKNPADT	20	0.627
9	A	170	194	**T**PNAPSITLKLGDYGEVTLDCEPRS	25	0.621
10	A	286	296	KCRLKMDKLAL	11	0.6
11	A	131	134	QPEN	4	0.555
12	A	35	38	ANDK	4	0.526
13	A	228	232	PSSTA	5	0.519

B.

No.	Chain	Start	End	Peptide	Number of residues	Score
1	A	205	210	RY**NDTW**	6	0.809
2	A	300	310	TTT**DSGKLITD**	11	0.72
3	A	177	199	DECDGAIIGTAVKGHVAVHSDLS	23	0.709
4	A	338	352	VMHDETTLVRSQVDA	15	0.672
5	A	280	294	CPGTKVTITEDCSKR	15	0.637
6	A	218	238	GEVKSCTWPETHTLWGDDVEE	21	0.623

Table 5.3. IEDB Ellipro-predicted linear epitope(s) for JEV E **(A)** and NS1 **(B)**. The parts of the epitopes that match with our shortlisted conserved peptides are highlighted in bold.

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A.

No.	Residues	Number of residues	Score
1	**A:S64, A:V65,** A:T66, A:D67, A:I68, A:S69, A:T70, A:V71, A:A72, A:R73, A:C74, A:P75, A:T76, A:T77, A:G78, A:E79, A:A80, A:H81, A:N82, A:E83, A:K84, A:R85, A:A86, A:D87, A:S88, A:S89, A:Y90, A:C92, A:K93, A:Q94, A:G95, A:F96, A:T97, A:D98, A:R99, A:G100, A:W101, A:G102, A:N103, A:G104, A:C105, A:G106, A:L107, A:F108, A:G109, A:K110, A:G111, A:S112, A:I113, A:D114, A:T115, A:C116, A:A117, A:K118, A:S227, A:P228, A:S229, A:S230, A:T231, A:A232, A:L239, A:E241, A:F242, A:E243, A:G244, A:A245, A:H246, A:A247, A:T248, A:K249, A:Q250, A:S251, A:V252, A:V253, A:A254, A:L255, A:G256, A:S257	78	0.743
2	A:E13, A:G14, A:A15, A:S16, A:G17, A:A18, A:T19, A:W20, A:V21, A:D22, A:A35, A:N36, A:D37, A:K38, A:P39, **A:S51, A:Q52,** A:Q131, A:P132, A:E133, A:N134, A:T148, A:S149, A:E150, A:N151, **A:S156, A:A157, A:V159, A:G160, A:A161, A:S162, A:Q163, A:T170,** A:P171, A:N172, A:A173, A:P174, A:S175, A:I176, A:T177, A:L178, A:K179, A:L180, A:G181, A:D182, A:G184, A:E185, A:V186, A:T187, A:D189, A:E191, A:P192, A:R193, A:S194, **A:K286**, A:R288, A:K290, A:M291, A:D292, A:K293, A:L294, A:A295, A:L296, A:K297, A:G298, A:T299, A:T300, A:Y301, A:G302, A:M303, A:C304, A:T305, A:E306, A:K307, A:F308, A:S309, A:F310, A:A311, A:K312, A:N313, A:P314, A:A315, A:D316, A:T317, A:G318, A:G320, A:E325, A:L326, A:S327, A:Y328, A:S329, A:G330, A:S331, A:D332, A:G333, A:S334, A:I337, A:P338, A:V340, A:S341, A:V342, A:A343, A:S344, A:L345, A:N346, A:D347, A:M348, A:T349, A:P350, A:V351, A:G352, A:R353, A:V361, A:A362, A:T363, A:S364, A:S365, A:A366, A:N367, A:S368, A:K369, A:E375, A:P376, A:P377, A:F378, A:G379, A:D380, A:S381, A:Y382, A:I383, A:V384, A:V385, A:G386, A:R387, A:G388, A:D389, **A:K390, A:Q391, A:I392, A:N393, A:H394, A:H395, A:W396, A:H397, A:K398, A:A399, A:G400, A:S401**	148	0.676

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B.

No.	Residues	Number of residues	Score
1	A:T286, A:T288, A:E289, A:D290, A:C291, A:S292, A:K293, A:R314	8	0.791
2	A:D177, A:E178, A:C179, A:D180, A:G181, A:A182, A:I183, A:I184, A:G185, A:T186, A:A187, A:V188, A:K189, A:G190, A:H191, A:V194, A:S196, A:D197, A:L198, A:S199, A:Y206, **A:N207, A:D208, A:T209, A:W210, A:G218, A:E219, A:K221,** A:S222, A:C223, A:T224, A:W225, A:P226, A:E227, A:T228, A:H229, A:L231, A:W232, A:G233, A:D234, A:D235, A:V236, A:K251, A:S252, A:K253, A:H254, A:R256	47	0.68
3	A:C280, A:P281, A:G282, A:T283, A:P296, A:V298, A:R299, A:T301, A:T302, **A:D303, A:S304, A:G305, A:K306, A:L307, A:I308, A:T309,** A:M339, A:H340, A:D341, A:E342, A:T343, A:T344, A:L345, A:V346, A:R347, A:S348	26	0.675
4	A:K284, A:D310, A:V338	3	0.661
5	A:D269, A:E270, A:N271, A:G272, A:E326, A:V350	6	0.557

Table 5.4. IEDB Ellipro-predicted discontinuous epitope(s) for JEV E **(A)** and NS1 **(B)**. The parts of the epitopes that match with our shortlisted conserved peptides are highlighted in bold.

Protein	Rank	Sequence	Amino acid start position	Score
JEV E	10	GD**KQINHHWHKAGSTL**	388	0.84
JEV E	17	**SAQVGASQAAKFTIT**P	156	0.76
JEV NS1	1	**GKLITDWCCRSCS**LPP	305	0.93
JEV NS1	12	**TWKLERAVFGEVK**SCT	209	0.77

Table 5.5. ABCpred determination of B-cell binding affinities of JEV E and NS1. The parts of the epitopes that match with our shortlisted conserved segments are highlighted in bold. A high score (maximum 1) indicates a good binding affinity.

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Protein	Peptide position	Sequence	Average antigenicity
JEV E	156-170	SAQVGASQAAKFTIT	1.039
JEV E	390-404	KQINHHWHKAGSTLG	1.001
JEV NS1	207-221	NDTWKLERAVFGEVK	1.015
JEV NS1	303-317	DSGKLITDWCCRSCS	1.063

Table 5.6. The antigenicity of the peptides predicted by Kolaskar and Tongaonkar method. The average antigenicity of the selected epitopes was found to be ≥ 1 and hence considered as putative immunodominant regions.

5.4.3. Docking studies

Our study on the placement of the selected epitope-based peptides on the E and NS1 proteins shows that all the four peptides lie on the surface of the proteins (Figures 5.2 and 5.3). The 3D structures of the peptides E_{156}, E_{390}, $NS1_{207}$ and $NS1_{303}$ were obtained by I-TASSER (Figure 5.4). AutoDock generated 10 dock configurations for the peptides E_{156} and $NS1_{207}$ with their corresponding best-binding HLA proteins HLA-DRB1*07:01 and HLA-DRB3*02:02, respectively. The most favored ones with the best binding scores are selected and shown in Figure 5.5 and their properties are enlisted in Table 5.7. This further confirms that the peptides can bind to the HLA molecules and can be presented to the Tc cell receptor for recognition.

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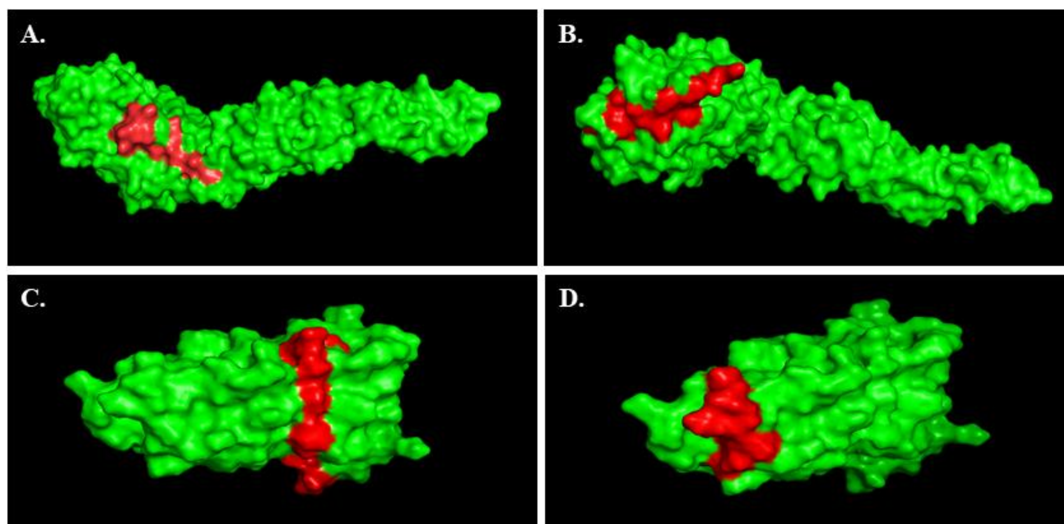

Figure 5.2. Display in space fill rendering of Japanese encephalitis virus (JEV) E (5MV1) and NS1 (5O19) by PyMOL. The final four identified peptides, as given in Table 5.8., are highlighted in red. Peptides highlighted are in terms of peptide stretch position numbers, E156 **(A)**, E390 **(B)**, NS1207 **(C)**, NS1303 **(D)**.

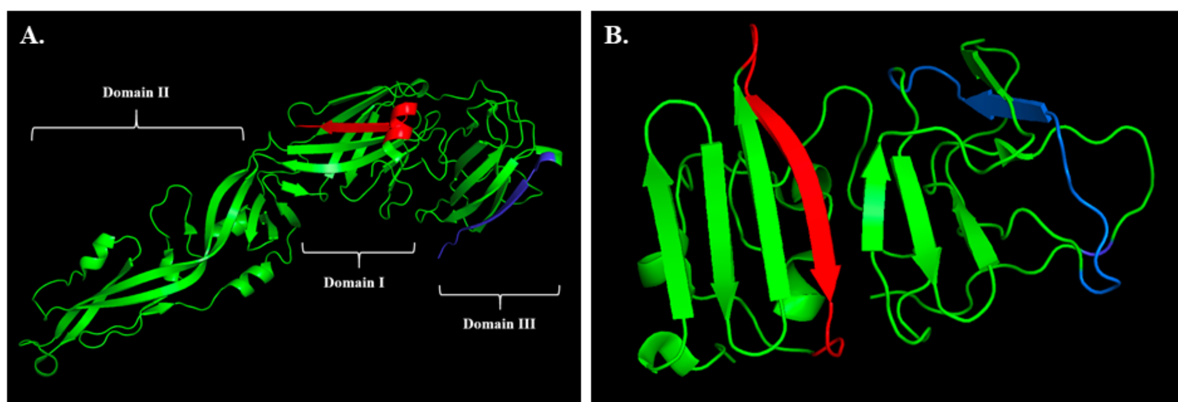

Figure 5.3. Display in space fill rendering of Japanese encephalitis virus (JEV) E (5MV1) and NS1 (5O19) by PyMOL. The final four identified peptides, as given in Table 5.8., are highlighted in red and blue. Peptide E156 (red) occurs in domain I, and E390 (blue) occurs in domain III of the E protein **(A)**. Peptides NS1207 (red) and NS1303 (blue) both occur in C-terminal β-ladder domain of NS1 protein **(B)**.

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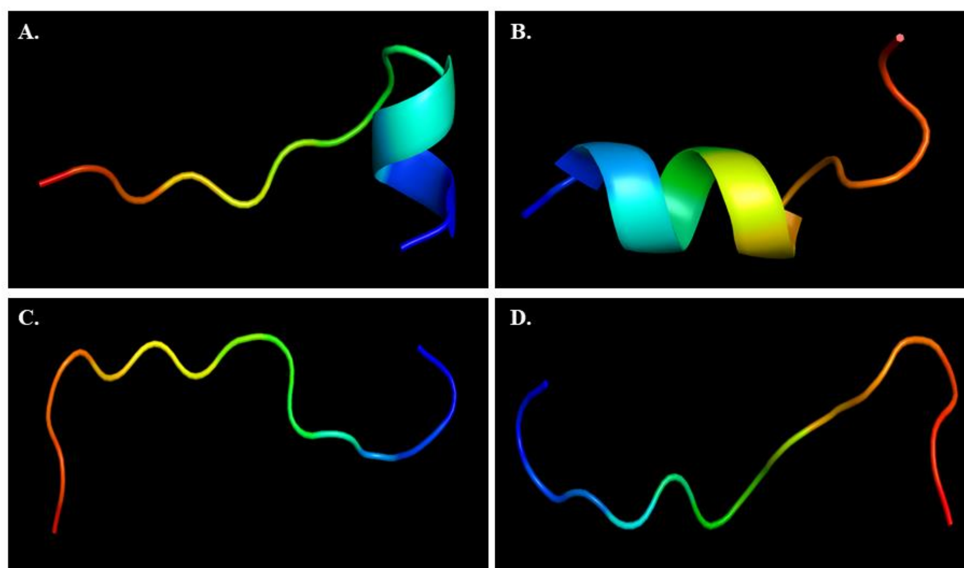

Figure 5.4. Three-dimensional structures of the peptides E_{156} **(A)**, E_{390} **(B)**, $NS1_{207}$ **(C)** and $NS1_{303}$ **(D)** obtained by I-TASSER.

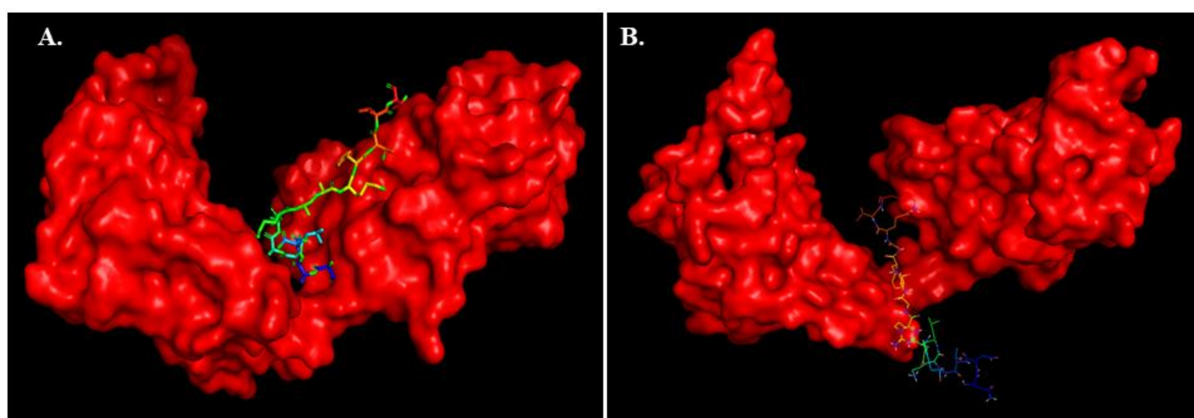

Figure 5.5. Best dock configurations for E156: HLA-DRB1*07:01 **(A)** and NS1207: HLA-DRB3*02:02 **(B)** generated by AutoDock.

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Properties	**E_{156}: HLA-DRB1*07:01**	**$NS1_{207}$: HLA-DRB3*02:02**
Binding energy	3.56	9.5
Ligand efficiency	0.03	0.08
Intermol energy	-6.02	-2.61
Desolv energy	-5.07	-2.49
Electrostatic energy	-0.94	-0.12
Total internal energy	-3.32	-5.45
Torsional energy	12.9	17.56

Table 5.7. Properties of the most-favored dock configuration for E_{156}: HLA-DRB1*07:01 and $NS1_{207}$: HLA-DRB3*02:02. The unit of energy is kcal/mol.

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Protein	Peptide name	Start position	End position	Peptide sequence	T-cell epitope percentile rank[a] HLA-DRB Consensus best		B-cell epitope			Average antigenicity
					Consensus best	Average[b]	Linear	Discontinuous	Binding affinity[c]	
JEV E	E_{156}	156	170	SAQVGASQAAKFTIT	20.00	41.14	No	Yes	0.76	1.039
JEV E	E_{390}	390	404	KQINHHWHKAGSTLG	17.00	59.00	Yes	Yes	0.84	1.001
JEV NS1	$NS1_{207}$	207	221	NDTWKLERAVFGEVK	21.00	41.71	Yes	Yes	0.77	1.015
JEV NS1	$NS1_{303}$	303	317	DSGKLITDWCCRSCS	9.50	67.21	Yes	Yes	0.93	1.063

[a] The percentile ranks are as given in Tables 2A and 2B.

[b] The averages are calculated from the ranks of all 7-allele HLA-DRB reference set values for each peptide.

Yes and No imply that the identified peptides form a part of the predicted linear and discontinuous epitopes or not.

[c] B-cell binding affinity was derived from the ABCpred server, as shown in Table 5.5.

Table 5.8. Summary of the 4 identified peptides on the E and NS1 proteins of JEV.

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5.5. Discussion

The present study aimed to design epitope-based peptides capable of explicitly detecting JEV infections, without cross-reacting with other co-circulating flaviviruses. To achieve this, we designed peptides based on several web-based bioinformatics servers and numerical characterization techniques. The amino acid sequences of JEV E and NS1 proteins chosen for the analysis spanned several sources worldwide, and all the five reported genotypes. At first, we shortlisted the conserved amino acid stretches in full E and NS1 proteins of JEV strains to avoid any inadvertent mutation, followed by the determination of their surface exposure and solvent accessibility. Our result suggests that the epitope-based peptides E_{156} is present in the β-barrel of DI; E_{390} is present in the globular domain DIII of E (Figure 5.3 A); $NS1_{207}$ and $NS1_{303}$ are located in the β-ladder domain of NS1 (Figure 5.3 B).

In order to be an effective immunogen, a peptide should induce both the B-cell mediated protective antibody response, and T-cell mediated cellular immune responses (Milich 1989). Therefore, in the present *in-silico* study, we selected four peptides that show good B-cell and T-cell binding affinities. The epitope-based peptides selected by us, mostly match with the multiple-epitope fragment of the JEV E protein designed by Wei et al., for generating protective efficacy against JEV infection in BALB/c mice (Wei, Huang et al. 2010). These peptides are indicators only to the eventual development of peptide-based ELISA against JEV by synthesis and purification of the peptides, confirmation of their immunogenicity, coating of ELISA plates and testing with several serum samples. These essential steps that are yet to be undertaken and remain for future studies.

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Chapter 6
Future Prospects

We have characterized the JEV live attenuated vaccine strain SA14-14-2 in BHK-21 cells and observed an enhanced replication following its serial passage in the cells. SA14-14-2 can be adapted and characterized in other permissive cell lines, e.g., Vero, Neuro2a, Microglial, C6/36 and CHO cells. With the help of docking and simulation studies, we can try to understand how the cell-culture-adapted SA14-14-2 will interact with the different cell-surface receptors and attachment factors of different cell lines. This will lead to the identification of the critical amino acid residues in the JEV polyprotein, which are more vulnerable to reversion and thus, design more stable vaccines. Also, we can decipher targets for the development of antivirals against JEV.

Using reverse genetics, we developed rNDV-based immunogens against JEV. Confirmatory challenge experiments in different animal models with virulent field strains of JEV remains for future studies, which will help to estimate the efficacy of the proposed rNDV-vectored vaccines in mice as well as pigs. NDV infects through the oculonasal route, which is an easier route of vaccine administration and can be economically produced on a large scale in embryonated chicken eggs. So, rNDV-based vaccines against JEV can be an ideal candidate for immunizing large and difficult to handle species like pigs. Aerosol vaccine delivery can be used for rNDV-based vaccines against JEV.

It is known that the co-synthesis of pre-membrane protein prM helps in the proper folding, membrane association, and assembly of the *Flavivirus* E protein (Konishi and Mason 1993). So, in future studies, we can express fusion/chimeric proteins, e.g., prM/E, or a combination of E with other non-structural proteins in the rNDV vector and compare the immunogenicity of different vaccine candidates. Also, the addition of cytokines in the rNDV

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vaccine construct has been shown to enhance the immunogenicity of rNDV vaccine candidates (Pan, He et al. 2016). rNDVs can be designed that express human/mice/pig IL-2 or IFN-γ along with the immunogenic proteins of JEV.

In addition to the cytokine profiling of rNDV-based immunogens against JEV by ELISA, the Th1 and Th2 activation by the immunogens can be further analyzed by flow cytometry and cell sorting. The safest and the most effective lead candidate can then be chosen for further downstream processing, clinical development and final production.

In the last chapter, we have proposed JEV E and NS1 epitope-based peptides. These peptides can be synthesized and checked for their immunogenicity by the production of monoclonal antibodies against them in mice models. We can develop peptide-based diagnostic kits for JEV and evaluate their cross-reactivity by using an array of JEV, WNV and Dengue serum samples. We can also develop a peptide-based vaccine against JEV by using the reverse vaccinology approach.

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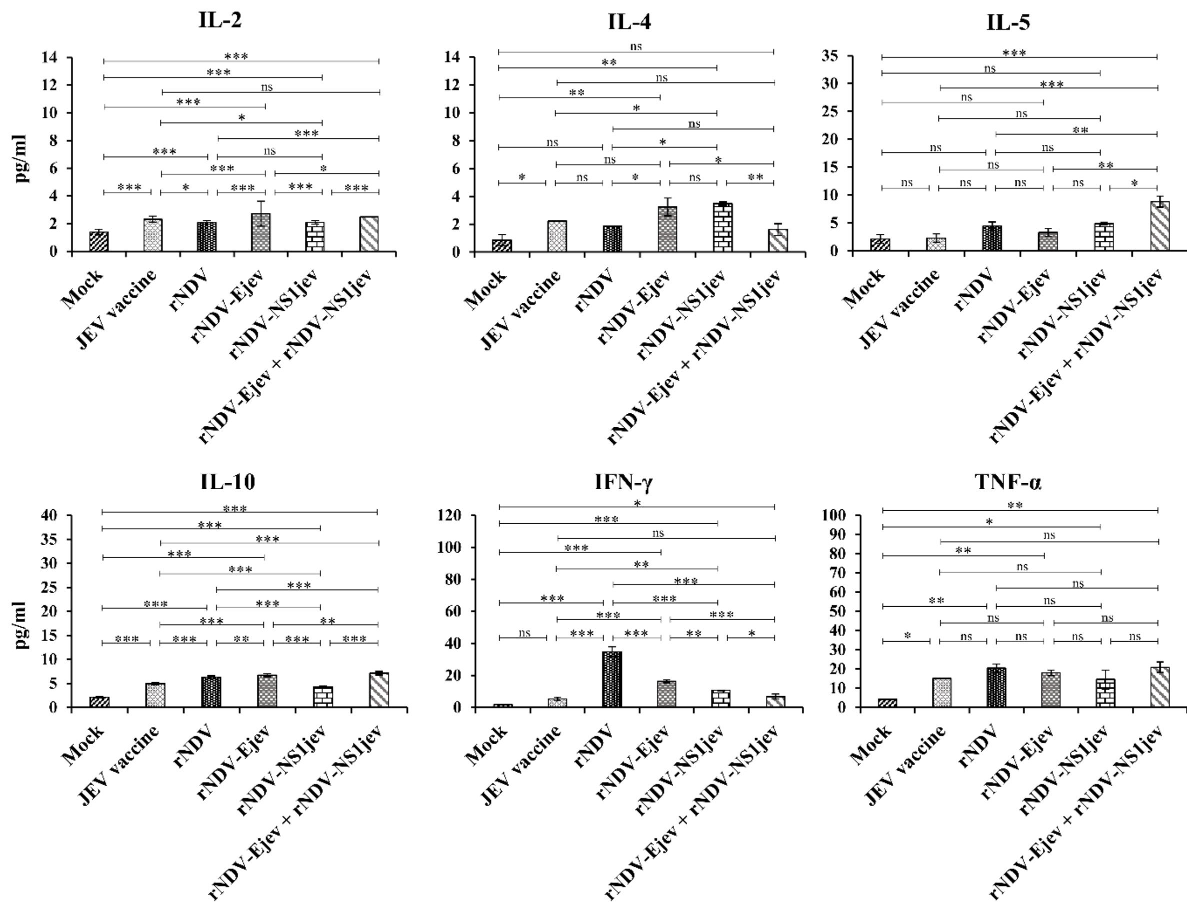
Supplementary Figure



Supplementary Figure S1. Detailed cytokine analysis by bead-based ELISA in the sera collected 14 days post-immunization (dpi) from the indicated groups. Values represent the mean of three mice sacrificed at 14 dpi, and data are expressed as (picogram) pg/ml ± standard deviation of the mean. The comparisons between all the 22 experimental groups are shown in the figure. The final p-value style was set to APA style. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

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Supplementary Tables

Mean between-group evolutionary distance calculation using complete F gene	Genotype II	Genotype IV	Genotype XIII		Genotype VII
			Sub-genotype XIIIa	Sub-genotype XIIIb	
NDV/Chicken/Pandu/2015	19.0%	16.6%	9.2%	11.2%	11.9%
Standard error	0.014	0.013	0.008	0.009	0.010

Supplementary Table S1. Mean between-group evolutionary distances calculated for Newcastle Disease Virus (NDV) isolate NDV/Chicken/Pandu/2015 against genotype II, IV, VII and XIII viruses in class II circulating in Southeast Asia. The number of base substitutions per site from averaging over all sequence pairs between groups, including genotype II, IV, VII and XIII, are shown. The analysis involved 57 nucleotide sequences. The numbers of sequences analyzed per group were as follows: genotype II, n = 8; genotype IV, n = 4; sub-genotype XIIIa, n = 12; sub-genotype XIIIb, n = 22; genotype VII, n = 10. Standard error estimate(s) were obtained by a bootstrap procedure (100 replicates). Analyses were conducted using the maximum composite likelihood model. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1654 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

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Sub-genotype or clade	Evolutionary distance for the indicated sub-genotype or clade			
	XIIIa	XIIIb	Tentative XIIIc	NDV isolate Cockatoo/India/7847/1982
XIIIa	-	0.004	0.007	0.003
XIIIb	3.7%	-	0.008	0.005
Tentative XIIIc	7.0%	8.0%	-	0.008
NDV isolate Cockatoo/India/7847/1982	2.4%	4.0%	6.4%	-

Supplementary Table S2. Estimates of Net Evolutionary Divergence between sub-genotypes of genotype XIII of NDV. The numbers of base substitutions per site from an estimation of the net average between groups within genotype XIII are shown. The analysis involved 40 nucleotide sequences. The numbers of sequences analyzed per group were as follows: sub-genotype XIIIa, n = 13; sub-genotype XIIIb, n = 22; Tentative sub-genotype XIIIc, n = 4. Standard error estimate(s) were obtained by a bootstrap procedure (100 replicates). Analyses were conducted using the maximum composite likelihood model. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1654 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

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A.

GenBank Accession No.	Source	Genotype
KY083694	India	_
FJ979831	India	_
HE565484	China	_
AB626541	Japan	_
HE565483	_	_
AB241119	Japan	_
DQ404096	China	GI
AB795032	Japan	GI
AB797319	Japan	GI
DQ355369	Japan	GI
AY377577	Japan	GI
GU108334	Japan	GI
JF499816	Taiwan	GI
D45363	Thailand	GI
HM204527	China	GI
FJ161967	China	GI
D45363	Thailand	GI
GQ260633	Taiwan	GI
DQ404104	China	GI
AY376464	Vietnam	GI
FJ185154	Vietnam	GI
AB231463	Japan	GI
DQ404094	China	GI
FJ185145	Japan	GI
LC095826	Japan	GI
U70401	Thailand	GI
U34928	South Korea	GI
U70388	Thailand	GI
AB213007	Japan	GI
AB112706	Japan	GI
AB238693	Japan	GI
AB306941	Japan	GI

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MF326270	China	GI
JQ429305	Indonesia	GII
U70421	Malaysia	GII
U70406	Indonesia	GII
JQ429306	Indonesia	GII
AY184213	Malaysia	GII
AF217620	Australia	GII
FJ943462	Japan	GIII
DQ404116	China	GIII
AY427794	Japan	GIII
AF112297	Japan	GIII
JN375542	Indonesia	GIII
AB028271	Japan	GIII
U70387	India	GIII
AY243842	China	GIII
GU253947	China	GIII
AY376462	Vietnam	GIII
U70414	Japan	GIII
U70417	Philippines	GIII
FJ979830	India	GIII
U70395	Sri Lanka	GIII
U70393	Thailand	GIII
KC526870	India	GIII
KC802022	India	GIII
Z34095	India	GIII
U70418	India	GIII
DQ404112	China	GIII
KF667303	Taiwan	GIII
JX018170	India	GIII
JQ434468	India	GIII
U70392	Nepal	GIII
KR265316	China	GIII
JX131374	India	GIII
KX965684	China	GIII

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U70408	Indonesia	GIV
U34929	South Korea	GIV
D45362	Japan	GIV
AY184212	Indonesia	GIV
JN587258	South Korea	GV
KJ420590	South Korea	GV
KJ420591	South Korea	GV
KM496505	South Korea	GV
KJ420592	South Korea	GV
KM496503	South Korea	GV
MF526897	South Korea	GV
MF526899	South Korea	GV
MF526901	South Korea	GV
JF915894	China	GV
HM596272	Malaysia	GV
AY585243	South Korea	_
EF623988.1	India	_
L48961	China	_
AF254452.1	Taiwan	_
AF075723.1	India	_
AF098735.1	Taiwan	_
AB051292.1	Japan	_
AF069076.1	Japan	_
NC_001437.1	Japan	_
AY508813.1	Japan	_
AB269326.1	Japan	_
AB241118.1	Japan	_
AY585242.1	Korea	_
AF045551.2	Korea	_
EU880214.1	China	_
KC196115.1	Laos	_
AB594829	Japan	_
MF002373	China	_
AF315119	China	_

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B.

GenBank Accession No.	Source	Genotype
AB241119	Japan	_
MF326270	China	GI
AF217620	Australia	GII
KR265316	China	GIII
JX131374	India	GIII
KX965684	China	GIII
AY184212	Indonesia	GIV
JF915894	China	GV
HM596272	Malaysia	GV
AY585243	South Korea	_
EF623988.1	India	_
L48961	China	_
AF254452.1	Taiwan	_
AF075723.1	India	_
AF098735.1	Taiwan	_
AB051292.1	Japan	_
AF069076.1	Japan	_
NC_001437.1	Japan	_
AY508813.1	Japan	_
AB269326.1	Japan	_
AB241118.1	Japan	_
AY585242.1	Korea	_
AF045551.2	Korea	_
EU880214.1	China	_
KC196115.1	Laos	_
AB594829	Japan	_
MF002373	China	_
AF315119	China	_
MH385014	China	GI
KP164498	India	GIII
KX779521	China	_
KF297916	China	GIII

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AB196926	–	–
LC461961	Indonesia	GIV
AY316157	–	–
AB830335	Japan	GI
AB853904	Japan	–
LC461957	Japan	GI
LC461958	Thailand	GI
LC461960	Philippines	GIII
AB196923	–	–
AB196924	–	–
AB196925	–	–
GQ199609	Korea	–
KF297915	China	GIII
KX779520	China	–
KX779522	China	–
MN639770	China	GIII
M18370.1	–	–
KF907505	Taiwan	GIII

Supplementary Table S3. The details of 100 full coding sequences (CDS) of JEV E **(A)** and 50 CDS of JEV NS1 **(B)** downloaded from GenBank. '—' denotes that the information is not available in GenBank.

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A.

Peptide Stretch no.	ASA peak location	Start position	End position	Peptide
1	42	35	49	ANDKPTLDVRMINIE
2	58	51	65	SQLAEVRSYCYHASV
3	77	70	84	TVARCPTTGEAHNEK
4	91	84	98	KRADSSYVCKQGFTD
5	132	125	139	AIGRTIQPENIKYEV
6	163	156	170	SAQVGASQAAKFTIT
7	199	192	206	PRSGLNTEAFYVMTV
8	217	210	224	SFLVHREWFHDLALP
9	235	228	242	PSSTAWRNRELLMEF
10	266	259	273	EGGLHQALAGAIVVE
11	280	273	287	EYSSSVKLTSGHLKC
12	316	309	323	SFAKNPADTGHGTVV
13	334	327	341	SYSGSDGPCKIPIVS
14	352	345	359	LNDMTPVGRLVTVNP
15	370	363	377	TSSANSKVLVEMEPP
16	397	390	404	KQINHHWHKAGSTLG
17	482	475	489	ARDRSIALAFLATGG

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B.

Peptide Stretch no.	ASA peak location	Start position	End position	Peptide
1	37	30	44	DRYKYLPETPRSLAK
2	55	48	62	KAHKEGVCGVRSVTR
3	83	76	90	NVLLKENAVDLSVVV
4	103	96	110	RYRSAPKRLSMTQEK
5	109	102	116	KRLSMTQEKFEMGWK
6	131	124	138	FAPELANSTFVVDGP
7	141	134	148	VVDGPETKECPDEHR
8	148	141	155	KECPDEHRAWNSMQI
9	182	175	189	STDECDGAIIGTAVK
10	192	185	199	GTAVKGHVAVHSDLS
11	200	193	207	AVHSDLSYWIESRYN
12	214	207	221	NDTWKLERAVFGEVK
13	224	217	231	FGEVKSCTWPETHTL
14	237	230	244	TLWGDGVEESELIIP
15	272	265	279	QGPWDENGIVLDFDY
16	279	272	286	GIVLDFDYCPGTKVT
17	299	292	306	GKRGPSVRTTTDSGK
18	310	303	317	DSGKLITDWCCRSCS
19	328	321	335	LRFRTENGCWYGMEI

Supplementary Table S4. JEV E **(A)** and NS1 **(B)** amino acid stretches with the least protein variability and the highest ASA values.

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Research Achievements

Journal Publications

https://scholar.google.com/citations?user=O8CYTC8AAAAJ&hl=en

Thesis

1) Evaluation of Japanese encephalitis virus E and NS1 proteins immunogenicity using a recombinant Newcastle disease virus in mice — 2020
Nath, B., et al. *Vaccine*, 38(7): 1860-1868.

2) Enhanced cytopathic effect of Japanese encephalitis virus strain SA14-14-2: Probable association of mutation in amino acid of its envelope protein — 2017
Nath, B., et al. *Microb Pathog.*, 111: 187-192.

3) Emerging variant of genotype XIII Newcastle disease virus from Northeast India — 2017
Nath, B. and Kumar, S., *Acta Trop.*, 172: 64-69.

4) *In-silico* approach to designing epitope-based peptides for viral detection systems against JEV
Nath, B., et al. (Submitted)

Other

5) Structure analysis of the nucleoprotein of Newcastle disease virus: An insight towards its multimeric form in solution — 2020
Nath, B., et al. *Int J Biol Macromol.,* 151: 402-411.

6) The emergence of porcine circovirus 2 infections in the Northeastern part of India: A retrospective study from 2011 to 2017 — 2018
Barman, NN., **Nath, B.,** et al. *Transbound Emerg Dis*., 65(6): 1959-1967.

7) Emergence of a genotype I variant of avian infectious bronchitis virus from Northern part of India — 2018
Jakhesara, S., **Nath, B.,** et al. *Acta Trop.*, 183: 57-60.

8) Molecular characterization of Newcastle disease virus strains isolated from different outbreaks in Northeast India during 2014-15 — 2015
Nath, B., et al. *Microb Pathog.*, 91: 85-91.

9) Potent Inhibition of Immunosuppressive Indoleamine 2,3-Dioxygenase 1 Enzyme by targeting the Heme and Apo-form
Pradhan, N., Akhtar, N., **Nath, B.,** et al. (Under review)

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Book Chapter

1) Reverse genetics and its usage in the development of vaccine against poultry diseases

Nath, B. et al. (Under review)

Awards

1) **Best Oral Presentation** 2020

IAVMI-CON 2020, 33rd Annual Convention of IAVMI*

*Indian Association of Veterinary Microbiologists, Immunologists & Specialists in Infectious Diseases

2) **Student Travel Award** 2018

37th Annual Meeting, American Society for Virology (ASV)

3) **International Travel Support (ITS)** 2018

Science and Engineering Research Board (SERB), DST, Govt. of India

4) **Best Poster Presentation** 2017

VIROCON 2017, 26th National Conference of Indian Virological Society

5) **7th Rank in Northeast India (7 states)** 2015

State Level Eligibility Test (SLET) for Lectureship

6) **Appreciation Award for Poster Presentation** 2015

IAVMI 2015, 29th Annual Convention of IAVMI

Conference Proceedings

International

1) **ASV, 37th Annual Meeting of American Society for Virology, Maryland, USA** 2018

Nath, B. and Kumar, S. *Recombinant Newcastle disease virus-based approach for the development of a vaccine against Japanese encephalitis virus*

National

2) **33rd Annual Convention of IAVMI, IVRI, Bareilly** 2020

Nath, B., et al. *Structure analysis of the nucleoprotein of Newcastle disease virus: An insight towards its multimeric form in solution*

3) **VIROCON, 26th National Conference of Indian Virological Society, Mangalore** 2017

Nath, B. and Kumar, S. Improved Japanese encephalitis virus vaccine using recombinant Newcastle disease virus as a vector

4) **57th Annual Conference of Association of Microbiologists of India, Guwahati** 2016

Nath, B. and Kumar, S. In vitro characterization of Japanese encephalitis virus strain SA14-14-2: adaptation and propagation in baby hamster kidney cells

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5) **29th Annual Convention of IAVMI, Guwahati** 2015

Nath, B. et al. Molecular characterization of Newcastle disease virus strains isolated from different outbreaks in Northeast India during 2014-15

Workshops

Attended

1) **Intellectual Property Rights** 2019

Ink Idée, Research Conclave 2019, IIT Guwahati

2) **Flow Cytometry Application in Basic, Applied and Clinical Biology** 2016

Flowcytometry Solutions Pvt. Ltd. and Bio-Rad, IIT Guwahati

3) **Flow Cytometry Data Analysis** 2015

Flowcytometry Solutions Pvt. Ltd. and Bio-Rad, IIT Guwahati

4) **Culture of Responsibility, Pathogen Inventory Management and Fundamentals of Working in Biosafety Cabinets** 2015

Indian Council of Agricultural Research, American Society for Microbiology, and Society for Biosafety, ICAR-NEH, India

Organized

3-day Annual Workshop on **Diagnostic Approaches in Virology**, Department of Biosciences and Bioengineering, IIT Guwahati for 3 consecutive years 2018-20

Membership

International

1) American Society for Virology (ASV) 2018-19

National

2) IAVMI (Life Membership)

3) Society for Biosafety, India (Life Membership)

Teaching assistantship

1) Applied Biology and Bioengineering Lab

2) Chemical Thermodynamics

3) Genetics

4) Frontiers in Biotechnology

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