Molecular and serological analysis of dengue virus (DV) isolates and investigation of DV-mediated pathogenesis towards identifying DV antivirals

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by

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वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद की एक इकाई
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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "Molecular and serological analysis of dengue virus (DV) isolates and investigation of DV-mediated pathogenesis towards identifying DV antivirals" submitted by Sri Himadri Nath who got his name registered on 9.08.2019 for the award of Ph.D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of Dr. Subhajit Biswas and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

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Declaration by the author

I hereby declare that the work presented in this thesis entitled "Molecular and serological analysis of dengue virus (DV) isolates and investigation of DV-mediated pathogenesis towards identifying DV antivirals" is the result of the research performed by me in the Division of Infectious Diseases and Immunology, CSIR-Indian Institute of Chemical Biology, Kol-32, West Bengal, India under the supervision of Dr. Subhajit Biswas, Principal Scientist.

In keeping with the general practice of reporting scientific observation, due acknowledgements have been made wherever the work described is based on the finding of the other investigators/scientists.

Himadri Nath. 20.07.2022

Signature of the author

Dedicated to my parents, sister and beloved.

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Abbreviations

Ab	Antibody
Ag	Antigen
ACE2	Angiotensin-converting enzyme 2
ADE	Antibody dependent enhancement
ALT	Alanine transaminase
ALP	Alkaline phosphatase
AST	Aspartate transaminase
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
cDNA	Complementary Deoxyribonucleic acid
cfDNA	Cell free Deoxyribonucleic acid
CC3	Cleaved Caspase 3
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DV	Dengue virus
DI	Domain I
DII	Domain II
DIII	Domain III
DHF	Dengue hemorrhagic fever
DMSO	Dimethyl sulfoxide
DSS	Dengue shock syndrome
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
E	Envelope
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
ELISA	Enzyme Linked Immunosorbent Assay
Fc	Fragment crystallizable region of antibody
Fab	Fragment antigen-binding region of antibody

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gE	Genome equivalent
HDL	High-density lipoprotein
HRP	horseradish peroxidase
HSP	Heat shock protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl β- d-1-thiogalactopyranoside
JEV	Japanese encephalitis virus
LFIA	Lateral flow immunoassay
M	Membrane protein
MCP	Monocyte chemoattractant protein
MEM	Minimum Essential Medium
MIF	Macrophage inhibitory factor
MTase	Methyltransferase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAT	Nucleic Acid Testing
NF	Nuclease free
NTC	Non-template control
NTP	Nucleoside triphosphatase
NS	Non-structural
sNS1	Soluble Non-structural protein 1
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PES	Polyethersulfone
prM	Precursor Membrane
qRT-PCR	Quantitative/Real Time Polymersae Chain Reaction
RNA	Ribonucleic Acid
RTPae	RNA Triphosphatase
RT	Room temperature
RT-PCR	Reverse Transcription Polymersae Chain Reaction
RdRP	RNA dependent RNA polymerase

SARS	Severe Acute Respiratory Syndrome
SDS	Sodium Dodecyl Sulfate
TCID50	Median Tissue Culture Infectious Dose
TGN	Trans-Golgi Network
Th2	Type II helper T cells
TLR	Toll like Receptor
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VEGF	Vascular endothelial growth factor
VNT	Virus neutralization test
WNV	West Nile Virus

Synopsis

Dengue is one of the most serious life-threatening vector-borne infectious diseases. It affects approximately 50 million people across the globe annually and results in more than 25,000 deaths. Dengue virus is a member of the family *Flaviviridae*, genus Flavivirus, and is transmitted to humans by the bite of female *Aedes* mosquitoes. The infection with any of the main four DV serotypes (1-4) can either be asymptomatic or manifest in three clinical forms of increasing severity: dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS).

Serum samples from DF patients were collected during 2017-18 for molecular level screening and establishment of laboratory culture. Serum samples were passaged in Vero (Green monkey kidney cell line) cells. Then nucleic acid level screening and DV serotyping were carried out. Representatives of three serotypes (DV1, 2 & 3) were isolated. DV type 2 was most prevalent as has been found in this study. qRT-PCR was done to determine the virus titer and to select suitable ones for stock preparation. To culture the clinical isolates, mosquito cell line C6/36 (*Aedes albopictus* larvae cells) was procured from NCCS. The culture of C6/36 and infection protocol was standardized. This was followed by three consecutive blind passages of all the selected virus strains in C6/36. After passage in C6/36, virus titer was determined using one step qRT-PCR, for four virus isolates and substantial increase in titer was observed. These four samples encompass the representatives of dengue virus types 1, 2 & 3.

Liver has been found to be one of the main organs to get affected in dengue virus infection. Hepatic dysfunction is an important feature of dengue as evident from various clinical reports, but the direct effect of dengue virus clinical strains on liver cells is not yet clear. So,

the effect of clinical dengue virus and non-structural protein-1 (NS1) on human liver cell line (Huh7) was investigated. Dengue virus NS1 is one of the main players of pathogenesis and immune modulation. To study its effect, NS1 of three serotypes (DV-1,2,3) were cloned in pcDNA3.1(+) vector.

We observed that both DV infection and NS1 expression can induce apoptosis in liver cell lines as observed from cleaved caspase 3 expression. Only NS1 expression was enough to induce apoptosis. Now, to access the effect of NS1 in comparison with whole DV, we standardized the infection and NS1 transfection in such a way that similar amounts of NS1 were secreted in both the cases. Under such conditions, cellular apoptosis was further evaluated using apoptotic DNA ladder assay and TUNEL assay. Surprisingly, DV infected cells were with very less apoptotic DNA breaks in comparison with NS1 transfected cells, although both were expressing cleaved caspase 3. So, it appeared that DV is slowing down the apoptosis which is an antiviral response. To understand this phenomenon, DV infected cells were treated with Camptothecin (chemical inducer of apoptosis, Topoisomerase I inhibitor) and apoptotic DNA pattern was analysed. It was observed that apoptotic DNA is much less in infected cells in comparison with only Camptothecin treatment. This shows that DV protects cellular DNA as a strategy to delay the apoptosis, so that the window for virus replication and progeny formation can be extended.

As NS1 was found to be a potent viral protein for pathogenesis, the expression and secretion pattern was studied in detail for a target to intervene the secretion of NS1. NS1 expresses in ER; then travels to plasma membrane (PM) through trans-Golgi network, forming dimer. Then three dimers assemble on PM to form soluble hexamar with a lipid core. We designed peptides to bind with the hydrophobic β-sheet platform of NS1 dimer with a view to prevent hexamer formation. We tested the peptides on DV infected cells but there was no statistically significant reduction in NS1 secretion.

In early days of COVID19, there was a report that two SARS-CoV-2 infected patients' serum samples came positive in dengue IgG and IgM strip tests. This raised the possibility of false positive results. So, we tested the opposite scenario i.e. effect of DV serum on SARS-CoV-2 strip tests. In order to confirm the absence of SARS-CoV-2 antibodies (Abs), we used DV serum samples which were archived in 2017. It was found that 5 of 13 samples were positive in SARS-CoV-2 Abs lateral flow-based strip tests. Now, in dengue endemic countries such as in India, 48% population is seropositive for dengue which can result in many false positive results in rapid SARS-CoV-2 Abs tests. This was the first report that dengue serum samples can cross-react with SARS-CoV-2 antigen. We further tested COVID-19 serum from highly dengue endemic region (Kolkata) in DV rapid Abs tests and ELISAs and found very high cross-reactivity as well. Further, the effect of SARS-CoV-2 Abs on DV infectivity was tested by means of virus neutralization (VNT) assay. COVID-19 serum samples, including those with no previous dengue history neutralized clinical DV. So, SARS-CoV-2 Abs tests are needed to be supplemented with other tests such as nucleic acid tests for proper diagnosis, especially in areas where both the viruses are co-endemic now.

1. Chapter 1

Review of Literature

1.1. Introduction

Dengue is arthropod-borne viral disease caused by one of the four dengue virus serotypes, DV-1, DV-2, DV-3, and DV-4. Dengue virus belongs to *Flavivirus* genus of *Flaviviridae* family. *Flaviviridae* is a well-known virus family which includes the first discovered human virus yellow fever virus. It also includes many highly pathogenic viruses like Zika virus, yellow Fever virus and Japanese encephalitis virus. Female *Aedes* mosquitoes serve as vector of DV. DV is a positive sense RNA virus. Infection with DV may be asymptomatic or mild fever, but in few cases it leads to life threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Dengue is endemic in more than hundred countries which mean one third of the world population is at risk (Bhatt *et al.*, 2013). At present there is no suitable vaccine for all or effective treatment strategies for DV infection. Current treatments are mainly symptom based and often fail in case of DHF and DSS. The prevention of dengue is mostly dependent on vector control and effective community involvement is very important.

1.2. History

Reference of dengue fever can be found as "water poison" linked with flying insects in medical literature of China in 992. The word "dengue" is from Swahili phrase means "cramp-like seizure". The first dengue epidemics with clinical recognition took place around 1780s in Asia, Africa and North America. Benjamin Rush from Philadelphia was the first to report dengue case clinically in 1789. Then Dr. Rush coined the term "break bone fever" considering the symptoms like myalgia and arthralgia. In India, first dengue like symptoms was recorded from Madras (Chennai). But DF epidemic with virus diagnosis occurred in Calcutta (Kolkata) in 1963-64. First dengue virus isolation was done in Japan around 1943 and then in Calcutta in 1944 (Gupta *et al.*, 2012).

1.3. Global Epidemiology

Dengue virus infection is endemic in more than 100 countries in Americas, Southeast Asia, the western pacific, eastern Mediterranean and Africa (Fig 1.1). This results in risk of 3.6 billion people worldwide (Bhatt *et al.*, 2013). As per CDC, dengue cases have increased 30 fold in the last fifty years (WHO Global strategy for dengue prevention and control. World Health Organization, Geneva2012). But the true disease burden is not well reported from many places of Indonesia, China, Brazil, Africa and India (Bhatt *et al.*, 2013).

Dengue illness is also responsible for high economic burden. As per one report, annually 2.9 million dengue cases with 5906 deaths in Southeast Asia caused an economic burden of $950 million (Shepard, Undurraga and Halasa, 2013). The societal and demographic changes in last 50-60 years are quite responsible for rapid global emergence of dengue. These changes include population growth at an unprecedented rate, increased population movement, unplanned urbanization and changing climate resulting in breakdown of vector control.

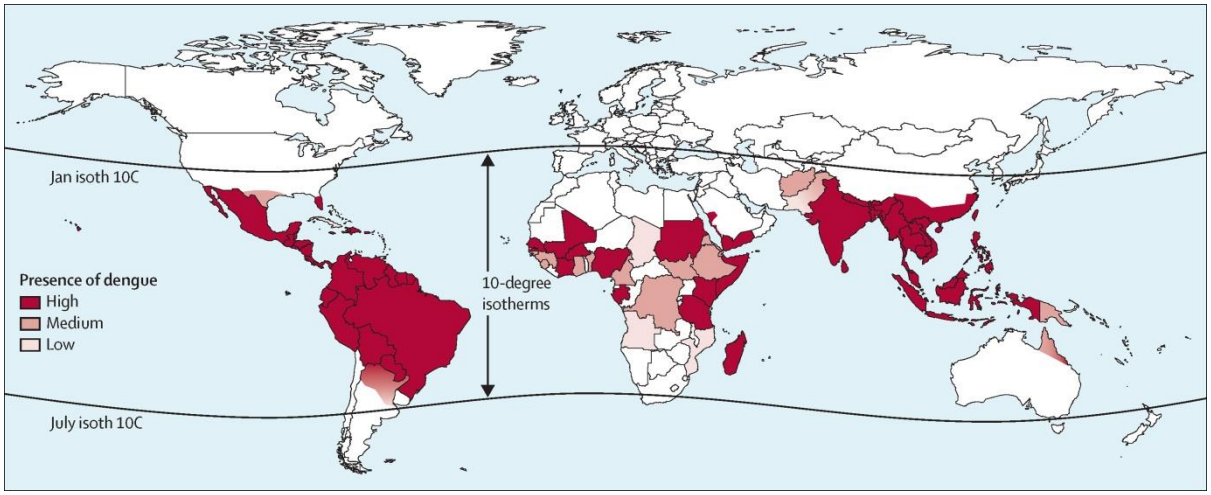
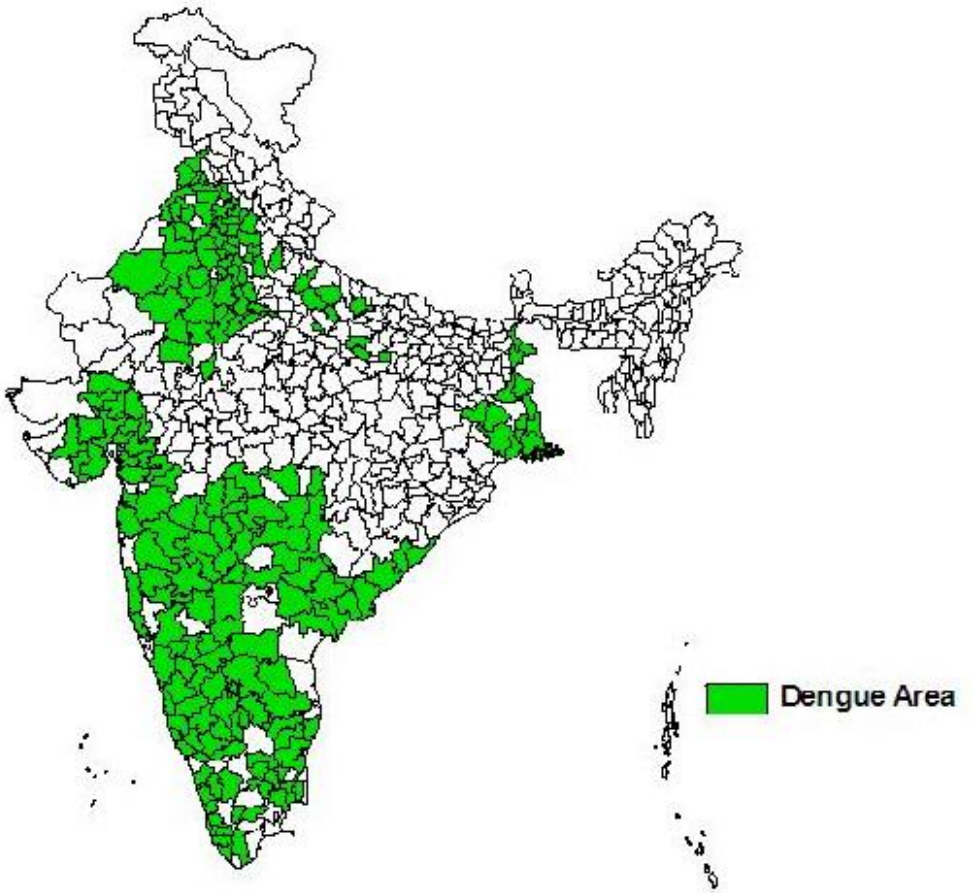


Figure 1.1. Global burden of dengue as of 2014, based on (Bhatt *et al.*, 2013)**,** Centers for Disease Control and Prevention Dengue map (http://www.healthmap.org/dengue/en/), WHO International travel and health interactive map (http://apps.who.int/ithmap/). Adapted from (Guzman and Harris, 2015).

1.4. Indian scenario

In India, every year more than one lakh people get infected which is confirmed with clinical diagnosis. But differential analysis and mathematical programming suggest that the actual number is much higher as most of the cases (80-90%) are asymptomatic. These sorts of predictions are much supported by serological study of DV in different parts of India. One of such study in 2017-18 conducted by ICMR scientists reported that overall DV seroprevalence in India is 48.7% (Murhekar *et al.*, 2019).

A



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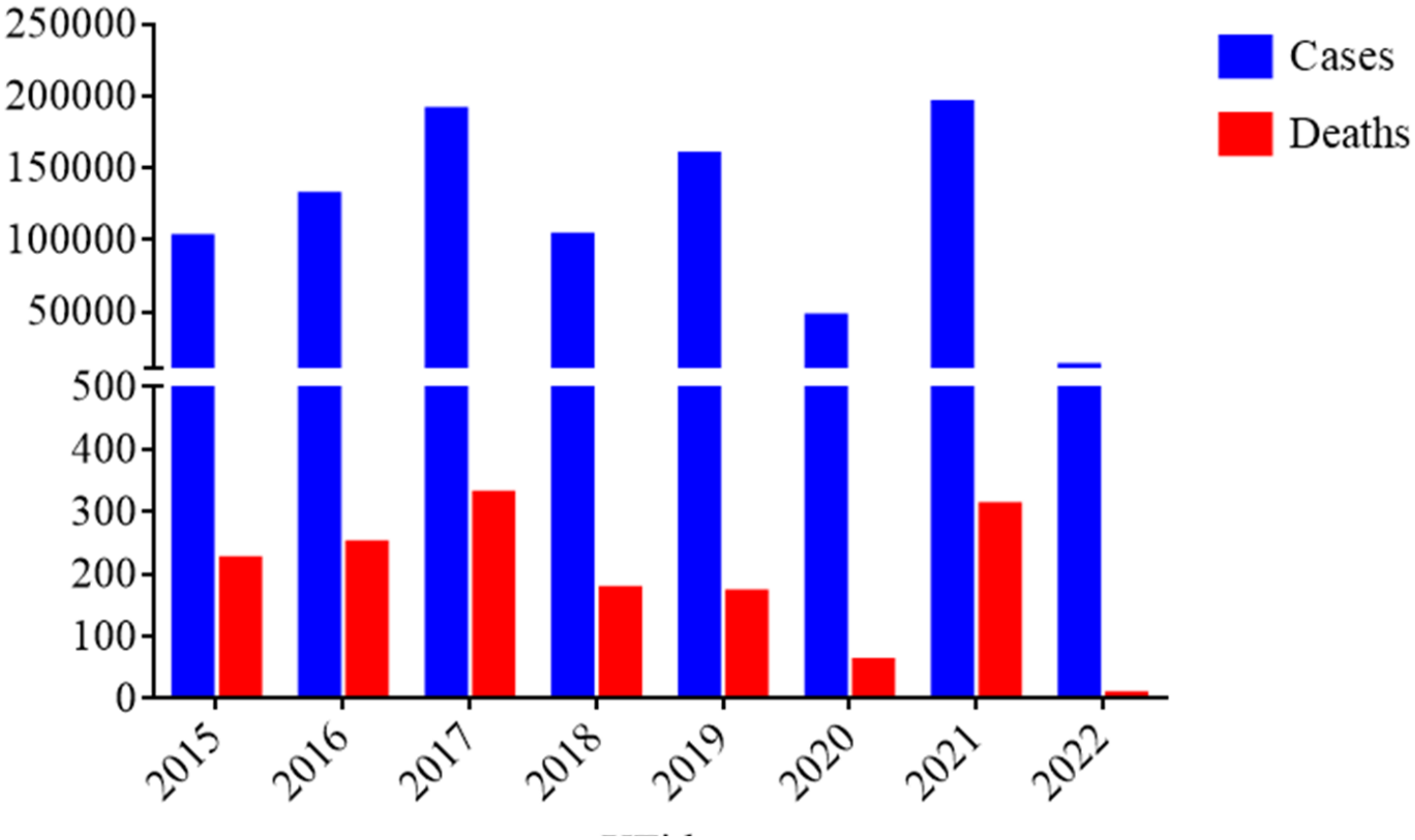


Figure 1.2. Dengue prevalence in India

(A) Areas of India where dengue fever is endemic. (B) Year wise dengue virus infection cases along with the deaths. In 2020 there were underreporting due to COVID-19. In case of 2022, the data as reported up to 31st May 2022.

Adapted from (Dengue/DHF situation in India, NVBDCP).

1.5. Vector

Dengue fever is an arthropod borne viral disease transmitted by female *Aedes* mosquitoes. Although Aedes aegyptai is the primary vector of DV but *Aedes albopictus, Aedes polynesiensis,* and *Aedes scutellaris* are also capable of transmission. The shape of *Aedes aegypti* is small in comparison with *Anopheles* mosquitoes. It is dark with white bands on its legs. *Aedes* mosquitoes are mostly found in tropical and sub-tropical regions within latitude 35^0 N to 35^0 S where the temperature is not below 10^0C in winter. In the life cycle of *Aedes* mosquitoes, there are four stages viz. eggs, larva, pupa and adult. Female mosquitoes lay eggs in stagnant water. Eggs are capable to hatch from few days to months till it gets submerged in water (Fig 1.3). After hatching, larvae stay in water and develop into pupae within five days. Pupae turn into adult mosquitoes in 2 to 3 days (Carrington and Simmons, 2014).

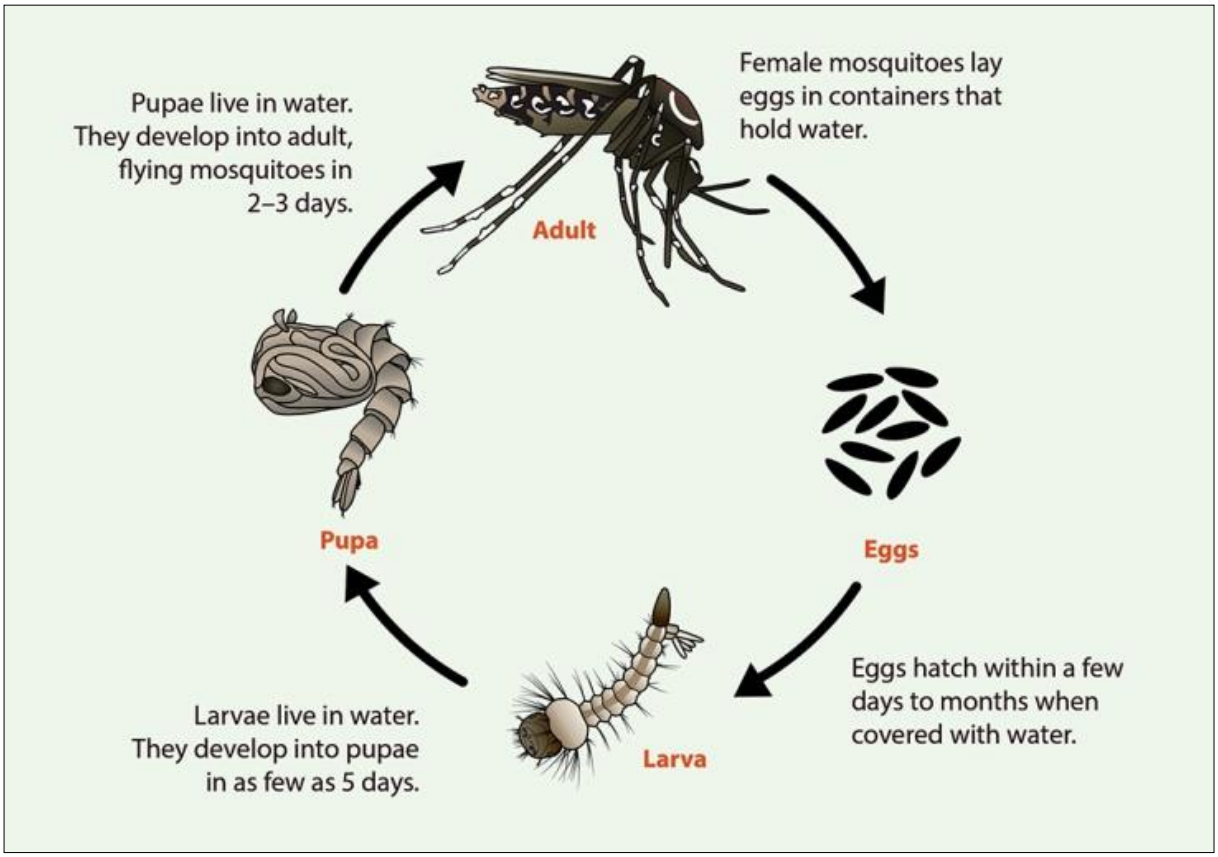


Figure 1.3. Life cycle of *Aedes* mosquitoes.
Female *Aedes* mosquitoes lay eggs on walls of wet water containers. Eggs can stay even for months in drought till it gets submerged in water. After hatching, the larvae feed on microorganisms to grow. Then the larvae metamorphosed to pupae which undergo changes without feeding until adult mosquito is formed.
Adapted from (*Life Cycle of Aedes aegypti and Ae. albopictus Mosquitoes, CDC*)**.**

1.6. Dengue virus transmission cycle

1.6.1. Sylvatic cycle

DV transmission cycle consists of two ecologically distinct hosts, sylvatic cycle and human cycle. Human is the only amplifying reservoir host in which DV shows clinical symptoms. There are few lower primates in which DV susceptibility has been observed but the duration and level of viraemia is much lower. The sylvatic cycle is constituted by these animals. DV can get transmitted from infected mosquito to its egg through transovarial route as well. The infection capability of sylvatic DV strains on human cells has been tested *in vitro* and potential infection has been reported (*Dengue guidelines for diagnosis, treatment, prevention and control : new edition*). So, it can be said that adaptive barrier is not sufficient to prevent the sylvatic DV strain infection in human. Now, as the ecological disturbances increase it is possible that sylvatic DV strains can infect human as well.

1.6.2. Human cycle

Dengue virus is transmitted to mosquitoes when a female *Aedes aegypti* bites an infected human during the viremic phase of sickness, which lasts 2-7 days. Once infected, a mosquito can transmit the virus to human for its lifetime. After taking blood meal, the epithelial cells of mosquito's midgut gets infected. The viruses then escape from the midgut epithelium into the haemocele, causing infection of the salivary glands. Afterwards, the infection spreads in other tisssues like foregut epithelium, nerve cells, epidermal cells, ovary, fat body, and hemocytes. It also replicates in the female mosquito's reproductive tract, where it can infect her eggs via transovarial virus transmission. Then, the virus is secreted in the saliva, from where it can be transmitted to another person during probing and blood feeding(Fig.1.4) (Carrington and Simmons, 2014).

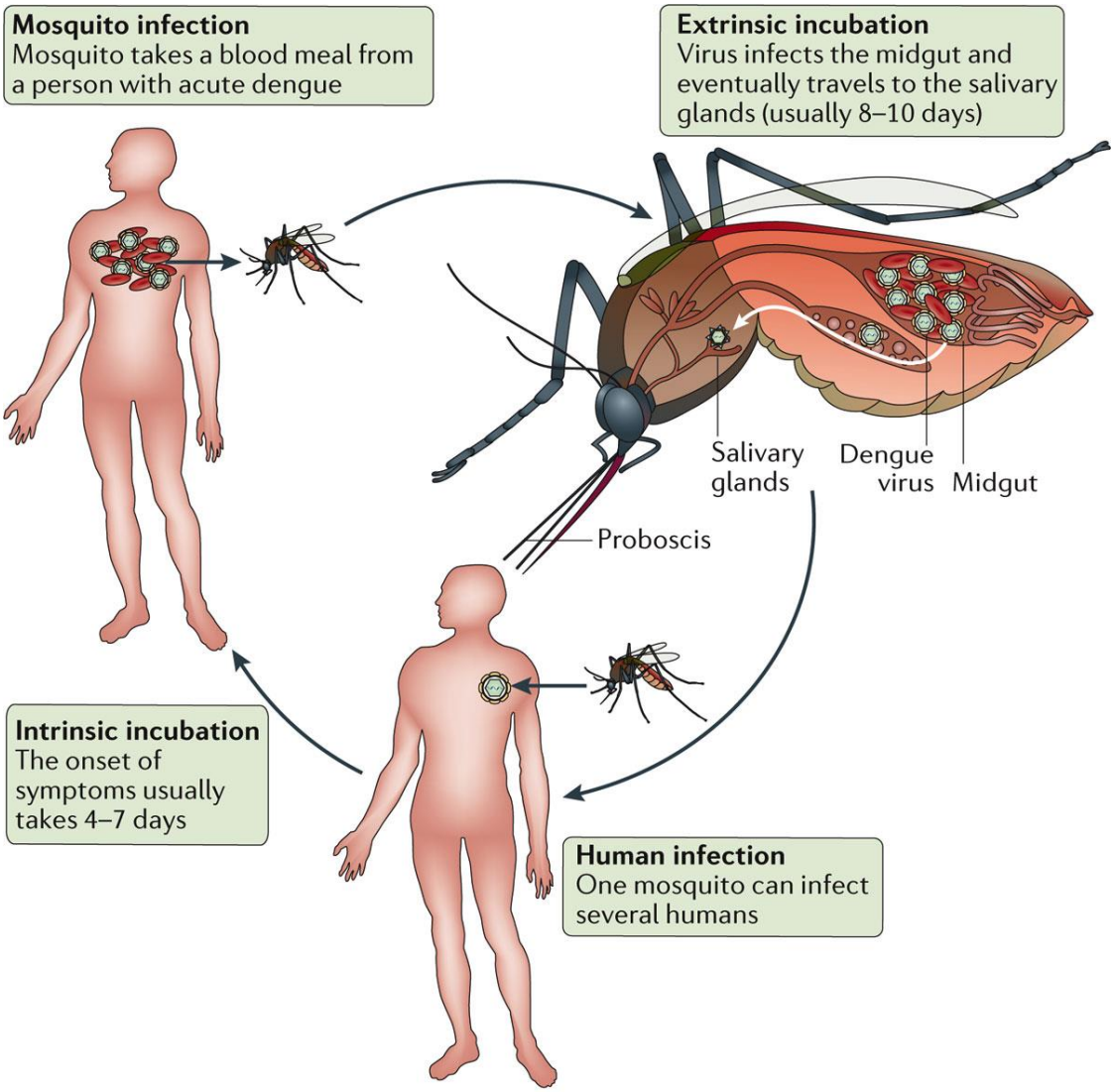


Figure 1.4. Life cycle of dengue virus in human and mosquitoes
Aedes mosquitoes get infected through feeding on a person during the viraemic phase of DV infection. Then DV infect the midgut cells and other tissues of mosquitoes before reaching the salivary glands. This is called as extrinsic phase. Infected mosquitoes can transmit DV to several humans as they bite. After the entry of the virus into the body, it takes 4 to 7 days to show the symptoms and to make a person capable of transmission. Although most of the cases are asymptomatic, still they can transmit the DV to mosquitoes.
Adapted from (Guzman *et al.*, 2016)

1.7. Disease characteristics of dengue

The clinical condition varies from a non-specific, acute viral fever (dengue fever, DF) to a severe dengue which includes lethal hemorrhagic condition called Dengue Hemorrhagic Fever (DHF)/Dengue Shock Syndrome (DSS). Dengue fever is known to be asymptomatic or subclinical in 50-90 percent of cases where people may not be aware about the infection. Only a small percentage of infected persons may develop severe dengue with complications like low platelet count, bleeding, organ damage and plasma leakage. The clinical demarcation of severe dengue was done in 1950s during epidemics in Thailand and Philippines. As per WHO classification, there are two broad categories: dengue (presence or absence of warning sign) and severe dengue. Further the sub-classification of dengue based on with or without warning symptoms is mentioned to assist the clinical practitioners regarding hospital admission for continuous observation for developing severe dengue (*Dengue guidelines for diagnosis, treatment, prevention and control : new edition*, no date).

1.7.1. Dengue

A patient can be suspected for DV infection when there is high fever (40^0C or 104^0F) in association with any two of the following symptoms in febrile phase which lasts for 2 to 7 days.

- Retro-orbital pain
- Joint and muscle pain
- Severe headache
- Nausea
- Vomiting
- Inflamed glands
- Rash

1.7.2. Severe dengue

A patient may enter into critical phase usually around 3-7 days after the onset of the illness. During critical phase which is around 24-48 hours, a small percentage of patients may have a sudden worsening of symptoms. Severe dengue is considered to be fatal due to its association with plasma leakage, accumulation of fluid, respiratory problem, severe bleeding, and organ malfunction (*Dengue guidelines for diagnosis, treatment, prevention and control : new edition*, no date). Warning signs that doctors usually look for are as follows-

- Rapid breathing and restlessness
- Fatigue
- Liver enlargement
- Severe abdominal pain
- Continuous vomiting
- Traces of blood in vomit or in stool

In presence of at least one or two warning signs as mentioned above, a close medical surveillance is essential to avoid further complications which may even lead to death. Medical attention should be continued in the convalescent phase as well.

1.7.2.1. Dengue hemorrhagic fever (DHF)

DHF is mostly seen in secondary infections as said above but in case of children it may happen during primary infection as well. Children often get the maternal anti-dengue antibodies which may cause the antibody dependent enhancement (ADE) during primary infection. In line of clinical course DHF can be divided into three phases: Febrile phase which is followed by leakage and convalescent phase at the end (Halstead, 2015).

The clinical signs of DHF are as follows-

- Acute febrile illness leading to high fever which may last for two to seven days.
- Episodes of hemorrhage may appear in at least one of the below ways-
    - Petechiae
    - Purpura
    - Ecchymosis
    - Epistaxis
    - Gingival bleeding
    - Mucosal bleeding
    - Hematemesis
- Tourniquet test positive and hepatomegaly
- Thrombocytopenia in which platelet count become <100,000/cu mm blood.

1.7.2.2. Dengue shock syndrome (DSS)

DSS can be defined as DHF associated with unstable narrow (<20mmHg) pulse pressure, cold, restlessness, clammy skin and cyanosis. As the shock gets worse, multiorgan damage and intravascular coagulation result in high mortality. Although the shock persists for a short period but this is the most critical time phase for the patient and close clinical observation is highly recommended. Usually a faster recovery is observed if a patient can overcome the shock period (Halstead, 2015).

1.8. Diagnosis of dengue

There are different methods to diagnose dengue virus infection. The appropriate method is chosen based on the time or stage of disease presentation by individual patient. For a patient in the first week of illness, can be tested using all the below mentioned ways.

1.8.1. Virus isolation and RT-PCR

The virus may be isolated from the serum if the blood is collected during the viraemia phase which is around 4-5 days after the onset of the fever (Fig.1.5). Alternatively, RNA can be extracted directly from the serum sample of the suspected patient. Then RT-PCR technique can be used to find virus sequence specific amplification (Lanciotti *et al.*, 1992). Although RT-PCR is considered as gold standard for the diagnosis of dengue infection, it involves specialized equipment with trained personnel which are costly and time consuming.

1.8.2. NS1 ELISA

In DV infection, the presence of abundant soluble Non-structural protein 1 (NS1) in serum has been well established. There are commercially available diagnostic test kits which can detect NS1 in serum using ELISA method. In this method results can be obtained within 2 hrs. Apart from being fast, these tests are comparatively easy to perform and cost effective (Chuansumrit *et al.*, 2008; Shan *et al.*, 2015). NS1 can be detected up to 6 to 7 days post infection (Fig.1.5).

1.8.3. Serological methods

In serological methods the presences of antibodies (IgG and IgM) against DV are detected. Antibodies can be detected either by ELISA or by lateral flow immunoassay (strip test). The IgM antibodies can be detected around one week after infection and used as an indication of recent infection. On the other hand, it takes some time to develop IgG antibodies but it

remains for years (Fig.1.5). The detection of IgG is an indication of past infection (Murphy and Whitehead, 2011).

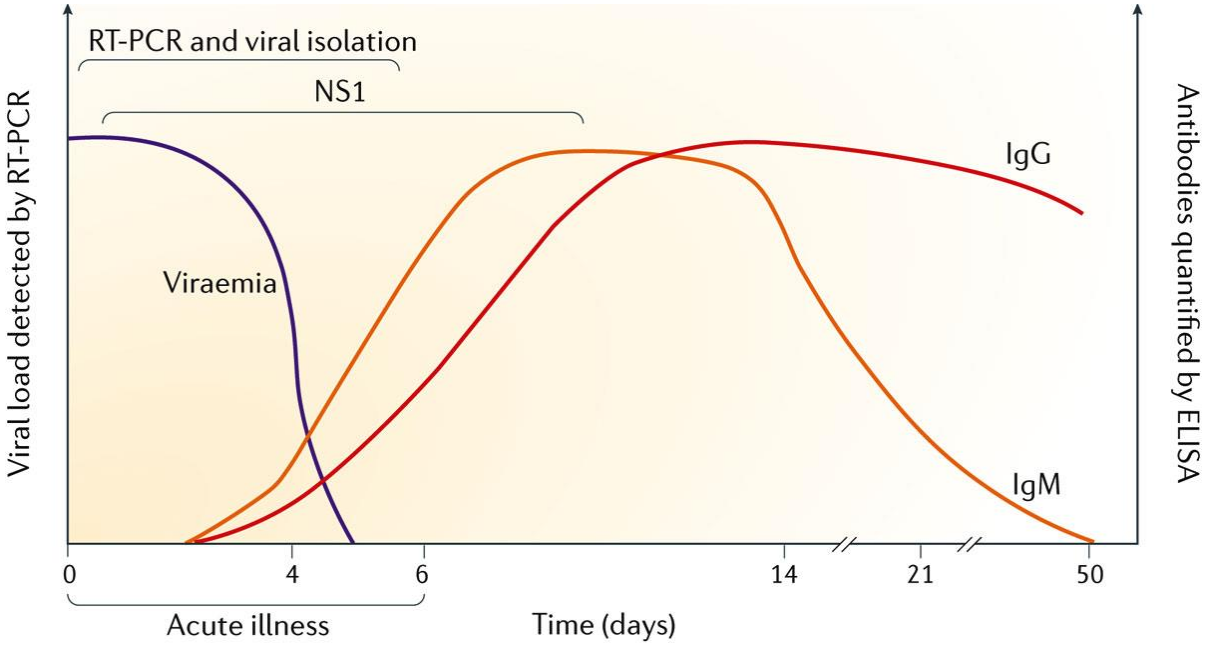


Figure 1.5. Viraemia and serological response in dengue virus infection.
Viraemia phase of DV infection lasts for around five days. During this period virus can be isolated from serum and RT-PCR can be done for nucleic acid level diagnosis. NS1 ELISA can be done within nine to ten days of the onset of acute illness. IgM is detectable on the first week of the infection but starts to decline after fifteen days. IgG appears in circulation at the end of second week of illness and stay detectable after two to three months, even up to a year. Adapted from (Guzman *et al.*, 2010)

1.9. Primary and secondary DV infection

1.9.1. Primary DV infection

If a person gets infected with any of the four serotypes of DV for the first time in his/her life then it is considered as primary infection. IgG and IgM antibodies appear after 3 to 5 and 6 to 10 days post primary infection respectively (Fig.1.5). The IgM antibodies get disappeared after 2-3 months of illness or asymptomatic infection. But the IgG antibody stays almost for lifetime (Guzman *et al.*, 2010). So, infection with one serotype provides life-long immunity against that particular serotype but not against the other serotypes. As antibody generated against one serotype cannot neutralize the other one hence, they have been designated as different serotypes (Swaminathan, Batra and Khanna, 2010).

1.9.2. Secondary DV infection

A second time infection of DV with a previously un-encountered serotype results in secondary infection or classical dengue fever. However, only 2-3% of such infections may lead to DHF which sometimes transform into DSS that mostly results in death (Swaminathan, Batra and Khanna, 2010). The primary infection results in moderate viraemia with long lasting immunity against that particular serotype. But that immunity is short and not very effective against other serotypes. Now, if a person gets infected with a second serotype which is different from the initial one then antibodies do appear in circulation but mostly non-neutralizing. So, instead of neutralization these antibodies bind with viral envelope antigen and promote virus entry into monocytes through Fc receptor. Thus during secondary infection non-neutralizing but cross-reactive antibodies promotes virus internalization and replication which is known as antibody dependent enhancement (ADE). Then immune clearance of these infected monocytes lead to the recruitment of T-cells which cause the release of different cytokines, activation of complement system and γ interferon secretion. There are

experimental evidences of ADE in non-human primates and mice (AG129). In those models DV titer increased after passive administration of anti- DV envelope monoclonal antibody at sub-neutralizing level. But, every severe case is not a secondary infection and a secondary infection may not essentially lead to DHF/DSS (Guzman and Vazquez, 2010; Murphy and Whitehead, 2011).

1.10. Treatment

In case of dengue fever, the treatment is not specific rather mostly symptom based. An infected person should take rest and stay hydrated, seeking medical attention may be necessary as well. Depending on the disease outcome, doctor may advise a patient to stay at home or may refer for in-hospital care.

Patients are normally advised to take antipyretics and pain killers to control the symptoms. Acetaminophen and Paracetamol are widely used to relif the symptoms. On the other hand NSAIDs (Aspirin, Ibuprofen) should not be taken during DV infection as there drugs reduces the symptoms by diluting the blood. Such blood thinning may be deleterious in a disease with a risk of hemorrhage.

1.10.1. Vaccination against dengue

Dengvaxia by Sanofi Pasteur was approved as first dengue vaccine in Dec 2015 in approximately 20 countries. CYD-TDV is a live attenuated, tetravalent, chimeric vaccine based on 17D strain of yellow fever virus. The envelope part of 17D strain is replaced with immune-dominant regions of four different dengue serotypes. The schedule consists of three doses at 0, 6 and 12 months respectively. As per Phase III trial vaccine efficacy is around 50 to 60 %. But the problem arises as analysis showed those clinical trial participants who were inferred to be seronegative at the time of first dose had a comparatively higher risk of having

more severe dengue than unvaccinated ones. The reason behind such observation is antibody dependent enhancement (ADE) which results due to the presence of non-neutralizing but cross-reactive antibodies. So, the vaccine administration clauses are modified as follows-

- As per WHO Dengvaxia can be used in age group of 9 to 45 years with previous dengue infection having a laboratory confirmation and living in dengue endemic areas.
- Children below 9 years should not get vaccinated.
- The vaccine is not yet licensed for people aged above 45 years.
- Immunocompromised children should not be administered.
- Dengvaxia is not yet approved for travelers.

(WHO 'Vaccines and immunization: Dengue', *Q&A Detail*, (April 2018), pp. 2–3.)

1.11. Dengue virus genome

Dengue virus particle is approximately 500Å in diameter. It has a positive sense RNA genome of ~10.7 Kb coding for three structural and seven non-structural proteins. The dengue virus genome contains only one open reading frame and forms a single polyprotein of around 3000 amino acid long. This large precursor polyprotein is acted upon by viral and host proteases during post translational modification. Capsid, precursor-Membrane & Envelope are the three structural proteins. Non-structural proteins are NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5. The 5' end of the genome contains m^7GpppAmN cap but there is no poly adenylate tail at the 3' end. The 5' cap increases the stability of the viral RNA and also helps to escape host immunity. There are two noncoding regions at the 5' and 3' flanking end of the genome. The length of 5' UTR (Untranslated region) is around 100 nucleotides (nt) whereas the 3' UTR is around 500-600 nt long. The 5' UTR forms bifurcating stem-loop structure which is believed to initiate the RNA replication through interaction with NS5 RdRp.

Similarly, 3’ UTR also forms stem loop (SL) secondary structures due to repetitive complementary sequences (Villordo, Alvarez and Gamarnik, 2010; Iglesias and Gamarnik, 2011).

Flavivirus genome cyclization is necessary for viral RNA synthesis and it is mediated via long distance interaction between elements of 5’ and 3’ UTR. It requires several complementary regions between the 5′ and 3′UTR to form a panhandle namely, the 5′–3′ cyclization sequence and 5′–3′ UAR (upstream of AUG region) sequences. The 5′ cyclization sequence is located within the ORF, while the 3′ cyclization sequence is located immediately upstream of the 3′SL. (Alvarez *et al.*, 2005). It is presumed that NS5 attached with 5’ SL comes to the proximity of 3’ site, which initiate the minus strand initiation. Now, it is important to note that melting of secondary structures at both ends is necessary for such interactions. On the other hand, abolishing the secondary confirmation leads to the occlusion of translation initiation site. In this way, virus can control the switch between translation of polyprotein and RNA replication (Alvarez *et al.*, 2005; Villordo, Alvarez and Gamarnik, 2010).

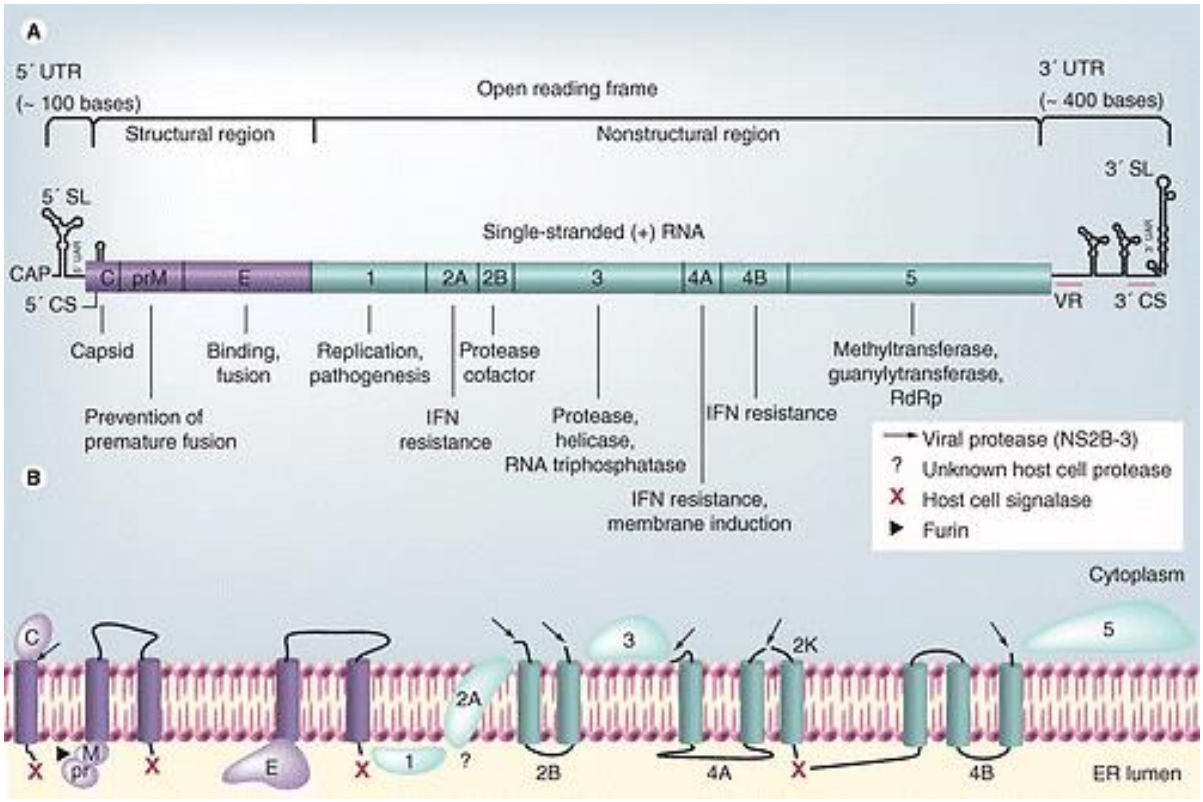


Figure 1.6. Dengue virus genome organization. (A) Organization of DENV genome with structural proteins (purple), nonstructural proteins (green) and SL structures at 5' and 3' UTR. Complementary sequences at the 5' and 3' end are highlighted.
(B) Dengue virus polyprotein in ER membrane. Post translational cleavage sites are marked based on different proteases. CS: Cyclization sequence; UTR: Untranslated region; M: Membrane protein; E: Envelope protein; IFN: Interferon; RdRp: RNA dependent RNA polymerase; VR: Variable region; SL: Stem loop; ER: Endoplasmic reticulum.
Adapted from (Bartenschlager and Miller, 2008).

1.12. Dengue virus structural proteins

1.12.1. Capsid protein

Capsid is a ~11kD highly basic protein. In the nascent form Capsid protein contains a hydrophobic end at C-terminal. This acts as an anchor during synthesis in ER and gets cleaved at two steps by NS2B-3 and signal peptidase respectively. DV capsid is important for interaction with RNA and formation of core nucleocapsid (Sangiambut *et al.*, 2008). There are reports of nuclear localization of capsid protein and this is more understandable from a motif located (85-100 aa of Capsid) which is a nuclear localization signal (Sangiambut *et al.*, 2008; Netsawang *et al.*, 2010).

1.12.2. Membrane Glycoprotein prM

The precursor of M or prM is a ~26kDa glycoprotein with 1-3 N-linked glycosylation sites. It also contains disulfide linked six conserved cysteine residues. Just like Capsid, prM also contains C-terminal hydrophobic domain which acts as an ER retention signal. The main function of prM is to regulate and assist the proper folding of Envelope (E) glycoprotein. prM prevents the untimely rearrangement of E in acidic environment during the transit of immature virions through secretory pathway. The pr domain has seven β strands and it fits over the E protein to form pr-E spikes over the immature virions (Wang, He and Anderson, 1999a). Thus inaccessibility of E fusion domain during the movement through secretory pathway prevents the fusion with the membrane of secretory vesicle. In these vesicle prM stay inaccessible due to steric hindrance. The acidic environment of trans-Golgi vesicles induces the rearrangement which exposes furin cleavage site of prM. But even after cleavage pr peptide do not get separated from virus particle immediately. Pr part gets separated only after exposure to the extracellular neutral pH environment and this results in formation of mature virions (Wang, He and Anderson, 1999a; Perera and Kuhn, 2008).

1.12.3. Envelope Glycoprotein

Envelope (E) is a ~53kDa glycoprotein found on the surface of virus particle. The maturation and folding of E depends on the association with prM. E is considered as fusion protein of class II which mediates membrane fusion along with interaction with the receptors. There are three domains in each E-protein, DI, DII and DIII. DII contains the fusion peptide and it is covered by pr peptide located in the hydrophobic hinge between DI and DIII, to prevent the fusion with secretory vesicle. DIII stays slightly up from the surface of the mature virion and it is considered to be involved in binding with the receptor (Gromowski, Barrett and Barrett, 2008). In line with this, it is observed that most of the neutralizing antibodies target the DIII domain (Lai *et al.*, 2008).

1.13. Dengue virus nonstructural proteins

1.13.1. NS1 Glycoprotein

Nonstructural protein 1 (NS1) is a ~48kDa glycoprotein which gets translocated into ER during its synthesis. The N-terminal processing of NS1 is done by host protease and at C-terminal end NS1-2A link gets cleaved by some uncharacterized host protease. NS1 has 12 conserved cysteine residues and three N-linked glycosylation sites. Soon after synthesis, NS1 form homodimers which have affinity for membrane association. But this affinity at peptide level is not very clear. NS1 expression over the infected cells is observed and it can invite antibodies followed by complement mediated lysis of the infected cells (Scaturro *et al.*, 2015). NS1 gets secreted from the infected cells and it is the most abundant viral protein in the serum of DV infected patient. NS1 capture ELISA is used for early detection of DV infection and it can give positive result even before RT-PCR detection level is reached (Chuansumrit *et al.*, 2008; Shan *et al.*, 2015). Secretory form of NS1 is hexameric and consists of three dimers with a detergent sensitive hydrophobic cavity. This cavity may carry

around 70 lipid molecules which is comparable with HDL buildup (Gutsche *et al.*, 2011). Due to the presence of such lipid rich structures NS1 can bind with cell membrane and internalize probably through interaction with glycosaminoglycans (Gutsche *et al.*, 2011). Due to the similarity with alpha-lipoprotein, NS1 has been suspected to interfere with the coagulation cascade by affecting the biogenesis of endogenous lipoprotein particles. Secreted NS1 is highly immunogenic and elicit antibody production. There are evidences that anti-NS1 antibodies are found to cross-react with host proteins (Lin *et al.*, 2002) and it can result in vascular leakage as well (Beatty *et al.*, 2015; Killingbeck *et al.*, 2015; Puerta-Guardo, Glasner and Harris, 2016). DV NS1 can bind and increase the turnover of C4 of classical complement pathway and helps to escape complement mediated lysis (Chen, Lai and Yeh, 2018).

1.13.2. NS2A and NS2B protein

NS2A is a comparatively small (~22kDa) protein and its C-terminal end is processed by NS2B-3 viral protease. DV2 NS2A has been found to affect IFN signaling as observed in some mutational studies (Muñoz-Jordán *et al.*, 2003).

NS2B is a cofactor of NS2B-3 serine protease. It is a small protein around 14kDa and has affinity for membrane. After forming complex with NS3, central peptide of NS2B interacts with the fold of serine protease and activates protease domain. There is a 40 amino acid segment from 1396 to 1435 which is essential for NS3 protease activity. This is a hydrophilic domain with surrounding hydrophobic regions (Leung *et al.*, 2001).

1.13.3. NS3 protein

NS3 is the second largest nonstructural protein of DV and it has enzymatic function in post translational processing of polyprotein and RNA replication. The N-terminal part contains the catalytic core of serine protease which considers the consecutive basic residues as substrate at

NS2A-NS2B, NS2B-NS3, NS3-NS4A, NS4B-NS5 junctions. Additionally it also process C-terminal end of mature capsid and NS4A (Leung *et al.*, 2001). The C-terminal end of NS3 has super group 2 RNA helicase-NTPase. RNA unwinding is mediated by NS3 using RNA stimulated NTPase (Cui *et al.*, 1998). This C-terminal region also shows RNA triphosphatase (RTPase) activity to dephosphorylate 5' end of whole genome before capping (Wengler and Wengler, 1993).

1.13.4. NS4A and NS4B proteins

Both NS4A and NS4B are comparatively small (16kDa and 27kDa) proteins with hydrophobic nature. NS4A has been reported to be associated with RNA replication and found to get co-localized with replication complex. NS4A synthesize components that keep the replication complex bound to the membrane bound. The interaction between NS1 and NS4A is quite important for replication (Lindenbach and Rice, 1999). NS4A is the least characterized DV nonstructural protein.

NS4B is found to co-localize with NS3 at the site of RNA replication. NS4A and NS4B both can block the IFN-I signaling. It is observed that first 125 amino acids of NS4B is capable enough to hinder the IFN-α/β signaling (Castillo Ramirez and Urcuqui-Inchima, 2015).

1.13.5. NS5 protein

NS5 is a conserved phosphoprotein with N-terminal methyl transferase domain and C-terminal RdRp domain. It performs a key function in RNA dependent RNA synthesis. The formation of 5' cap of viral RNA involves four major steps; (i) one phosphate group will be removed from 5' triphosphorylated RNA, (ii) 5' guanosine cap will be added from a guanosine triphosphate by a guanylyltransferase, (iii) N7 of the guanylyl cap will be methylated by a methyltransferase, (iv) Methylation of 2' O of the second residue will be done by the same methyltransferase (Davidson, 2009).

As mentioned before NS3 has RTPase activity and performs the first step. This also suggests that NS5 and NS3 both work together during capping of RNA. Although it was elusive for long time, recent data suggest that N-terminal domain of NS5 is capable of acting as guanylyltransferase. Finally, the N-terminal methyl transferase domain of NS5 can add methyl group to both N7 and 2'-O position. Mutation studies on NS5 capping domain revealed that N7 methylation is essential for viral translation whereas 2'-O methylation helps to evade host innate antiviral response (Egloff *et al.*, 2002).

The C-terminal RdRp domain of NS5 is quite conserved and has resemblance with other RNA polymerases. The RNA polymerase activity of NS5 has been proved *in vitro* and it is used as a model for screening new RdRp inhibitors. It is believed that NS5 binds with the 5'SL and then circularize to reach the 3' terminal to start the negative strand synthesis (You and Padmanabhan, 1999; Iglesias and Gamarnik, 2011).

1.14. Dengue virus RNA replication

The DV RNA replication starts with the synthesis of negative strand of whole genome which is then used as a template for the synthesis of positive strand. The negative strand is detectable as early as 3hrs post infection (Lindenbach and Rice, 1997). Viral RNA synthesis is unique due to its asymmetric nature in which positive strand accumulation is ten times more than the negative one (Cleaves, Ryan and Walter Schlesinger, 1981). After viral RNA replication, metabolic labeling has been done with actinomycin D. There are three main species of viral RNA that have been reported in DV infection. The first type is the positive sense (plus-strand) genome, second is the replicative form consisting of double stranded RNA and lastly the replicative intermediate form which contains double stranded and newly synthesized RNA (Wengler, Wengler and Gross, 1978; Cleaves, Ryan and Walter Schlesinger, 1981). During the replication subgenomic RNAs have been found in infected cells. Mostly these RNAs are 0.2 to 0.6 kb in length and quite linear with 3' non coding region (Urosevic *et al.,* 1997).

1.15. Assembly and release of particles from DV infected cells

The virus assembly starts with the interaction between basic capsid dimer and genomic RNA and formation of nucleocapsid precursor in cytoplasm. This is followed by budding of the nucleocapsids into ER lumen containing E-prM complex. E-prM complex formation should occur simultaneously to confirm the folding of E (Wang, He and Anderson, 1999b). Electron microscopy studies on DV infected cells revealed that vesicle containing the replicase complex and the site of virus budding in ER both are the part of a continuous network. There are some pores within the replication vesicle from which newly synthesized RNAs get released just beside the DV budding area in ER (Welsch *et al.*, 2009). It is believed that some sort of co-ordination work occurs between these two machineries to reduce the chance of

defective genome packaging. After packing of mature virion, it gets transported through trans golgi secretary pathway to reach the cell surface. During this transport, there is a change of pH which is responsible for E-prM shuffling, followed by prM cleavage(Wengler and Wengler, 1989; Randolph and Stollar, 1990; Welsch *et al.*, 2009).

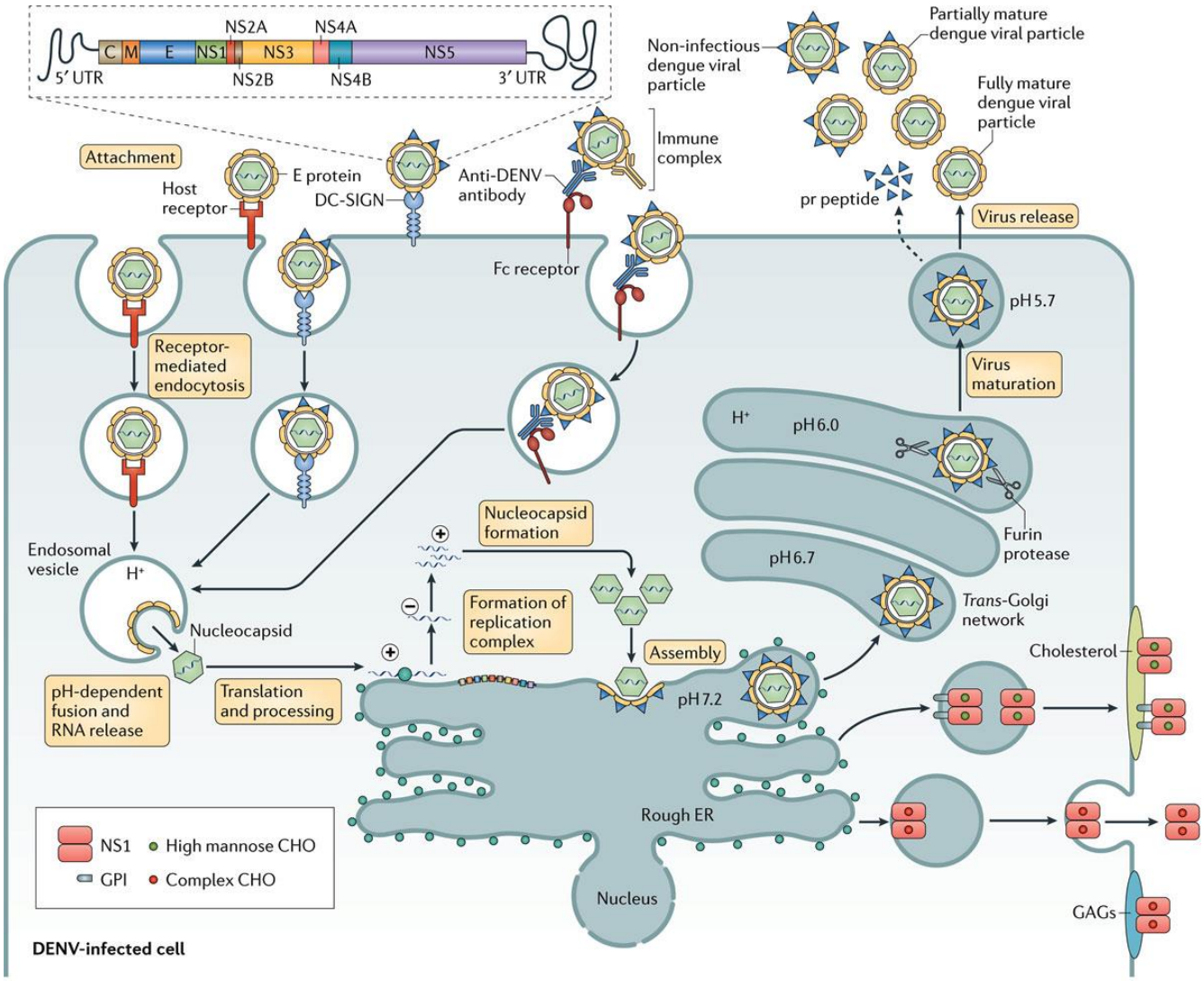


Figure 1.7. Schematic diagram of dengue virus replication in a cell.
Viral particles attach with host cell via interaction between envelope protein (E) and some less characterized receptors which are followed by receptor mediated endocytosis. In case of secondary infection, Fc receptors mediate viral entry. Inside endosomal vesicle pH dependent rearrangement of E protein leads to fusion of endosomal and viral membrane. This leads to release of nucleocapsid which then dissociates to release viral RNA with 5' cap. Translation of viral RNA leads to formation of polyprotein which is enzymatically cleaved by NS2b-3 viral protease or host proteases. The nonstructural proteins assemble around ER to form the replication complex. NS5 along with other viral and host proteins synthesize negative strand RNA which is followed by positive strand synthesis. NS1 forms in the ER as monomer which then dimerizes and moves to the cell surface. + and – signs stand for positive and negative sense RNA; C: Capsid; DC-SIGN: dendritic cell specific ICAM3-grabbing non-integrin; UTR: Untranslated region. Adapted from (Guzman *et al.*, 2016).

1.16. Dengue virus pathogenesis

1.16.1. Role of NS1 in Dengue virus pathogenesis

DV NS1 has been reported as a major viral factor responsible for endothelial cell disruption and vascular leakage. DV NS1 can elicit hyper immune response through cytokine production mainly by macrophages and PBMCs. TLR4 performs a key role in this immune activation (Modhiran *et al.*, 2015; Pang, Zhang and Cheng, 2017a). Endothelial permeability has been reported to increase with NS1 in a dose dependent manner in mouse model. But endothelial permeability returns to normal upon anti-NS1 Ab treatment. NS1 also disrupts the glycocalyx of vascular epithelial cells causing leakage in body cavity. NS1-mediated secretion of pro - inflammatory cytokines also contributes to endothelial permeability. DENV NS1 can activate alternate complement pathway, specifically targeting liver cells, resulting in the release of inflammatory cytokines. This results in plasma leakage and fluid accumulation, resulting in DSS (Glasner *et al.*, 2018). It was also observed that anti-DV antibodies can cross react with endothelial cell surface thereby initiating the complement activation which is followed by membrane attack complex formation (Puerta-Guardo, Glasner and Harris, 2016). There are reports stating that increased plasma NS1 level is co-related with severe dengue (Libraty *et al.*, 2002).

1.16.2. Role of anti-NS1 antibodies

In addition to NS1 antigen, anti-NS1 antibodies are considered to play a major role in pathogenesis of DV infection. Endothelial cells secrete cytokines in an uncontrolled way in presence of anti-NS1 Abs. Lin et al. have shown that anti-NS1 antibodies attach with the NS1-GPI complex on cell membranes and this activates signal transduction pathways through phosphorylation of tyrosine and other proteins. DV NS1 Abs can activate NF-kB pathway which initiate the transcription of various pro-inflammatory cytokines (Lin *et al.*, 2005).

Among them three main cytokines are MCP-1, IL-6 and IL-8. IL-6 and IL-8 have been associated with clinical features of DHF. This study also reported that DV infected endothelial cells secreted more MCP-1 upon treatment with anti-NS1 Abs. MCP-1 up-regulates ICAM-1 expression of NS1-Ab treated HMEC-1 cells. So, endothelial cells get activated with expression of ICAM-1 which may attract the immune cells and results in endothelial cell damage (Lin *et al.*, 2005). Mehta VK et al. observed IL-6 and IL-8 levels in serum and CSF is related with the neurological complication of dengue (Mehta *et al.*, 2017). Similarly significantly higher levels of IL-6 and IL-8 were reported in DHF than DF. It is thought that IL-8 is more relevant in terms of DHF as IL-8 is related with thrombocytopenia and ALT level (Priyadarshini *et al.*, 2010). As a pro-inflammatory cytokine IL-6 is actively involved in pathogenesis of DV along with IL-1 and TNF-α. Another study reported that >50% of DHF patients who died had a above 200pg/ml IL-8 (Chaturvedi *et al.*, 2000).

Liver is often get affected in severe dengue disease and NS1-Abs are found to cause liver injury in murine model (Lin *et al.*, 2008). They have also noticed NS1-Abs bind with the vascular endothelium of portal and central veins of mouse liver. Further histopathological study of liver from NS1 immunized mice showed fatty liver, hepatic fibrosis, vesicle formation and necrotic bodies. Serum levels of ALT and AST were much higher in mice administered with NS1-Abs (Lin *et al.*, 2008). Autopsy and liver histopathology of DHF indicated that kupffer cells and hepatocytes might be a target of DV for replication (Huerre *et al.*, 2001). So, in severe dengue there are multiple ways through which liver damage can occur.

NS1 has some immunogenic sequence homology with some host proteins and it renders some self-antigens as target of NS1-Ab. Among structural proteins prM and E proteins also have some homology and elicit self-reacting Abs (Wan *et al.*, 2013). DV infection induced autoantibodies can cross-react with integrin, plasminogen and platelets (Chuang *et al.*, 2016).

In DHF and DSS patients, anti-endothelial cells and anti-platelets Abs are much higher than only DF patients (Wan *et al.*, 2013).

1.16.3. Antibody dependent enhancement (ADE)

The chances of Dengue Hemorrhagic Fever (DHF) and Dengue Shock syndrome (DSS) are higher in case of heterotypic secondary dengue infection than primary infection (Guzman, Alvarez and Halstead, 2013). Numerous evidences show that if an infant is infected with a different dengue serotype from its mother which is possible when the woman was previously infected with a different serotype then it might be fatal for the child. About 5% of the children hospitalized due to dengue infection comprises of such situations in South-East Asian countries (Halstead *et al.*, 2002). Some researchers assumed that this situation was due to the incapability of the antibodies produced during primary infection to neutralize the dengue virus in the subsequent infection with a different serotype. This is known as antibody-dependent enhancement (ADE) and was first observed in 1973, where PBMCs cultured from a dengue-infected individual showed rapid growth of DV than a non-infected person.

A 100-fold increase in viral replication was observed in rhesus monkeys that were injected with anti-DV antibodies prior to viral inoculation (Goncalvez *et al.*, 2007; Chaichana *et al.*, 2014; Pang, Zhang and Cheng, 2017b). A proper documentation on ADE of dengue virus is obtained from Cuban epidemic where the most circulating serotype during 1977-1979 was DV-1 and during 1981 and 1997 it was DV-2. Though the exact mechanism of ADE is unclear but it can be concluded that pre-existing non-neutralizing antibodies from the primary infection cross-reacts and binds to the virus particles in the subsequent heterotypic dengue infection thus forming virion-antibody immune complexes rather than neutralizing them. The Fcɣ-receptor recognizes these virus-immune complexes which lead to its uptake by phagocytosis thereby causing enhancement of viral replication.

Apart from Fcɣ-receptor dependent viral replication another mechanism by which increase in virus replication occurs is called intrinsic ADE. According to this mechanism, the antiviral genes are inhibited after the uptake of the dengue virus inside the cell by Fcɣ-receptor because of the suppression of the antiviral response produced by the Type-1 IFN. In another instance, overproduction of IL-10 shifts the immune response towards TH-2 which down regulates the antiviral effect as well as decreases viral clearance (Halstead *et al.*, 2010; Ubol *et al.*, 2010; Wan *et al.*, 2013). Moreover, increased release of cytokines occurs during Fcɣ-receptor mediated ADE which causes higher vascular permeability due to endothelial cell dysfunction in the vascular layer (King, Anderson and Marshall, 2002; Brown *et al.*, 2011).

The ADE associated with DV infection makes it difficult to design an effective vaccine against dengue. There are few studies stating the ADE associated with the DV vaccine (Hadinegoro *et al.*, 2015; Martínez-Vega *et al.*, 2017). Based on the possibility that DV vaccine can prime seronegative individuals as first infection, policy makers of health administration have not yet ready to incorporate DV vaccine in routine vaccination schedule (*PAHO/WHO | XXIV Technical Advisory Group (TAG) Meeting on Vaccine-preventable Diseases*; Martínez-Vega *et al.*, 2017). The observation of neutralizing antibody against DV is the basis of the vaccine research. But if a vaccine induces antibody response similar to initial infection and those antibodies are cross-reacting with other serotypes, the approach is needed to be reassessed (Ferguson *et al.*, 2016; Katzelnick *et al.*, 2017).

1.17. Objectives

1. Molecular and serological characterization of Dengue virus (DV) serotypes prevalent in Kolkata during 2017-18.

2. Passage of clinical isolates of Dengue virus in suitable cell lines, to establish indigenous virus strains for future work.

3. In-depth evaluation of Dengue virus-mediated cellular apoptosis.

4. Analysis of DV mediated pathogenesis, towards deciphering target for intervening strategies.

2. Chapter 2

Molecular characterization of DV circulating in Kolkata and virus stock preparation

2.1. Background

Dengue is a mosquito borne disease endemic in India in which Kolkata is a metropolitan city where DV infections have been reported every year. We thought to use clinical DV samples in our study and considered Kolkata as our site for sample collection. Initially serum samples were collected from Calcutta National Medical College (CNMC) and stored in -80^{0}C. As the volume of the samples were low i.e. 200-300ul, it was inoculated in Vero cells. Then RNA was extracted from cell culture and nucleic acid level screening was done. As a part of the first and second objective, different circulating dengue serotypes of Kolkata were investigated followed by establishment of the clinical DV stocks for further study.

2.2. Experimental methods

2.2.1. Serum sample collection

Serum samples with proper information of patients were collected during 2017-2018. Samples were selected based on presence of dengue like symptoms in the patients and NS1 antigen positive results. Biosafety committees of Calcutta National Medical College (CNMC) and CSIR-Indian Institute of Chemical Biology, Kolkata have approved this study. Written consents were taken from all the patients in their native language but the samples were used as anonymous during this study.

2.2.2. Ethics statement

This study was performed in accordance with the ethical standards (at par with the 1964 Helsinki declaration and its later amendments) of the review boards of all relevant institutions. Ethical approval for the research was granted by the respective Institutional Ethical Committee of CSIR-IICB and Calcutta National Medical College, Kolkata. All experiments were carried out in accordance with the relevant guidelines and regulations.

2.2.3. Virus inoculation in Vero Cells

Vero cells were obtained from NCCS, India. Cells were cultured in DMEM (D5796, Sigma) supplemented with 10% FBS (Gibco) and Pen-Strep and L-Glutamine mix (Sigma). Cells were grown at 37^0C with 5% CO_2. During passage, cells were washed with PBS (1X) and detached with Trypsin-EDTA (1X) (Gibco). For culture, Thermo-Nunc flasks and culture plates were used. The volume of serum samples were low 200-300ul and mosquito cell line culture was not established in our lab initially. So, all samples were passaged once in Vero (Green monkey kidney) cells and then cell extract was prepared for RNA extraction. Vero is considered a suitable cell line for virus culture as it has spontaneous deletion in Type 1 interferon gene (Desmyter, Melnick and Rawls, 1968; Mosca and Pitha, 1986). Serum samples were filtered using Millipore 0.22 µm PES syringe filters. 70% confluent monolayer Vero cells were infected with filtered serum (inoculum volume made up to 800µl in MEM for T-25 flask of C6/36 cells); adsorption was done for 2h under normal cell culture conditions with intermittent shaking at every 15minutes. Cells with virus were incubated for 120h. During harvesting T-25 containing cells were frozen at -80^0C in 1ml supernatant. These cells in supernatant were rapidly thawed and centrifuged at 13,000 rpm for 15min at 4^0C to pellet the cellular debris. The resultant clear supernatant was aliquoted and stored at -80^0C as virus stocks.

2.2.4. Isolation of Viral RNA

RNA extraction was carried out using “High Pure Nucleic Acid Kit” (Roche Applied Science, Cat no. 11858874001) following manufacturer’s protocol. RNA was eluted in a sterile nuclease free micro centrifuge tube using 40µl of elution buffer. Purity and quantification of isolated viral RNA was estimated using Nanodrop and stored at -80°C.

2.2.4. Reverse Transcription & PCR

Virus serotyping is done as per Lanciotti *et al*. (Lanciotti *et al.*, 1992).Two primers D1 (Forward) and D2(Reverse) were used to amplify a 511bp product (genome position 134-644) which is homologous to genomic RNA of four serotypes. Here downstream consensus primer D2 is used to get cDNA from viral RNA using RT enzyme (Superscript III, Invitrogen). This is followed by PCR using D1 & D2 primers (GoTaq, Promega). There are other four serotype specific primers which are used for identification of serotypes. For serotyping a second round amplification is done on first round 1:10 diluted D1&D2 product (511bp). The table 2.1 is depicting about the primer combinations and amplified band lengths. For the second round D1 was used as forward primer and TS1, TS2, TS3, TS4 were used as reverse primers, are used in a single PCR mix. Because of the position of priming with each Dengue virus type specific primers (Table2.2), the size of the resulting DNA bands is characteristic of each Dengue virus type.

D2 primer was used for reverse transcription as DV is a positive strand RNA virus-

1. Primer – D2 (20uM)- 2µl
2. dNTP (10µM) mix- 1.5µl
3. RNA – 10ul of each RNA was used.
4. The above mixture was heated at 65^0C for 5min.

Then it was incubated on ice for 1 min and centrifuged the tube briefly.

2^{nd} Step:

4µl 5X first stand buffer.

1µl 0.1(M) DTT

1µl RNase IN

1.5µl of Superscript III RT (200units/µl)

Then it was mixed by pipetting gently up and down.

* Gene specific reaction temperature 55^0C for 60 minutes followed by inactivation of the reaction by heating at 70^0C for 15 minutes. After that D1 and D2 PCR was done using 10µl of cDNA.

1. NF water- 12µl *5= 60µl
2. Go Taq PCR MIX- 25µl *5=125µl
3. D1 PRIMER (20µM)- 1.5µl *5=7.5µl
4. D2 PRIMER(20µM)- 1.5µl *5=7.5µl
5. Template – 10µl cDNA

95^0C for 5 mins 1 cycle

94^0C for 30 sec, 55^0C for 1 min, 72^0C for 1 min -- 35 cycles.

72^0C for 7 mins --- 1cycle

1. NF water- 12µl
2. Go Taq PCR MIX- 25µl
3. D1 PRIMER (20µM)- 1.5µl
4. TS PRIMER (20µM)- 1.5µl
5. Template – 10µl DNA (1:10 DIL OF 1^{ST} ROUND) (10µl of 1^{st} round PCR product was mixed in 90µl of NF water)

(TS primer includes TS1, TS2, TS3, TS4)

Total reaction volume 50 µl.

95^0C for 5 mins – 1 cycle

94^0C for 30 sec, 55^0Cfor 1 min, 72^0C for 1 min ----- 25 cycles.

72^0C for 7 min --- 1cycle

Gel Electrophoresis was done with 1% Agarose Gel containing SYBR safe view dye.

Name of serotype	Band size in Gel electrophoresis	Specific PCR
DV	511bp	D1 & D2 (First round PCR)
DV1	482bp	D1 & TS1 (2nd round PCR)
DV2	119bp	D1 & TS2 (2nd round PCR)
DV3	290bp	D1 & TS3 (2nd round PCR)
DV4	392bp	D1 & TS4 (2nd round PCR)

Table 2.1. Combination of primers along with their respective amplified product size. (Lanciotti *et al.*, 1992)

Primer	Sequence	Genome position
D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'	134-161
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	616-644
TS1	5'-CGTCTCAGTGATCCGGGGG-3'	568-586
TS2	5'-CGCCACAAGGGCCATGAACAG-3'	232-252
TS3	5'-TAACATCATCATGAGACAGAGC-3'	400-421
TS4	5'-CTCTGTTGTCTTAAACAAGAGA-3'	506-527

Table 2.2. Sequence of primers used for serotyping of samples. (Lanciotti *et al.*, 1992)

2.2.5. DV NS1 Ag ELISA

NS1 Ag is the most abundant viral protein in the serum of DV infected patients. It is secreted from the DV infected cell and often linked with severity. We tried to find out the level of NS1 secretion of the serum inoculated Vero cells. NS1 Platelia ELISA kit (BioRad) was used for this purpose. It is a sandwitch ELISA in which anti-NS1 Ab is coated on the wells. Samples with NS1 are mixed with another anti NS1 Ab (against different epitope) which is also HRP tagged. Then TMB substrate is used to develop colorimetric reaction. Sample OD at 450nm is directly proportional to the NS1-Ag present. Cell supernatants of serum passaged Vero cells were used for ELISA, 50ul of each supernatant was used.

2.2.6. Real time PCR

Quantitative RT-PCR was standardized using primer sets as described by Lanciotti *et al.*,1992. For example, D1 and TS1 primers amplify 482bp fragment which is cloned in TOPO-TA plasmid (Invitrogen). Then serial dilution of insert containing plasmid was done to prepare the standards with known copies of plasmids. SYBR based Luna one step RT-PCR (NEB) was used. RT-PCR was done in Quant studio5 real time machine. Amplified products were run in gel to confirm specific bands. RT-PCR amplified products were checked in 1% agarose gel to see the specific product. This was to ensure that the quantification of RT-PCR is based on the specific product only.

Thermal cycling details:

55^0C for 30mins. – 1cycle, (95^0C for 1min- 1cycle, 95^0C for 10sec, 55^0C for 30sec, 72^0C for 45sec-reading)-40 cycles, 72^0C for 5mins – 1cycle, (95^0C for 15sec, 60^0C for 1min-Reading, 95^0C for 1sec) Melt curve

2.2.7. Inoculation of clinical DV in C6/36 cell line

C6/36 which is an *Aedes albopictus* larvae cell line was obtained from NCCS, India. C6/36 cells were cultured in MEM (Sigma), supplemented with 10% FBS (Gibco), Pen-Strep and L-Glutamine (Sigma) and Fungizone (Gibco). C6/36 cells were grown at 28^0C with 5% CO_2. C6/36 cell line is considered as an ideal host for DV replication. 70% confluent monolayer of C6/36 cells was infected with cell lysate of passage 1 (inoculum volume made up to 800µl in MEM for T-25 flask of C6/36 cells); adsorption was done for 2h under normal cell culture conditions with intermittent shaking at every 15minutes. Cells with viruses were incubated for 120h. Three such passages were given in C6/36. During harvesting T-25 containing cells were frozen at -80^0C in 1ml supernatant. These cells in supernatant were rapidly thawed and centrifuged at 13,000 rpm for 15min at 4^0C to pellet the cellular debris. The resultant clear supernatant was aliquoted and stored at -80^0C as virus stocks.

2.3. Results

2.3.1. Nucleic acid level screening of serum samples

Serotypes of serum samples passaged in Vero cells were determined and tabulated in table 2.4. Total 24 serum samples were passaged in Vero cells and followed by RNA extraction was done (Table 2.3). After nucleic acid level serotyping only 12 samples were found to be positive for dengue virus. Among these twelve samples eight were DV type 2, two of each DV type 1 and 3. We did not find any DV type 4, although our number of samples was low.

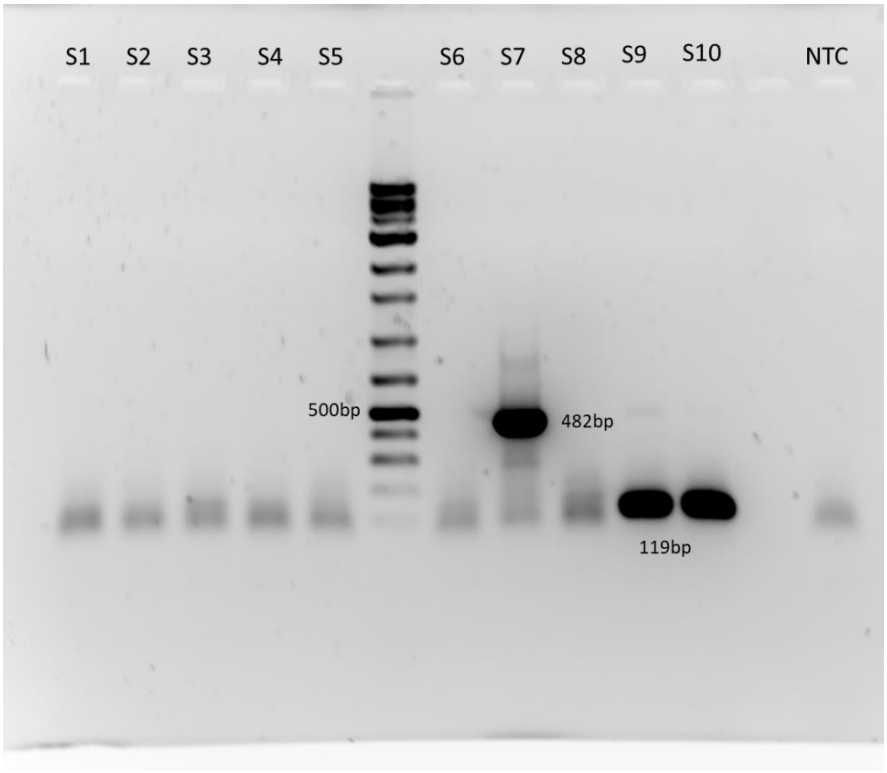
Sample Name	RNA Conc.(ng/ul)	A260/A280
S1,P1	409.9	2.46
S2,P1	300.7	2.98
S3,P1	308.6	2.86
S4,P1	326.8	2.81
S5,P1	334.9	2.79
S6,P1	355.4	2.86
S7,P1 (DV1-HNSBI,P1)	357.9	2.78
S8,P1	311.2	2.80
S9,P1	328.1	2.81
S10,P1	343.5	2.80
S11,P1	236.2	3.0
S12,P1	275.7	2.65
S13,P1 (DV2-HNSBI,P1)	260.6	2.89
S14,P1	196	3.12
S15,P1	253.6	2.99
S16,P1	275.6	2.70
S17,P1	260.2	2.88
S18,P1 (DV3-HNSBI,P1)	329.5	2.63
S19,P1 (DV1-HNSBII,P1)	347	3.33
S20,P1	360.8	3.24
S21,P1	404.8	3.00
S22,P1	366.5	3.14
S23,P1	383.7	3.16
S24,P1	380.7	3.14

Table 2.1. RNA quantities of Vero cells inoculated with serum samples.

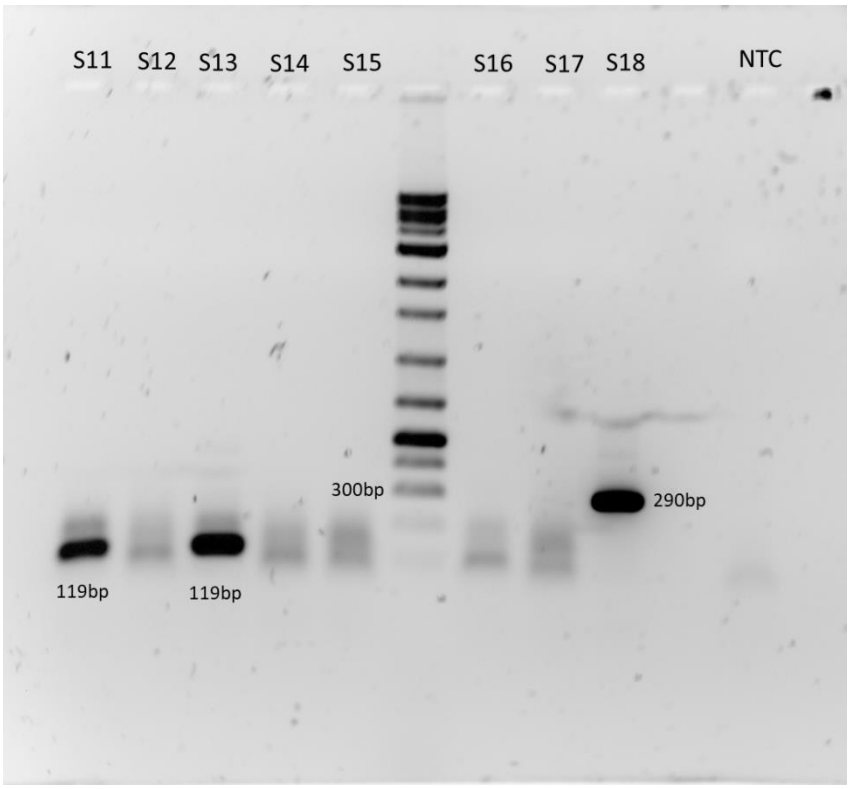
Sl. No	Name of Sample	Serotype
1	**S7,P1**	DENV type 1
2	**S9,P1**	DENV type 2
3	**S10,P1**	DENV type 2
4	**S11,P1**	DENV type 2
5	**S13,P1**	DENV type 2
6	**S18,P1**	DENV type 3
7	**S19,P1**	DENV type 1
8	**S20,P1**	DENV type 2
9	**S21,P1**	DENV type 2
10.	**S22,P1**	DENV type 3
11	**S23,P1**	DENV type 2
12	**S24,P1**	DENV type 2

Table 2.4. List of samples against their respective DV serotypes

A


S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
NTC
500bp
482bp
119bp

B


S11
S12
S13
S14
S15
S16
S17
S18
NTC
300bp
290bp
119bp
119bp

C

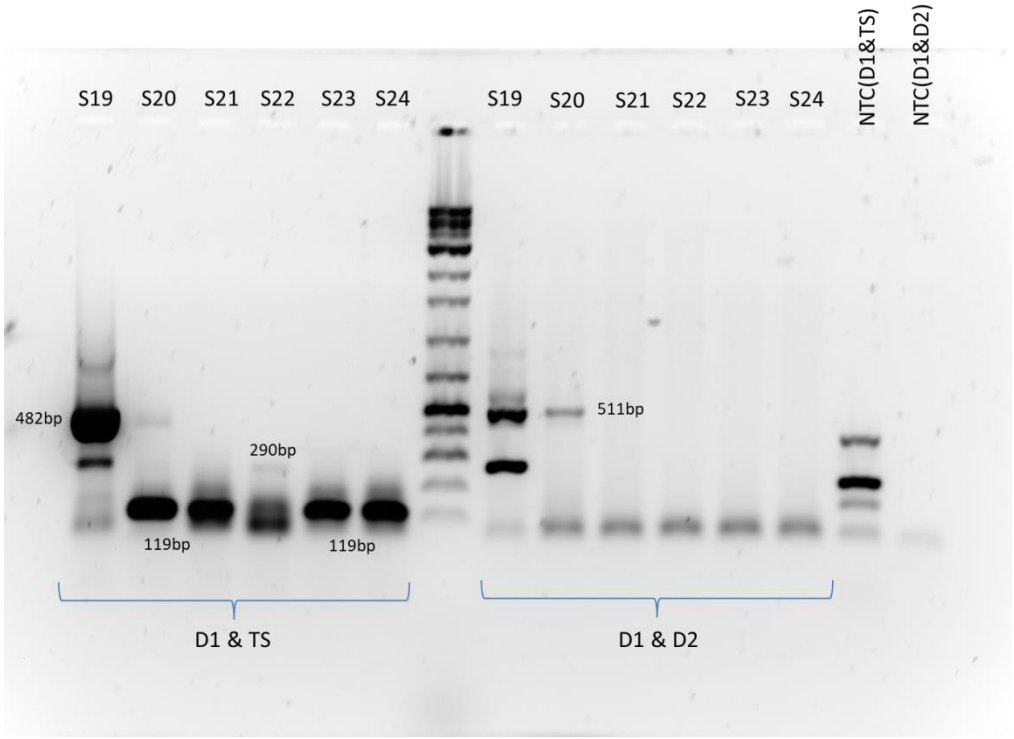


Figure 2.1. Gel Electrophoresis of RT-PCR amplified products.
For (A) and (B) panel gel electrophoresis of D1 and TS (TS1, TS2, TS3, TS4) primer specific RT-PCR products have been shown. For panel (C), amplified products of both rounds of nested PCR have been presented. For details of RT-PCR, please see 2.2.4. in the methods section.

2.3.2. Result of NS1-Ag ELISA

Cell supernatants of all virus infected cells were positive for NS1 Ag. Few samples were beyond the detection limit.

2.3.3. Quantitative RT-PCR of DV infected Vero cell lysate

Extracted RNA from passage 1 in Vero cells, were used in quantitative RT-PCR. The amplified products were checked by agarose gel electrophoresis. It was found that there were seven samples with prominent bands and have a high titer. These samples were chosen for stock preparation.

Sample Name	Serotype	Copy No.	Visible band in Gel Electrophoresis
S7 (DV1-HNSBI,P1)	DENV1	8.8 X 10^6 gE/ml	Yes
S9,P1	DENV2	1.4 X 10^5 gE/ml	Yes
S10,P1	DENV2	1.6 X 10^5 gE/ml	Yes
S11,P1	DENV2	2 X 10^5 gE/ml	Yes
S13,P1 (DV2-HNSBI)	DENV2	3.1 X 10^5 gE/ml	Yes
S18,P1 (DV3-HNSBI)	DENV3	1.7 X 10^6 gE/ml	Yes
S19,P1 (DV1-HNSBII)	DENV1	6.6 X 10^8 gE/ml	Yes
S20,P1	DENV2		No prominent band
S21,P1	DENV2	7.1 X 10^5 gE/ml	No prominent band
S22,P1	DENV3		No prominent band
S23,P1	DENV2		No prominent band
S24,P1	DENV2		No prominent band

Table 2.5. DV titers in samples (passage 1) as found in quantitative RT-PCR.
The detection of size (base pair) specific amplified product (band in gel electrophoresis) is mentioned at the rightmost column. Visibility of RT-PCR specific band further confirms the quantification.

2.3.4. Quantitative RT-PCR of DV infected C6/36 cell lysate

Based on the gE of DV as in Table 2.5, seven samples were chosen for inoculation in C6/36 cell line. The selection criterion was definitive band in gel electrophoresis to be sure about the desired product amplification. We planned three consecutive passages in mosquito cell line to increase the virus titer. At the end of three passages RNA was extracted from cell lysate and quantitative RT-PCR or real time PCR (qRT-PCR) was done. We found four viruses of three different serotypes appreciable titer and definitive amplification.

Sample Name	Serotype	Copy No.	Visible band in Gel Electrophoresis
DV1-HNSBII,P4	DENV1	2.5 X 10^9 gE/ml	Yes
DV1-HNSBI,P4	DENV1	4.1 X 10^{10} gE/ml	Yes
DV3-HNSBI,P4	DENV3	1.5 X 10^9 gE/ml	Yes
DV2-HNSBI,P4	DENV2	1.8 X 10^8 gE/ml	Yes

Table 2.6. DV titers in samples (passage 4) as found in qRT-PCR

2.4. Inference

As part of the first and second objectives, serum samples from dengue endemic area had been studied in detail. Nucleic acid level screening along with serotype characterization were done. The serum samples were passaged in cell lines to establish clinical DV stocks. After first passage in Vero cells seven samples representing three different serotypes were chosen to passage in C6/36 cell line. After three consecutive passages in C6/36 cells, four samples were standardized with decent gE copy numbers. These samples were used in further experiments to understand DV mediated pathogenesis in terms of apoptosis.

3. Chapter 3

Study of Dengue virus mediated apoptosis and NS1 as a target for antiviral

3.1. Study of dengue virus mediated apoptosis in cell culture

3.1.1. Background

Dengue virus infections affect multiple organ systems, the commonest being the liver (Fernando *et al.*, 2016). Hepatic dysfunction is an important feature of dengue as evident from various clinical reports (Dissanayake and Seneviratne, 2018). Liver involvement in dengue cases may vary from asymptomatic elevation of hepatic transaminases to severe manifestations in the form of acute liver failure (Samanta, 2015). Autopsy of dengue patients in Myanmar showed damage to liver with moderate to severe sinusoidal congestions involving midzonal and centrilobular areas (Aye *et al.*, 2014). In a study comprising of 240 patients, hepatic dysfunctions in the form of deranged total bilirubin (19.5%), AST (97.7%), ALT(93.9%), ALP (32.6%) and albumin (29.1%) were observed (Chhina *et al.*, 2008).

DV NS1 (48 KDa) is secreted from infected mammalian cells as hexamer and widely used as a diagnostic marker (Chuansumrit *et al.*, 2008). NS1 is essential for virus replication (Lindenbach and Rice, 1999; Youn *et al.*, 2012) and the secreted form contributes to complement fixation (Avirutnan *et al.*, 2010) and pathogenesis. NS1 has been shown to act as PAMP and activate TLR4, leading to induction and release of pro-inflammatory cytokines and chemokines (Modhiran *et al.*, 2015). Circulating levels of soluble NS1 (sNS1) vary from 0.04-0.6 µg/mL for DF, 0.6-2.5 µg/ml with reports of up to 15 µg/mL in case of DHF and persist for up to 4-6 days from the onset of fever (Libraty *et al.*, 2002). These data are based on NS1 levels measured in the bloodstream of infected patients. In mouse model, sNS1 was found to accumulate in liver and the hepatocytes appeared to be the major target cells *in vivo* (Alcon-LePoder *et al.*, 2005). Internalization and stability of sNS1in human hepatocyte cell lines like HepG2 and Huh7 had also been reported (Alcon-LePoder *et al.*, 2005). Antibodies against NS1 have been found to promote apoptosis of liver cells in mouse model (Lin *et al.*,

2008). But the direct effect of clinical DV infections on liver cells in terms of apoptosis was not clear. So, we have studied apoptosis in Huh7 cells upon infection with DV clinical isolates of serotype 1, 2 and 3 not passaged more than four times in cell culture. NS1 secretion in case of these clinical isolates in hepatocyte cells has been studied in details. African green monkey kidney cell line (Vero) is also used in this study.

3.1.2. Experimental methods

3.1.2.1. Cell culture

Vero and Huh7 cells were obtained from NCCS, India. Cells were cultured in DMEM (D5796, Sigma) supplemented with 10% FBS (Gibco) and Pen-Strep and L-Glutamine mix (Sigma). Monolayers of cells during experiments were maintained using DMEM supplemented with 1% FBS. Cells were grown at 37^0C with 5%CO_2. During passage, cells were washed with PBS (1X) and detached with Trypsin-EDTA (1X) (Gibco). For culture, Thermo-Nunc flasks and culture plates were used. C6/36 cells were cultured in MEM (Sigma), supplemented with 10% FBS (Gibco), Pen-Strep and L-Glutamine (Sigma) and Fungizone (Gibco). C6/36 cells were grown at 28^0C with 5%CO_2.

3.1.2.2. Viruses

Serum samples from dengue fever patients were collected from Calcutta National Medical College & Hospital, Kolkata-700014 with proper information and prior written patient consent. This study was approved by the Ethics Committee on Human Research of CSIR-Indian Institute of Chemical Biology, Kolkata. All serum samples were confirmed as Dengue virus-positive by means of NS1 diagnostic ELISA test (Platelia, Biorad). Serum samples were filtered using Millipore 0.22 µm PES syringe filters. 70% confluent monolayer Vero cells were infected with filtered serum (inoculum volume made up to 800µl in DMEM for T-25 flask of Vero cells); adsorption was done for 2h in 37^0C incubator with intermittent shaking

at every 15 minutes. Cells with virus were incubated for 120h. During harvesting T-25 containing cells were scraped in 1ml supernatant. These cells in supernatant were freeze-thawed and centrifuged at 13,000 rpm for 15 minutes at 4^0C to pellet the cellular debris. The resultant clear supernatant was aliquoted and stored at -80^0C as virus stocks. In case of inoculation in C6/36 cell line the protocol is same as above except MEM was used as culture media and incubated at 28^0C. Three such passages were given in C6/36. Virus serotyping was done as described by Lanciotti et al (Lanciotti *et al.*, 1992). Virus titer was determined using SYBR-based one step qRT-PCR with Luna Universal One Step qRT-PCR reagent (NEB). QuantStudio 5 (Applied Biosystems) was used to run the qPCRs. Primers as described by Lanciotti were used in qRT-PCRs.

3.1.2.3. TUNEL assay

TUNEL assay was done as per protocol of APO-BrdU™ TUNEL Assay Kit with Alexa Fluor™ 488 Anti-BrdU (Thermo, Life Tech). Adherent cells were trypsinized and centrifuged along with floating cells. The pellet was resuspended in 600µl 1X PBS. Then 4.4 ml of 70% chilled ethanol was added, keeping the cells suspended by mild vortexing. Cells in 70% ethanol were stored at -20^0C overnight. Next day, cells were centrifuged at 300g at 4^0C for 5mins and the supernatant was decanted. Cells were then washed twice with 1ml wash buffer. Thereafter, the pelleted cells were suspended in 50 µl reaction mix and incubated at 37^0C for 70mins. During incubation, tubes were tapped at every 15mins interval to keep the cells in suspension. After incubation cells were rinsed twice with 1ml of rinse buffer. Then 100 µl of antibody mix (5 µl in 145 µl rinse buffer) was added to each tube, keeping the cells suspended by tapping. Cells with antibody were incubated at room temperature (~25^0C) for 30mins. The cells were then washed once with 700 µl rinse buffer. After that, cells were suspended in 500 µl Propidium iodide (PI)-RNaseA solution and transferred to FACS tube. Cells were allowed to incubate with PI for 30mins, followed by FACS analysis in BD

LSRFortessa. Analysis of data was done using the BD FACSDiva 8.0.2 software. In TUNEL data analysis, gating for virus infected cells was based on uninfected cell control. In case of transfection experiments, gating was based on mock transfection control.

3.1.2.4. Apoptotic DNA Ladder assay

Adherent cells in T-25 flasks were detached using trypsin-EDTA and pelleted by centrifugation. Pelleted cells were lysed using cell lysis buffer (1X PBS, 0.2% TritonX100). RNaseA (Invitrogen) was added (2 µl) to the cell lysate (about 200-300 µl) and the resultant mix was incubated at RT for 5mins. This was followed by addition of 20 µl Proteinase K and incubation at 56^0C in heat block for 2h. Then equal volume of Phenol-Chloroform-Isoamyl alcohol (25:24:1) (HiMedia) was added and mixed by inverting. This was followed by centrifugation at 13,000rpm for 15mins. Of the three layers visible, upper most transparent layer was aspirated carefully to a new 1.5 ml Eppendorf tube. Isopropanol (1.2 times the volume of aspirated supernatant) was added to the supernatant and mixed well by inverting. 1 µl of Glycogen (Invitrogen) was added to the tube before storing it at -20^0C for 2hr. The tube was then centrifuged at 13,000rpm for 15mins at 4^0C to obtain the DNA pellet. Supernatant was discarded and pellet was washed using 70% ethanol twice. Pellet was air dried and suspended in 40µl nuclease-free (NF) water (Ambion). DNA quantity and quality were ascertained using Nanodrop One (Thermo). Equal quantities of cellular DNA from different conditions of the experiments were subjected to gel electrophoresis in 1.4% agarose gel with SYBR safe dye (Invitrogen). Agarose gel electrophoresis was done at 50V (5V/cm) for 3hr. Gel was observed in Gel Logic (Carestream) under UV transillumination.

3.1.2.5. NS1 Ag ELISA

ELISA was done as per protocol of Bio-Rad Platelia Dengue NS1 ELISA. In case of quantitative ELISA, serial dilutions of recombinant NS1 Antigen (Ag) (Bio-Rad) was used. Dilutions of Ag were made in PBS (1X). ELISA reading was taken in iMark plate reader

(BioRad)**.** Standard curve was generated from ODs of known dilutions of NS1-Ag. From the equation of that curve, soluble NS1 quantity of unknown samples were determined. Separate ELISAs were performed for each experiment.

3.1.2.6. Western Blot

Monolayer of Vero or Huh7 cells was subjected to Camptothecin (4µM) treatment for 12-13h in case of NS1 transfection or Dengue virus infection. Lysis buffer (1% TritonX100 in PBS, 10U/ml DNase1-Cat. No. D2821, Sigma with Proteinase inhibitor (Pierce, Thermo)) was directly applied on adherent cells and kept on ice for 10mins. Lysate was aspirated into 1.5ml tube and centrifuged at 13,000rpm for 15mins at 4^0C. Clear supernatant was aspirated and used for Western blotting. Protein quantification was done using BCA assay kit (Pierce). Cell lysate was separated by means of 5% (Stacking) and 15% (Resolving) SDS PAGE gel electrophoresis**,** using running buffer (25mM Tris, 192mM Glycine, 0.1% SDS: pH 8.3) at constant voltage of 90V for 2.5h. To observe the relative position of protein in gel, two protein ladders, namely PageRuler (Thermo) and Precision Dual Colour ladder (Bio-Rad) were used. For subsequent analysis proteins were transferred onto Nitrocellulose membrane using Trans-Blot Turbo Transfer System (Bio-Rad). Membrane was blocked using TBST with 5% skimmed milk for two hours at room temperature. Anti-Caspase 3 Ab (CST-9662) (1:1000) and anti-cleaved Caspase 3 Ab (CST-9661) (1:1000) were used to detect the target proteins of interest. Primary anti GAPDH Ab (CST-8884S), HRP conjugate (1:2000) was used as housekeeping protein control. Strips of membranes corresponding to target proteins (Caspase3, CC3 or GAPDH) were incubated with Primary antibodies, overnight at 4^0C with fresh blocking buffers. Strips were incubated with secondary anti-rabbit IgG-HRP (Abcam-97051) (1:2000) for 1.5h. The membranes were treated with ECL substrate (Bio-Rad) prior to visualization of the stained protein bands in Azure Biosystem Chemi Doc system or

ChemiDoc (BioRad). Densitometric analysis was done using ImageJ and Image Lab software (Bio-rad).

3.1.2.7. Cloning and expression of NS1 genes of Dengue virus types 1, 2, 3

RNA from virus was extracted using High Pure Viral Nucleic acid extraction kit (Roche). Using gene specific reverse primers of target genes, cDNAs were synthesized using Superscript-III (Invitrogen). Respective cDNA was used as template to PCR-amplify the desired target. PCR products were confirmed by agarose gel electrophoresis using 0.8-1% agarose and purified using PCR purification kit (Qiagen). In case of NS1 gene, for all three serotypes, the gene corresponding to signal sequences of 23-29 amino acids upstream of NS1 protein was included for NS1 gene amplification for cloning purposes. Primers for cloning contained appropriate restriction enzyme cutting sites, for instance, HindIII and BamHI restriction sites in forward and reverse primers respectively in case of DV1 NS1 gene (plus signal sequence). In case of DV2 NS1, NheI and XhoI restriction sites were incorporated in forward and reverse primers respectively. For, DV3 NS1, forward primer contained NheI and reverse primer contained BamHI restriction sites. Restriction enzymes (NEB) digestion was carried out for respective PCR products and the expression vector pcDNA3.1 (+). Ligation was done using T4 DNA ligase (Promega), followed by transformation in XL1-Blue cells. Plasmid was purified using Purelink-Midi plasmid purification kit (Invitrogen). Plasmid transfection was done using Fugene HD (Promega), following manufacturer's instructions.

DNA sequences deposited in GenBank	Accession numbers
DV1-HNSB-P4 NS1 gene sequence	MT072226
DV2-HNSB-P4 NS1 gene sequence	MT072227
DV3-HNSB-P4 NS1 gene sequence	MT072228
Oligonucleotide primer sequences (5'-3')	**Name of primers**
CGG CTA GCA GCA TGG GRT TGA ATT CAA ARA AYA CWT CC	DV3NS1F
CGC GGA TCC TTA BGC TGA GRC TAA AGA CTT TAC C	DV3NS1R
CGC GCT AGC GCC ATG AAT TCA CGY AGC ACC TC	DV2NS1F
CGC CTC GAG TTA RGC TGT RAC CAA AGA ATT G	DV2NS1R
CGA AGC TTA GCA TGA GGA RCA CGT CMC TYT CGA TG	DV1NS1F
CGC GGA TCC TTA TGC AGA GAC CAT TGA CCT GAC	DV1NS1R

Table 3.1. Dengue virus NS1 cloning primers and deposited sequence accession numbers

3.1.2.8. MTT assay

Huh7 cells were seeded as monolayer in 96 wells plate. Cells were infected with DV or transfected with NS1 gene construct. One column of cells was left uninfected/un-transfected, as positive control while another column was not seeded with cells but filled with media only, as negative control. Media was removed 48h post infection/transfection, from each well and the cells were washed using 1X PBS. Then 90 µl DMEM (Phenol red-free) and 10 µl MTT solution were added to each well. The plate was incubated at 37^0C and 5% CO_2 for four hours in a humidified CO_2 incubator for the formation of Formazan crystals in the living cells. Media was discarded from all wells; 100 µl DMSO was added to each well and the contents of the wells were mixed properly to dissolve the crystals. The plate was incubated for 30mins and absorbance was measured at 590nm wavelength.

% viable cells= (OD of sample-OD of blank)/ (OD of control-OD of blank) x 100

3.1.2.9. Immunofluorescence experiments

For DAPI-staining, monolayer of Huh7 cells was cultured on 22mm cover slips and treated (DV infection or NS1 gene transfection) or left untreated as needed. Media was removed at 48h post-treatment and cells were fixed with ice-cold 70% ethanol. This was followed by one wash with nuclease free water (Ambion). Cover slips were then soaked using lint free paper and mounted with Prolong Diamond antifade mountant with DAPI (Invitrogen). Images were taken using EVOS FL cell imaging system.

In case of DV envelope immunofluorescence, monolayer of Huh7 cells was grown on six chambered slide (Genetix). DV1, DV2 or DV3 were inoculated at 10 virus copies/cell. Virus adsorption was done for 2h. At 96h post infection, media was removed and cells were washed twice with PBS. Cells were subsequently fixed using 4% PFA in PBS for 10min at RT. Cells were again washed twice with 1XPBS. Cells were permeabilized with 0.1% Triton X100 in

1XPBST for 15mins at RT. Then blocking was done with 1%BSA and 22.52mg/ml Glycine in PBST for 45mins at RT on rocker. Cells were incubated with diluted (1:40) primary Ab (Ab41349) in 1% BSA in PBST for one hour at RT on a rocking platform. The antibody solution was then removed by decanting and the cells were washed three times with PBST, each wash for 5mins. The cells were then incubated with diluted (1:500) secondary Ab (Ab150113) in 1% BSA in PBST for 30mins at RT. Secondary Ab was removed and cells were washed four times with PBST, each of five min duration, on rocking platform. The slides were allowed to get dry and mounted with Prolong Diamond antifade mountant with DAPI (Invitrogen). Images were taken using Fluoview 10i, Confocal microscope (Olympus).

3.1.3. Results

3.1.3.1. Dengue virus and NS1 only, both can induce Cleaved Caspase3 (CC3)

Secreted NS1 from DV1-HNSB-P4-infected (10 virus copies or genome Equivalents (gE) /cell) and NS1-plasmid-transfected Huh7 cells in six well plates (approx. 10^6 cells at confluence), were measured in multiple experiments (Table 3.2). It was estimated that 1.0 µg pcDNA3.1-NS1 expression construct results in expression of 2.55 (± 0.59) µg/ml DV1-NS1 in the cell supernatant at 96h post-transfection. This was equivalent to 2.53 (± 0.79) µg/ml, expressed at 96h post-infection when the same number of cells were infected with the aforesaid virus inoculum (10 gE/cell). One more NS1-plasmid quantity i.e. 3.3 µg was tested, expressing 4.36 (± 0.33) µg/ml NS1 under similar conditions. The expression levels of CC3 rose with increasing levels of NS1 secretion, as observed in case of 1.0 µg and 3.3 µg NS1 plasmid transfected cells (Fig.3.1 a, b).

DV1-HNSB-P4 infected Vero cells expressed less NS1 compared to Huh7, 0.4 (± 0.09) µg/ml at 62h post infection. Still such cells also showed higher CC3 levels compared to uninfected cells (Fig.3.1 c, d). So, NS1 alone can induce apoptosis as observed from increased expression of CC3 over cell control in transfection experiments. Positive correlation between NS1 expression levels and CC3 in transfected cells, confirmed the above proposition. CC3 expression is considered a hallmark of apoptosis progression, so infected cells were supposed to undergo apoptotic DNA breaks. TUNEL and apoptotic DNA ladder experiments were performed to confirm this.

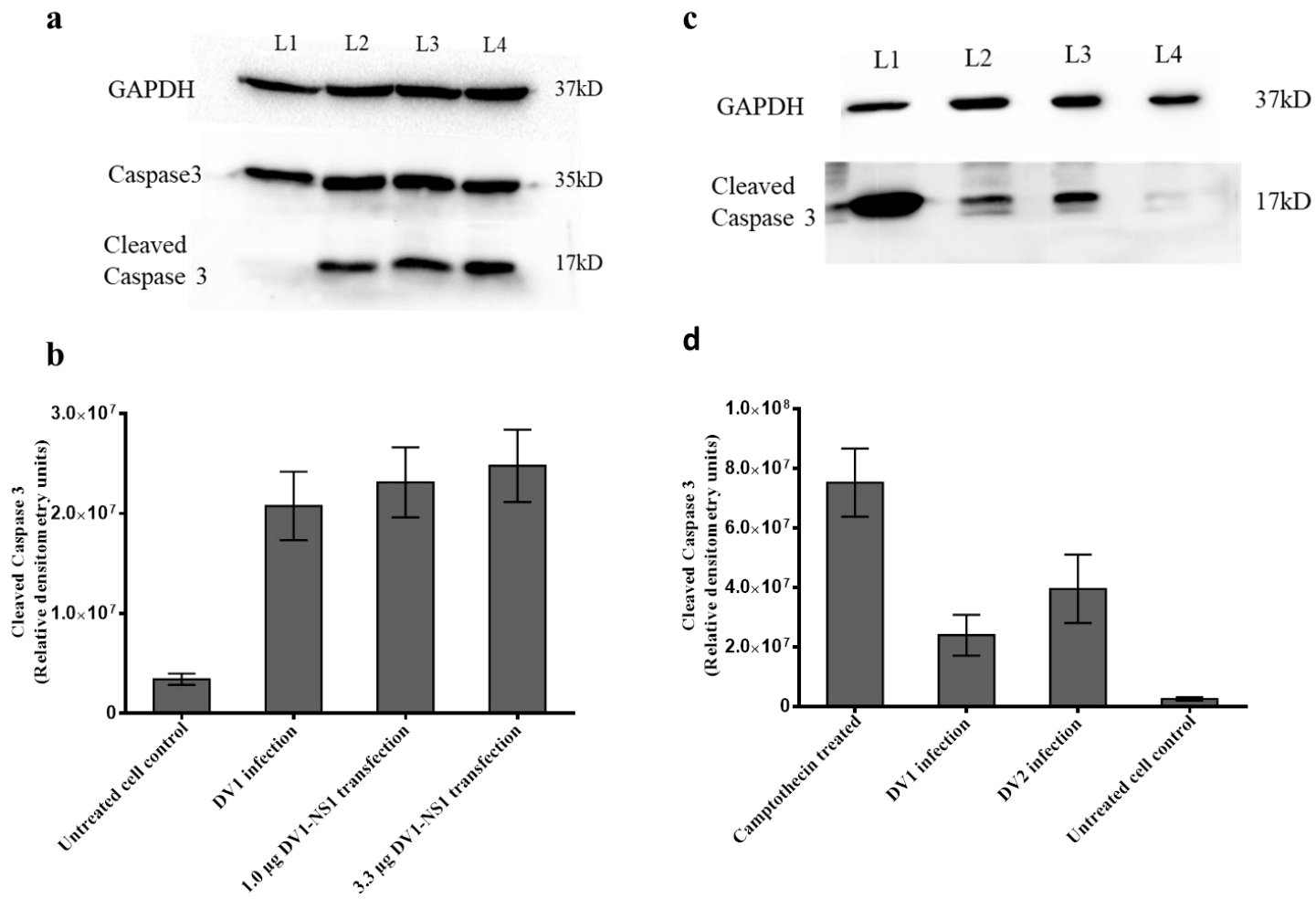


Figure 3.1. Dengue virus and NS1 only, both can induce Cleaved Caspase3. (**a**) Western blots data of Caspase 3 and Cleaved Caspase3 (CC3) expression in monolayer of Huh7 cells. Lane1: Untreated cell control; Lane2: DV1 infected cells (10 virus copies/cell); L3: 1.0 µg DV1-NS1-plasmid construct transfection; L4: 3.3 µg DV1-NS1-plasmid construct transfection. (**b**) Densitometry of bands from (a) for CC3. (**c**) Western blots data of CC3 expression in monolayer of Vero cells. Lane1: Cells treated with Camptothecin (4.0 µM) for 12h; Lane2: DV1 infected cells (10 virus copies/cell); Lane3: DV2 infected cells (10 virus copies/cell) R1; Lane4: Cell Control. (**d**) Densitometry of bands from (c) for CC3. GAPDH is as loading control. Both graphs show the average quantification from three independent experiments and error bars indicate SD.

Experiment with relevant Figure number	Type of cells & type of culture plate	Sample identification	Infection of DENV/ Transfection of NS1	NS1 in Inocu-lum (µg)	Yield of NS1 (µg) excluding NS1 in inoculum	Fold change of NS1 (µg) over inoculum	Yield NS1 conc. (µg/ml)	Mean yield (µg/ml)	SD	Virus copies/cell	Transfected plasmid (µg)
Fig.3.1a WB	Huh7, 6well	DV1-HNSB-P4(R1)	Infection of DV1-HNSB-P4	0.48	10.23	21.26	3.41	**2.53**	**0.79**	10	NA
	Huh7, 6well	DV1-HNSB-P4(R2)	Infection of DV1-HNSB-P4	0.48	8.71	18.10	2.90			10	NA
	Huh7, 6well	DV1-HNSB-P4-NS1(R1)	Transfection of DV1-HNSB-P4-NS1	NA	8.9	NA	2.97	**2.55**	**0.59**	NA	1
	Huh7, 6well	DV1-HNSB-P4-NS1(R2)	Transfection of DV1-HNSB-P4-NS1	NA	9.35	NA	3.12			NA	1
	Huh7, 6well	DV1-HNSB-P4-NS1(3.3)	Transfection of DV1-HNSB-P4-NS1	NA	14.22	NA	4.74	**4.36**	**0.33**	NA	3.3
Fig. 3.2a TUNEL	Huh7, 6well	DV1-HNSB-P4(R1)	Infection of DV1-HNSB-P4	0.2	6.57	32.25	2.19	**2.53**	**0.79**	10	NA
	Huh7, 6well	DV1-HNSB-P4(R2)	Infection of DV1-HNSB-P4	0.2	4.81	23.59	1.60			10	NA
	Huh7, 6well	DV1-HNSB-P4-NS1(R1)	Transfection of DV1-HNSB-P4-NS1	NA	5.68	NA	1.89	**2.55**	**0.59**	NA	1
	Huh7, 6well	DV1-HNSB-P4-NS1(R2)	Transfection of DV1-HNSB-P4-NS1	NA	6.65	NA	2.22			NA	1
	Huh7, 6well	DV1-HNSB-P4-NS1(3.3) (R1)	Transfection of DV1-HNSB-P4-NS1	NA	12.48	NA	4.16	**4.36**	**0.33**	NA	3.3
	Huh7, 6well	DV1-HNSB-P4-NS1(3.3) (R2)	Transfection of DV1-HNSB-P4-NS1	NA	12.54	NA	4.18			NA	3.3
Fig. 3.2c TUNEL	Vero, 6well	DV1-HNSB-P4(R1)	Infection of DV1-HNSB-P4	0.2	1.11	2.04	0.37	**0.40**	**0.09**	10	NA
	Vero, 6well	DV1-HNSB-P4(R2)	Infection of DV1-HNSB-P4	0.2	0.98	2.04	0.33			10	NA
	Vero, 6well	DV1-HNSB-P4(R3)	Infection of DV1-HNSB-P4	0.2	1.5	2.04	0.50			10	NA
	Vero, 6well	DV1-HNSB-P4-NS1(R1)	Transfection of DV1-HNSB-P4-NS1	NA	2.7	NA	0.70	**0.73**	**0.04**	NA	3.3
	Vero, 6well	DV1-HNSB-P4-NS1(R2)	Transfection of DV1-HNSB-P4-NS1	NA	2.18	NA	0.73			NA	3.3
	Vero, 6well	DV1-HNSB-P4-NS1(R3)	Transfection of DV1-HNSB-P4-NS1	NA	2.32	NA	0.77			NA	3.3

Table 3.2. Relative quantification of sNS1 in different experiments

3.1.3.2. Apoptotic DNA break is evident in the NS1 transfected cells

Based on the expression of CC3, the apoptotic DNA break of NS1 transfected and Camptothecin treated Vero cells were tested. We observed apoptotic ladder pattern in case of Camptothecin treated cells as expected. Similarly, we also noticed substantial amount of ladder pattern in case of only NS1 transfected cells in comparison with control (Fig. 3.2).

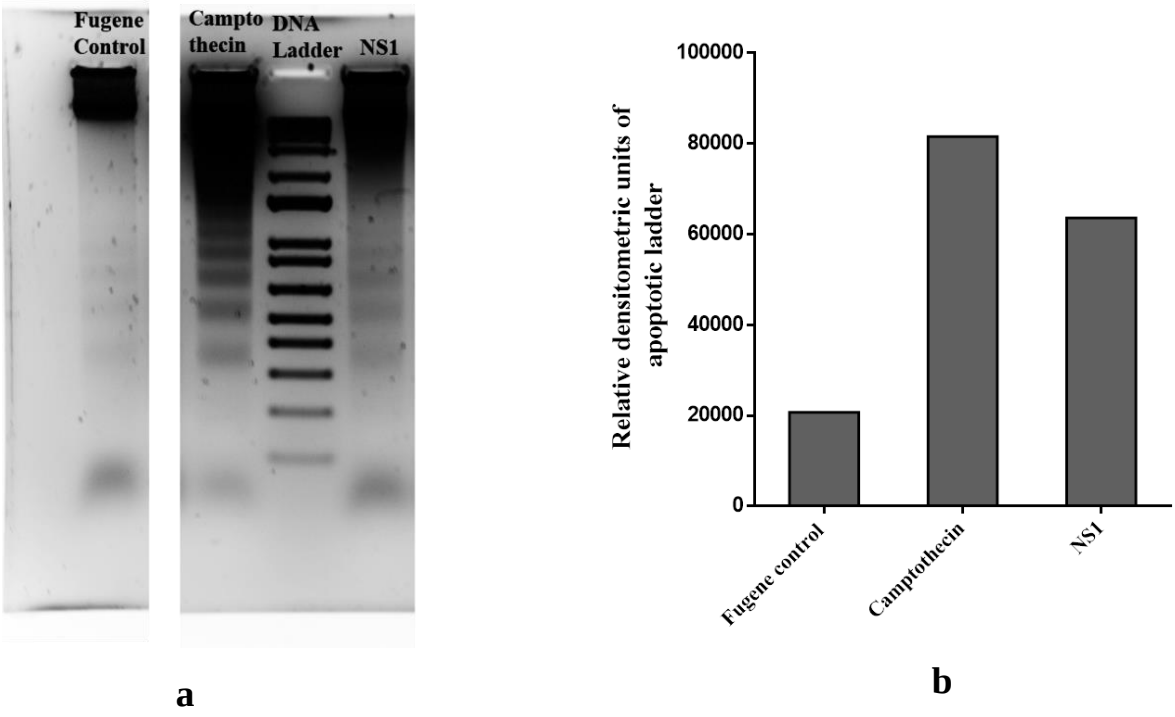


Figure 3.2. Apoptotic DNA ladder pattern in NS1 transfected and Camptothecin treated cells.

(a) Gel Electrophoresis image, from left, Lane 1 contains only Fugene treated Vero cells' DNA; Lane 2 contains whole genomic DNA of Camptothecin (4.0 µM) treated Vero cells; Lane 3 contains DNA molecular weight marker; Lane 4 contains genomic DNA of DV1-NS1 (1.0 µg plasmid) transfected Vero cells. Equal amount of DNA was loaded in each well (b) Relative densitometric analysis of lanes of gel image as shown in (a).

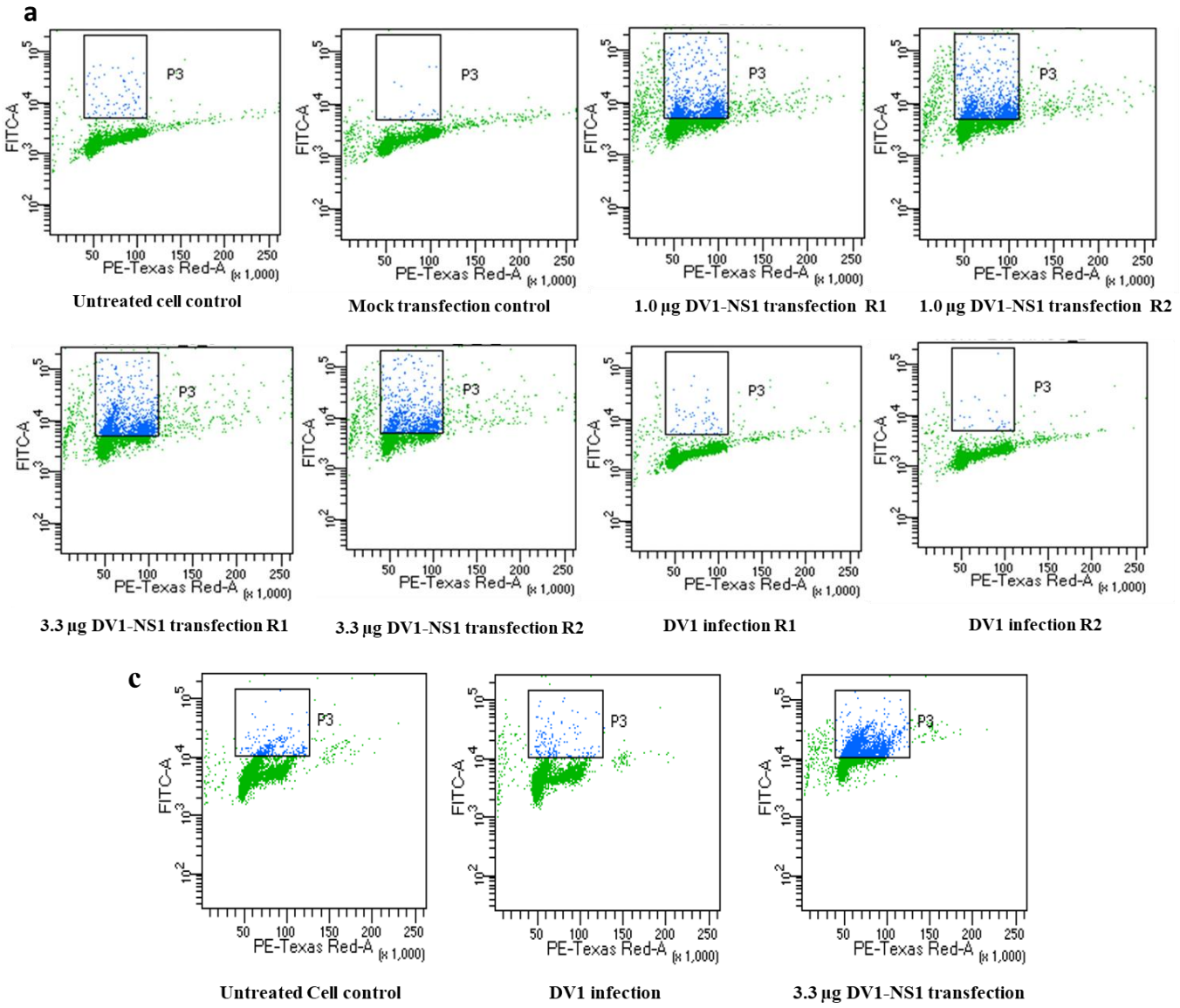
3.1.3.3. Dengue virus shows control over apoptotic DNA breaks in DV infected cells

DV1-NS1 plasmid transfected Huh7 cells showed high percentage of TUNEL positivity, 29.17 (± 2.48) % for 1.0 µg plasmid and 36.67(±2.26) % for 3.3 µg plasmid. Increase in percentage of DNA nicks was positively linked to increasing plasmid transfection, confirming apoptosis induction by NS1. Surprisingly, DV1-HNSB-P4 infected cells, expressing similar concentration of sNS1 2.53 (± 0.79) µg/ml like 1.0 µg NS1-plasmid transfected cells (2.55 (± 0.59) µg/ml), showed insignificant levels of apoptotic DNA nicks 0.97 (± 0.32) %. In fact, the infected cells behaved very similar to untreated cell and mock transfection controls, 2.1 (± 0.40) and 1.5 ± (0.8) % respectively (Fig.3.3 a, b). So, DV1-HNSB-P4 infected liver cells expressed similar level of CC3 compared to NS1-transfected cells (Fig.3.1 a, b) but no subsequent DNA breakage was observed even up to 96h post-infection (Fig.3.3 a, b). This observation suggests that DV counteracts NS1 mediated-apoptosis of infected cells, as evident from the absence of DNA breaks.

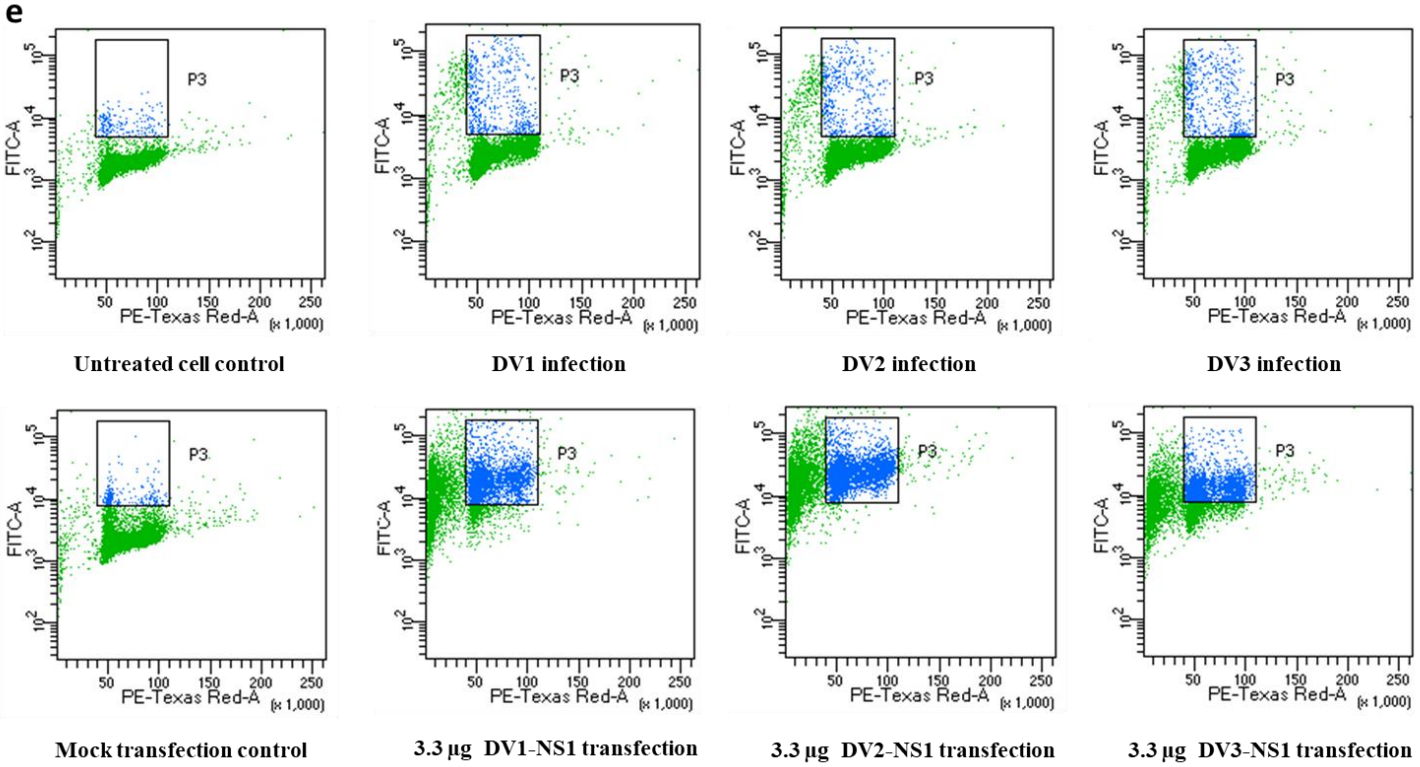
Similar study was also conducted multiple times using Vero cells. Again, there were more apoptotic DNA nicks in case of NS1 transfected Vero cells, confirming that only NS1 expression in cells was enough to induce apoptosis. DV1-HNSB-P4-infected Vero cells showed minimum TUNEL positivity (Fig.3.3 c, d) as observed in case of Huh7 cells.

The aforesaid phenomenon was found true for both DV2 and DV3. In case of DV2 clinical isolate, DV2-HNSB-NS1-transfected cells showed more apoptotic DNA breaks than only virus infected cells (10 virus copies/cell). 3.3µg DV2-NS1 plasmid transfected cells were more TUNEL positive (48.77 ± 2.55 %) than infected cells (7.83 ± 1.07 %) (Fig.3.3 e, f). Interestingly, this was observed with even higher levels of sNS1 production by DV2 infected (2.71 ± 0.08 µg/ml) than NS1-transfected cells (1.94 ± 0.11 µg/ml) (Table 3.2). So, despite higher level of NS1 expression in infected cells, apoptotic DNA breaks were lower compared to DV2-NS1 transfected cells. In case of DV3, the concentration of sNS1 in transfected cell

supernatant was 1.56 (± 0.16) µg/ml in case of 3.3 µg DV3-HNSB-NS1 plasmid transfection. DV3-HNSB-P4 infected cells secreted 1.21 (± 0.15) µg/ml sNS1 (Table 3.2). Again, for DV3, NS1 expression in transfected cells resulted in 41.87 (±2.32) % apoptotic DNA breaks, whereas DV3 infected cells expressing equivalent concentration of sNS1 showed only 6.77 (±1.55)% TUNEL positive (apoptotic) cells (Fig.3.3e,f).


a
P3
FITC-A
PE-Texas Red-A (x 1,000)
Untreated cell control
Mock transfection control
1.0 µg DV1-NS1 transfection R1
1.0 µg DV1-NS1 transfection R2
3.3 µg DV1-NS1 transfection R1
3.3 µg DV1-NS1 transfection R2
DV1 infection R1
DV1 infection R2
c
Untreated Cell control
DV1 infection
3.3 µg DV1-NS1 transfection
b
Apoptotic cells % (TUNEL positive)
Untreated cell control
Mock transfection control
1.0 µg DV1-NS1 transfection
3.3 µg DV1-NS1 transfection
DV1 infection
d
Apoptotic cells % (TUNEL positive)
Untreated cell control
DV1 infection
3.3 µg DV1-NS1 transfection

e


P3
FITC-A
PE-Texas Red-A (x 1,000)
Untreated cell control
DV1 infection
DV2 infection
DV3 infection
Mock transfection control
3.3 µg DV1-NS1 transfection
3.3 µg DV2-NS1 transfection
3.3 µg DV3-NS1 transfection

f

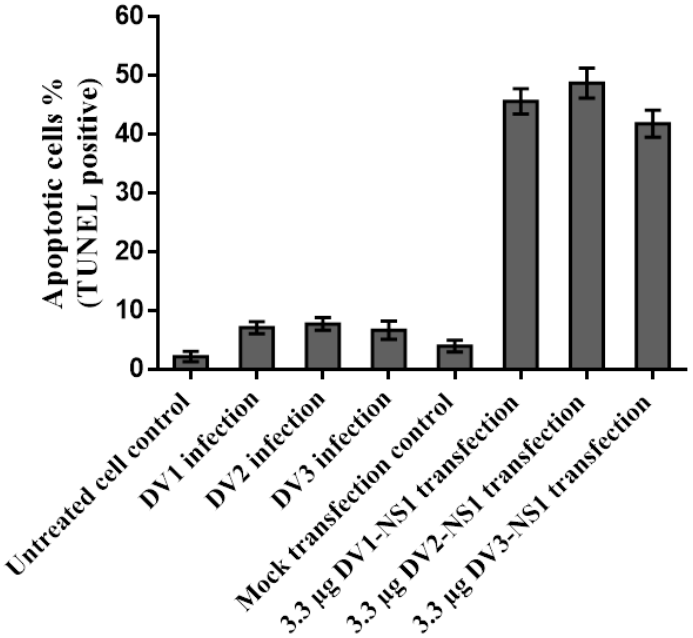

Apoptotic cells %
(TUNEL positive)
60
50
40
30
20
10
0
Untreated cell control
DV1 infection
DV2 infection
DV3 infection
Mock transfection control
3.3 µg DV1-NS1 transfection
3.3 µg DV2-NS1 transfection
3.3 µg DV3-NS1 transfection

Figure 3.3. Dengue virus shows control/check over apoptotic DNA breaks in DV infected cells.

(a) TUNEL assay representative data from monolayer of Huh7 cells, infected with DV1-HNSB-P4 (10 virus copies/cell) or transfected with 1.0 µg or 3.3 µg pcDNA3.1 (+)-DV1-NS1 construct/well of six well plate (as mentioned in figures). Cells were processed for TUNEL assay after 96h post infection or transfection. (**b**) Column graph of percentage of differentially treated cells that were found to be positive in TUNEL assay as in (**a**). (**c**) TUNEL assay data from monolayer of Vero cells with DV1 infection (10gE copies/cell) and transfection with 3.3 µg pcDNA3.1 (+)-DV1-NS1 construct (as mentioned in figures). (**d**) Column graph of percentage of differentially treated cells that were found to be positive in TUNEL assay as in (**c**). (**e**) Representative result of TUNEL assay from monolayer of Huh7 cells infected with 10 virus copies (DV1, DV2 or DV3) /cell, and cells transfected with DV1-HNSB-P4-NS1, DV2-HNSB-P4-NS1 or DV3-HNSB-P4-NS1 plasmid construct (3.3 µg) in each well of six well plate. (**f**) Column graph of TUNEL positive percentage of cells as in (**e**). Y axis of scatter plots (a, c, e) represents DNA breaks (BrdU labelling) and X axis represents DNA content (Propidium iodide staining). Column graphs show the average quantification from three replicates per condition and error bars indicate SD.

3.1.3.4. Dengue virus exerts inhibitory effect over Camptothecin induced apoptotic DNA breaks in infected cells

Ladder assay was performed on repeated occasions with Camptothecin (4.0 µM) treated Vero cells as positive control. Camptothecin treated cells showed distinct ladder pattern resulting from DNA fragmentation which occurs at late apoptosis (Fig.3.4 a). NS1 transfected (1.0 µg) Vero cells' DNA also showed apoptotic DNA ladder being consistent with CC3 expression (Fig.3.1 c,d). In this experiment, DV1-HNSB-P4 infected Vero cells were also treated with Camptothecin (4.0 µM) to promote apoptotic DNA breaks. Again, DV1-HNSB-P4 infected cells did not show apoptotic DNA ladder pattern even at 62h post-infection (point of cell harvesting), supporting the TUNEL assay results. Replication of virus was confirmed by 5-fold increase in sNS1 in supernatant over inoculum (Table 3.2).

DV1-HNSB-P4 infected cells together with Camptothecin (4.0 µM) treatment produced relatively reduced ladder pattern in comparison to only Camptothecin treated cells (Fig.3.4 a, b). So, this competitive assay showed that Dengue virus is actually opposing the apoptosis (by Camptothecin) and therefore, protecting the cellular DNA from fragmentation. This data conclusively proved that DV prevents cellular DNA breakage which is considered a salient feature of late apoptosis. Furthermore, it is observed that DV protects cellular DNA from damage even in presence of Camptothecin i.e. under high levels of CC3, as previously documented (Fig.3.1 c, d).

DAPI staining revealed that nuclear morphology of DV1 infected Huh7 cells was quite intact and similar to that of uninfected cells. In contrast, 1.0 µg DV1 NS1 plasmid transfected cells showed signs of nuclear damage in comparison to mock transfected (Fugene HD only) cells (Fig.3.4 c). We have already demonstrated that 1.0 µg DV1 NS1 plasmid transfected and DV1 infected (10 virus copies/cell) cells secrete comparable amounts of sNS1 (Fig.3.1 a,b). It is therefore, expected that there should be similar levels of damage of nuclear morphology for

both conditions. But nuclear morphology of DV1-infected cells was just like that of untreated cells, again providing direct visual evidence of Dengue virus's control/check over apoptotic DNA damage and cytotoxic effects of NS1.

3.1.3.5. Dengue virus keeps the infected host cell metabolically active reducing sNS1 mediated cytotoxicity

Metabolic activity of DV infected Huh7 cells (DV1, 2, 3) and their respective NS1 transfected cells were tested using MTT assay. Cells infected with DV at 10 virus copies/cell showed 89% viability in case of DV1; 86% with DV2 and 80% with the DV3 clinical isolate at 48h post-infection. Parallel sets of cells were transfected with respective NS1 constructs in amounts estimated to secrete equivalent amount of sNS1. It was observed that cell viability at 48h post-transfection was lower by 10-20% compared to DV infected cells, supporting the previously observed protective role of virus infection over NS1 cytotoxicity (Table 3.3).

% of viable cells in infected cells (10 virus/cell)	**R1**	**R2**	**R3**	**R4**	**Mean (SD)**
DV1	73.57	110.08	80.79	91.34	88.95 (15.87)
DV2	90.94	90.55	81.77	82.35	86.40 (5.02)
DV3	89.97	67.71	87.23	73.37	79.57 (10.74)
% of viable cells in NS1 plasmid (0.11µg) transfected cells	**R1**	**R2**	**R3**	**R4**	**Mean (SD)**
DV1-NS1	67.11	62.44	73.33	57.76	65.16 (6.65)
DV2-NS1	63.65	72.30	84.49	72.64	73.27 (8.56)
DV3-NS1	69.87	70.91	67.63	76.71	71.28 (3.87)

Table 3.3. Percentage cell viability or metabolic activity as observed in MTT assay. Metabolic activities of infected cells were compared with uninfected controls. For NS1 transfected cells, metabolic activity was compared with mock Fugene HD transfected cells. For each type of treatment, there were four replicates, denoted as R1, R2, R3 and R4.

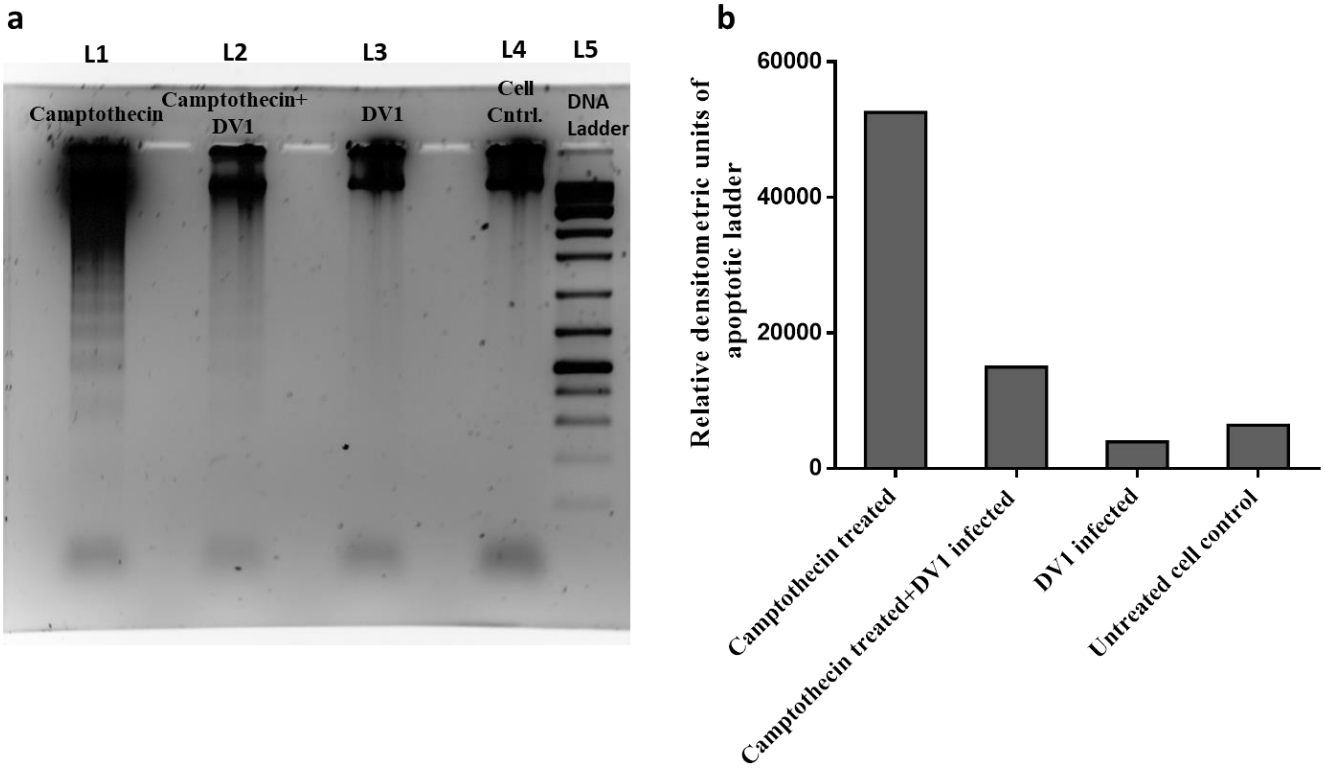

a
L1
L2
L3
L4
L5
Camptothecin
Camptothecin+ DV1
DV1
Cell Cntrl.
DNA Ladder
b
Relative densitometric units of apoptotic ladder
60000
40000
20000
0
Camptothecin treated
Camptothecin treated+DV1 infected
DV1 infected
Untreated cell control
c
DAPI
DAPI
DAPI
Untreated control
DV1 infected
Mock transfected
DV1-NS1 transfected

Figure 3.4. Dengue virus exerts inhibitory effect over induced apoptotic DNA breaks and counterbalances NS1 mediated nuclear damage.

(**a**) Gel Electrophoresis image, from left, (L1) Lane1 contains whole genomic DNA of Camptothecin (4.0 µM) treated Vero cells; (L2) Lane2 contains DNA from Camptothecin (4.0 µM) treated DV1-HNSB-P4 infected (10 virus copies/cell) Vero cells; (L3) Lane3 contains DNA of only DV1-HNSB-P4 infected cells; (L4) Lane 4 contains DNA from untreated cells. Equal amount of DNA was loaded in each well. (L5) Lane5 contains DNA molecular weight marker. (**b**) Relative densitometric analysis of lanes of gel image (**a**). (**c**) Monolayer of Huh7 cells, grown on 22 mm coverslip were infected with DV1-HNSB-P4 (10 virus copies/cell). For transfection, 1.0 µg of plasmid containing the DV1-NS1 construct was used. 1.0 µg plasmid was chosen to keep the secreted sNS1 level similar to that in case of infected ones. In case of mock transfection, only Fugene was used without plasmid. Nuclei were stained with DAPI at 48h post infection or transfection. Images (40X) are representatives from multiple replicates from two different experiments. Scale bar, 100 µm. DV1: DV1-HNSB-P4, Cell Cntrl.- Untreated Cell Control.

3.1.3.6. Release of mature virions as an evidence of successful virus replication in cell culture experiments

Secreted NS1 levels in experiments were measured using quantitative NS1 ELISA as described in methods. Individual ELISA, with standards, was performed for each experiment. In case of infection with DV1-HNSB-P4, DV2-HNSB-P4 and DV3-HNSB-P4, increase in sNS1 was expressed in microgram and as fold-increase over inoculum- sNS1 level (Table 3.2). Increase in sNS1 level was evidence of successful viral replication and NS1 production during every experiment. Interestingly, Camptothecin treatment did not have any significant effect on sNS1 production for virus-infected cells (Table 3.2).

Under same experimental conditions, Huh7 cells in six well plates were infected with the aforesaid three DV serotypes at 10 virus copies/cell. Inoculum was removed after virus adsorption and cell monolayer was washed with 1XPBS three times as done in case of all infections. Infected cells were harvested at 96h post infection and RNA was extracted. RNA from infected cell supernatant was also extracted to find the DV titer in it. Virus copy number was determined in cells and supernatant, using one step qRT-PCR(SYBR) and specific band size was confirmed in gel electrophoresis.

The means of intracellular virus copies were $4X10^7$ ($\pm 1.6X10^7$), $6X10^6$ ($\pm 7X10^4$) and $2X10^7$ ($\pm 10^6$) for DV1, DV2 and DV3 respectively. Similarly, supernatants of those infected cells also showed high virus titers, suggesting successful virus replication and release of mature virions. Mean total virus titers in DV1, DV2 and DV3 infected cell supernatants (3ml) were $4X10^7$ ($\pm 2X10^7$), $7X10^6$ ($\pm 5X10^6$) and $3.5X10^7$ ($\pm 10^7$) respectively. So, infected cells were secreting equal or more virus than intracellular titers (Table 3.4). The supernatant from the infected cells had been successfully used to infect fresh monolayer of cells, suggesting that the released virions in the supernatant were infectious, confirming their maturity and infectivity (data not shown).

	Intracellular virus copies Mean (±SD)	Virus copies in total supernatant (3ml) Mean (±SD)	Total yield of virus copies Mean (±SD)
DV1	4×10^7 ($\pm 1.6 \times 10^7$)	4×10^7 ($\pm 2 \times 10^7$)	8×10^7 ($\pm 4 \times 10^7$)
DV2	6×10^6 ($\pm 7 \times 10^4$)	7×10^6 ($\pm 5 \times 10^6$)	1.3×10^7 ($\pm 5 \times 10^6$)
DV3	2×10^7 ($\pm 10^6$)	3.5×10^7 ($\pm 10^7$)	5.5×10^7 ($\pm 10^7$)

Table 3.4. Virus copy number by qRT-PCR of RNA, extracted from infected Huh7 cells and supernatants.

3.1.3.7. Immunofluorescence assay of Dengue virus

To further confirm virus replication, immunofluorescence assay of DV infected Huh7 cells was done using anti-DV envelope primary Ab (DE1, Abcam) and goat anti-mouse IgG (Alexa fluor 488) secondary Ab. The presence of high virus titer was supported by viral envelope staining in more than 70% of the Huh7 cells. Nuclei of heavily infected cells were quite intact, again supporting DV-induced cell survival for increased virus replication (Fig. 4), opposing the deleterious effects of NS1 production and secretion. Primary Ab was developed based on envelope glycoprotein sequence of DV2 (Strain-16680) and worked best for DV2 staining, then DV1 but was found not effective for DV3 staining.

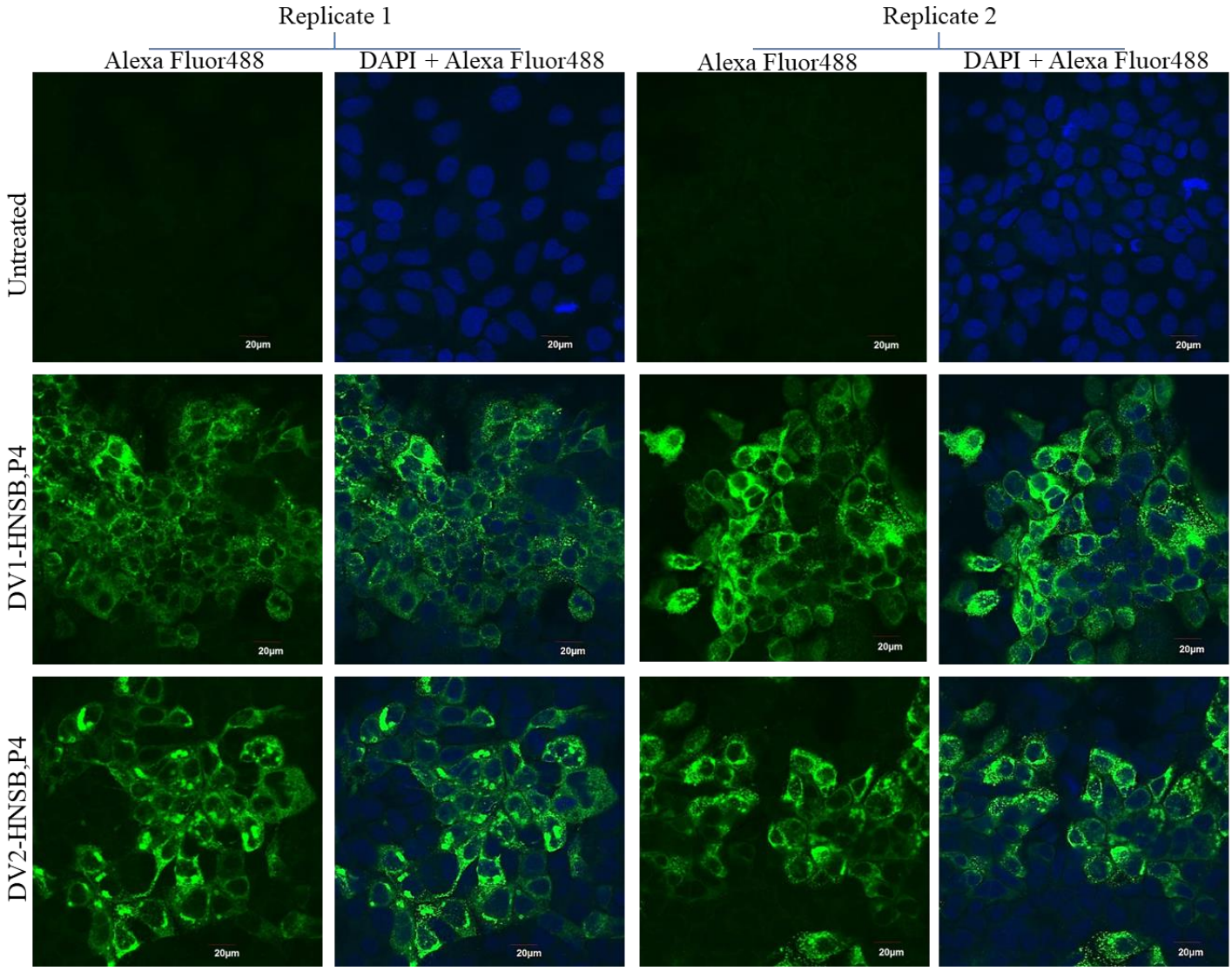


Figure 3.5. Immunofluorescence of viral envelope in DV1 or DV2 infected Huh7 cells.

Monolayer of Huh7 cells was infected with DV1-HNSB-P4 or DV2-HNSB-P4 (10 gE copies/cell). At 96h post infection cells were fixed and treated with anti-DV envelope Ab and Alexa fluor 488 tagged secondary Ab. Nuclei of cells were stained with DAPI. Images (60X) are representative of multiple replicates from two different experiments. Scale bar, 20 µm. Two sets of images had been presented row-wise for each condition, each set comprising of again two images. For each set, one image shows only Alexa Fluor 488 staining (left one) and the other image of the same view, with DAPI and Alexa Fluor 488 (right one).

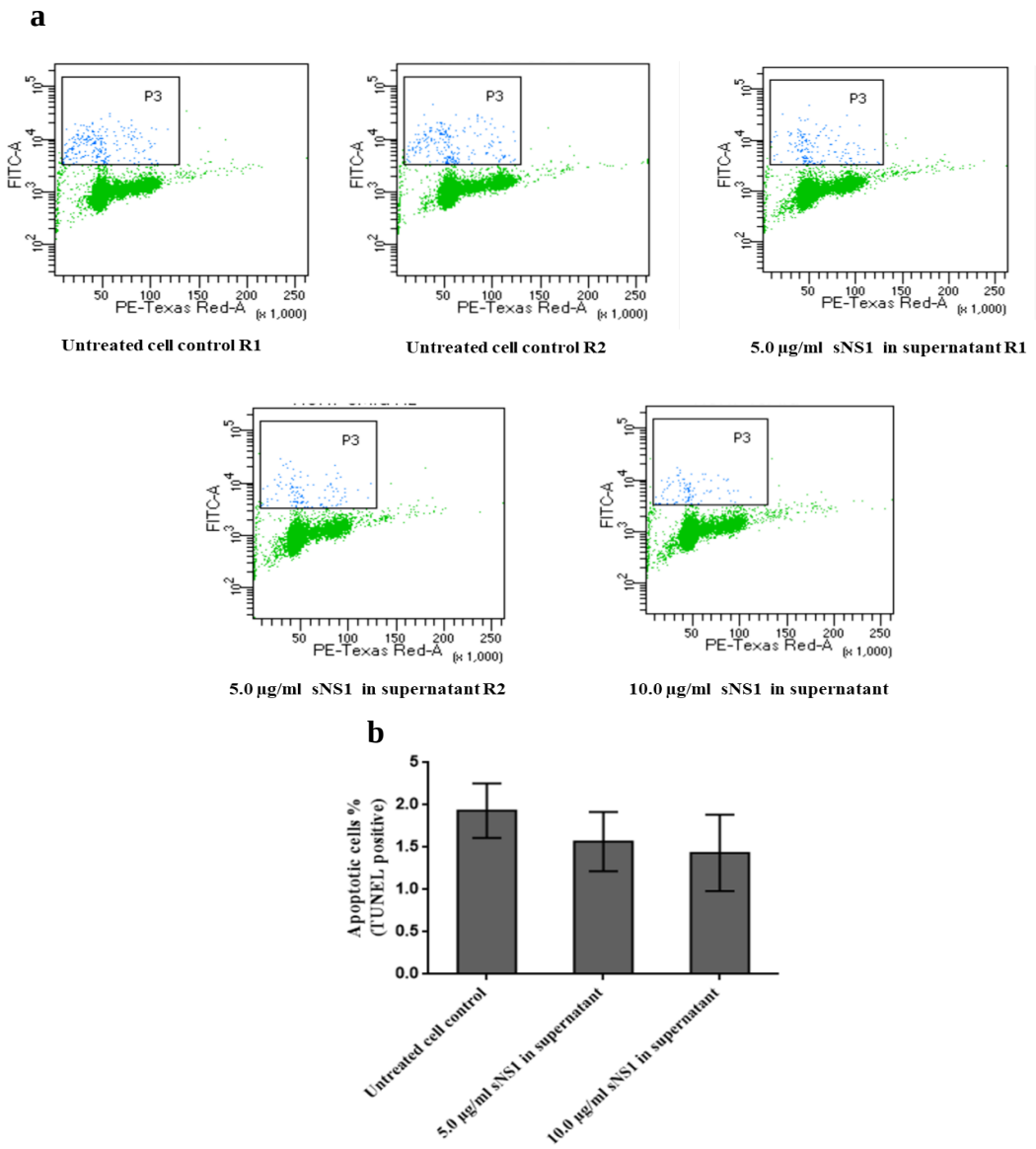


Figure 3.6. Soluble NS1 (sNS1) in supernatant of fresh Huh7 cells, could not induce apoptosis

(**a)** Representative TUNEL assay data from experiments where monolayer of Huh7 cells in six well plates were treated with 5.0 µg/ml or 10 µg/ml sNS1 in supernatant for 96h. (**b**) Column graph of percentage of sNS1 treated cells that were found to be positive in TUNEL assay as in (**a).** Y axis of scatter plots **(a)** represents DNA breaks (BrdU labelling) and X axis represents DNA content (Propidium iodide staining). Column graphs show the average quantification from three replicates per condition and error bars indicate SD.

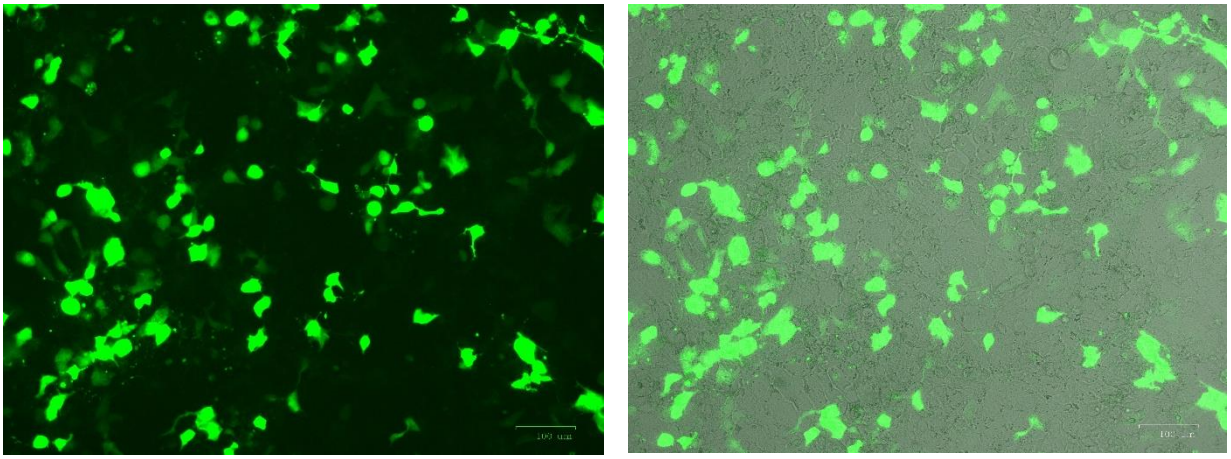


Figure 3.7. Huh7 cells transfected with pcDNA3-EGFP plasmid to find transfection efficiency

Huh7 cells were transfected with 1.0 µg pcDNA3-EGFP plasmid using Fugene HD. Images were taken at 48h post-transfection. Left panel shows only GFP expressing cells. The right panel shows merged image of phase contrast view of cells including the GFP expressing cells. The transfection was done along with NS1 transfection in same conditions. Scale bar 100 µm.

3.1.4. Discussion and Inference

We have ascertained that dengue virus-infected cells (Huh7 and Vero) and only NS1 gene-transfected cells, expressing equivalent amounts of sNS1 in the culture supernatant, both can induce cleaved caspase 3 (CC3). In case of transfection, sNS1 accumulation in the supernatant could not be due to a small fraction of cells as transfection with pcDNA3 EGFP (in similar amount to NS1-pcDNA3 plasmid) under identical conditions showed 60-70% cells to express GFP (Fig. 3.7). In case of transfection or virus infection (Fig. 3.5) (at 10 virus gE/cell), 60-70% cells were involved; both conditions produced around 2-3 µg/ml NS1 in the supernatant by 96 h post-infection which is comparable to physiological NS1 concentrations observed in clinical cases. In this study, we intended to examine NS1 at concentrations matching real case scenarios.

CC3 is a definitive marker of late apoptosis events that include subsequent fragmentation of cellular DNA. Our data suggest that virus-infected cells induced similar level of CC3 as observed for NS1-transfected cells (Fig.3.1 a, b). So apparently, NS1 contributes one major part in inducing apoptosis of DV-infected liver cells, the other part possibly comes from other pro-apoptotic factors like Capsid, Membrane, Envelope, NS2A and NS2B (Okamoto *et al.*, 2017). The TUNEL assay results further confirmed that NS1 alone is capable of inducing apoptosis, resulting in DNA nicks, producing positive TUNEL signals (Fig.3.3), apoptotic DNA ladder (Fig.3.2 a, b) and damage of nuclear morphology (Fig.3.4 c). In conclusion, our studies produced direct evidence that NS1 alone is capable of inducing apoptosis in the expressing liver cells at levels comparable to those found in real life DV clinical cases (Libraty *et al.*, 2002). We found no evidence that sNS1 (5.0 or 10.0 µg/ml) when transferred to fresh cell monolayers can induce apoptosis in such cells (tested at 96h post-transfer) by FACS analysis (Fig. 3.7).

It had been previously reported that DV infection in mouse neuroblastoma cell line, initiated survival signal via activation of PI3K and Akt phosphorylation. This survival signal was shown to prevent DV induced CC3 expression up to 24h (Lee, Liao and Lin, 2005). However, it is evident from our findings that DV infection actually results in CC3 expression, detectable even at 96h post infection.

This obviously raised the question whether DV-infected cells really undergo usual apoptosis as part of host defence against viral infection? We found that although DV infection induces the apoptosis mechanism of host defence in infected cells, cellular DNA is protected from apoptotic damage and fragmentation (Fig.3.3, 3.4). DV1 infected Huh7 cells expressed CC3 in similar levels as observed in case of 1.0 µg plasmid transfected cells. So, apoptotic DNA breaks as measured by TUNEL assay was supposed to be comparable in case of DV-infected cells and 1.0 µg plasmid-transfected cells. But it was found that virus-infected cells harboured much less apoptotic DNA breaks than transfected ones, in case of DV 1, 2 and 3 (Fig.3.3 a-f). This was further confirmed by the fact that DV infected cells, despite Camptothecin treatment, repeatedly showed reduced laddering pattern compared to only Camptothecin treated cells (Fig.3.4 a, b). Camptothecin is a Topoisomerase-I inhibitor and is known to induce apoptosis through DNA damage (Pommier, 2006). It was therefore, apparent from our observations that DV infection counteracts apoptosis-mediated DNA breakage in Huh7 and Vero cells, even up to 96h post-infection. These findings point towards the ability of DV infection to delay the onset of apoptosis by preventing DNA breaks, besides inducing the early-stage host survival signal via PI3K-Akt pathway activation (Lee, Liao and Lin, 2005). The results of the MTT assays revealed that DV infection also keeps the host cell metabolically active counter balancing host antiviral response i.e. apoptosis. On the other hand, NS1 expression and secretion from cells in absence of whole virus led to substantial metabolic damage or mortality of cells (Table 3.3).

There are several reports presenting evidences of DV-mediated apoptosis of liver cells (Marianneau *et al.*, 1997; Thongtan, Panyim and Smith, 2004; Thepparit *et al.*, 2013). In these studies, experiments were carried out with laboratory strains of Dengue virus. Our studies involved three clinical isolates of Dengue virus, representing serotypes 1, 2 and 3, circulating in Kolkata during 2017. DV isolates with low passage number (passage number 4 for all three isolates) were used in all assays in order to maintain them closer to the wild-type circulating isolates and to mimic the clinical scenario as much as possible (Diamond *et al.*, 2000).

Our observations perhaps explain why majority (≥80%) of DV infections in humans are asymptomatic/self-limiting (ten Bosch *et al.*, 2018) despite the fact that sNS1 on its own is a virotoxin capable of causing cytotoxicity/apoptosis of liver cells. The results also prove that sNS1-mediated cytopathic effects are suppressed/ameliorated in case of virus-infected cells. These observations encouraged us to propose that DV disease outcome/severity may depend on the "tilt" of the balance between DV infection-mediated prolongation of cell viability (by check of cellular apoptosis and resulting DNA damage) and induction of apoptosis by proapoptotic virus proteins like NS1. In this context, it is interesting to note that symptomatic to severe DV cases have been implicated in case of the following conditions/pre-disposing factors, namely (I) Antibody dependent enhancement (ADE) in case of secondary infection (Katzelnick *et al.*, 2017); (II) Level of sNS1 and viraemia (Libraty *et al.*, 2002; Nunes *et al.*, 2018); case fatality was high when NS1 antigenaemia was high as observed in case of DV2 infections. In contrast, higher case fatality was linked to higher viraemia in case of DV3 infections (Libraty *et al.*, 2002; Nunes *et al.*, 2018); (III) Co-infection with different serotypes (Dhanoa *et al.*, 2016); (IV) Immune status or comorbidities of the host. It appears that the above factors decide the fate of DV infection affecting the aforesaid "tilt" of the balance between asymptomatic condition (self-limited recovery) and disease progression.

Virus replication in Huh7 and Vero cells was confirmed in infected cells. Alongside high intracellular virus titer, infected liver cells also secreted equivalent or more mature virions, suggestive of successful virus infection of cells resulting in high viraemia. Mature virions were released keeping the cells intact as evident from high virus copy numbers in infected cell supernatants (Table 3.4). Additionally, DV replication in individual experiments was confirmed by fold increase in sNS1 level in supernatants (Table 3.2).

It has been observed that clinical isolates of DV are not plaque-forming at early passages in cell (Raut *et al.*, 2019). The same appears to hold true for our clinical isolates. Enveloped DV in infected cells was also visualized by immunofluorescence using Ab against DV envelope glycoprotein (Fig. 4). The nuclei of DV-infected Huh7 cells were similar to those of the uninfected cells and were in sharp contrast to the damaged nuclei in case of NS1-transfected cells (Fig.3.4 c). This observation again emphasized DV mediated survival of infected host cells in the presence of NS1 virotoxin in the background.

Apoptotic DNA fragmentation is triggered by the nuclease DNA fragmentation factor 40 (DFF40). DFF40 expression and folding are regulated by DFF45. The latter acts as a nuclease inhibitor prior to DFF40 activation mediated by execution caspases like Caspase3. Activated execution caspases cleave DFF45 which then dissociate from DFF40, leaving DFF40 as the functional nuclease (Zhou *et al.,* 2001). It is still not clear how DV exactly protects infected cells' DNA. The explanation appears to be not straightforward and warrants further in-depth investigation, it may be due one or more viral factors or modulated host factors or a combination of both.

Our finding also corroborates well with the fact that Dengue virus infection is associated with increased cell free DNA (cfDNA) in plasma. There are several reports that cfDNA increases in DV infected patients and is associated with disease severity (Ha *et al.*, 2011). In this article

the authors have said cellular apoptosis to be the source of this high cfDNA, but the mechanism was not clear; as apoptosis was supposed to produce fragmented DNA, to be removed soon via salvage pathways and excretion. But if DV infection protects cellular DNA from apoptotic breaks, that will result in larger DNA fragments which may stay in plasma for longer period. Our results possibly explain this cfDNA in Dengue infections more rationally. Disease severity is positively linked to DV titres; more virus replication means infection of more cells of the body; more cells rupture to release progeny virions and "protected" cfDNA- this perhaps explains why more cfDNA in plasma has been recorded as a biomarker of DV disease severity (Phuong *et al.*, 2019).

DV infection of cells definitely induces apoptosis as a response of host defence (Lee, Liao and Lin, 2005). Adding to this, we have shown NS1 alone is capable of inducing apoptosis, besides other well-known pro-apoptotic factors like virus capsid protein. So, it is a viral strategy against host defence that DV infection "protects" cellular DNA from apoptosis-induced fragmentation, to keep the host cell metabolically active even with toxic NS1 expression as observed. This would allow the virus a window to replicate for a relatively longer period using the host cellular machinery (we recorded up to 96h post-infection) followed by budding of mature virions (Table 3.4). This strategy is advantageous from the point of DV infection biology. In a nutshell, virus-mediated "protection" of cellular DNA allows more time and space for virus replication within infected cells, in the face of ensuing apoptosis, thereby allowing the virus to reach higher titres to establish successful viraemia in the host.

Successful virus transmission and propagation in an endemic region occurs when the virus is present in high titres in circulation so that the mosquitoes could take in adequate number of infectious virus particles during blood meal, thereby ensuring effective virus transmission and persistence in the host and vector populations.

3.2. NS1 secretory pathway as a target for antiviral design

3.2.1. Background

Dengue virus NS1 is a 48kDa multifunctional glycoprotein. It is the most abundant viral protein in the DV infected patients' sera. NS1 is secreted from the infected cells and the most abundant viral protein in the serum of DV infected patient. NS1 capture ELISA is used for early detection of DV infection and it can give positive result even before reaching RT-PCR detection level (Chuansumrit *et al.*, 2008; Shan *et al.*, 2015). NS1 is very important for viral replication. Deletion of NS1 from DV genome can completely abrogate the replication but ectopically expressed NS1 can rescue the virus without NS1. Secreted NS1, plasma membrane bound NS1 and intracellular all have roles in immune evasion (Akey *et al.*, 2015).

During synthesis NS1 moves towards ER lumen where it gets dimerize rapidly with addition of carbohydrate chain. After forming NS1 dimer, it can be targeted to different destinations: plasma membrane and followed by extracellular compartment or replication complex. Three NS1 dimers on cell membrane come in close proximity to form a hexamer which is barrel shaped with a hydrophobic lipid containing core (Fig. 3.8). The hexamer has a hydrophilic outer layer which makes it soluble (Akey *et al.*, 2015). Recently high resolution three dimentional structure of NS1 has been solved as well (Akey *et al.*, 2014). The NS1 dimer has three domains: first, two β hairpins interwined to form the β-roll; second, Wing domain consisting of α/β subdomain with an incomplete connector sitting against the β-roll; third, nine antiparallel β strands from each NS1 monomer (total eighteen) align forming a continuous β-sheet which runs the length of the dimer. The β-roll and connector domain make a hydrophobic face which interact with transmembrane viral proteins in ER. On cell membrane NS1 dimers remain attached with the hydrophobic β-roll face (Fig. 3.9). In case of

hexamer β-roll of the NS1 dimers face the interior lipoparticle where it gets associated with central lipid core (Akey *et al.*, 2014).

Secreted NS1 is highly immunogenic and elicit antibody production. There are evidences that anti-NS1 antibodies are found to cross-react with host proteins (Lin *et al.*, 2002) and it can result in vascular leakage as well (Beatty *et al.*, 2015; Killingbeck *et al.*, 2015; Puerta-Guardo, Glasner and Harris, 2016). DV NS1 can bind and increase the turnover of C4 of classical pathway and helps to escape complement mediated lysis (Chen, Lai and Yeh, 2018). So, we thought that if we can prevent the secretion of NS1 that can be an option to reduce the severity of DV infection. For this we targeted the interaction between the amino acids of the tetramer and the dimer of NS1. If these interactions can be competitively inhibited by small synthetic peptides that would lodge themselves between these interacting amino acids, hampering their interaction and association, then the assembly can be inhibited. We designed small peptides targeting the hydrophobic face of NS1 dimer which is attached with plasma membrane and supposed to form hexamer. Our proposition was to prevent the formation of soluble hexamer rendering NS1 dimer on cell surface. This can reduce the pathogenesis caused by the soluble NS1 and simultaneously cell surface NS1 can attract antibodies resulting in ADCC and destruction of infected cells. Considering the immunological point this strategy can also be helpful to restrict the disease progression in patients.

The synthetic peptides were synthesised by identifying the amino acids that are interacting from both the NS1 dimer structures and then inserting a flexible glycine-serine polypeptide that would attenuate the interaction between these amino acids.

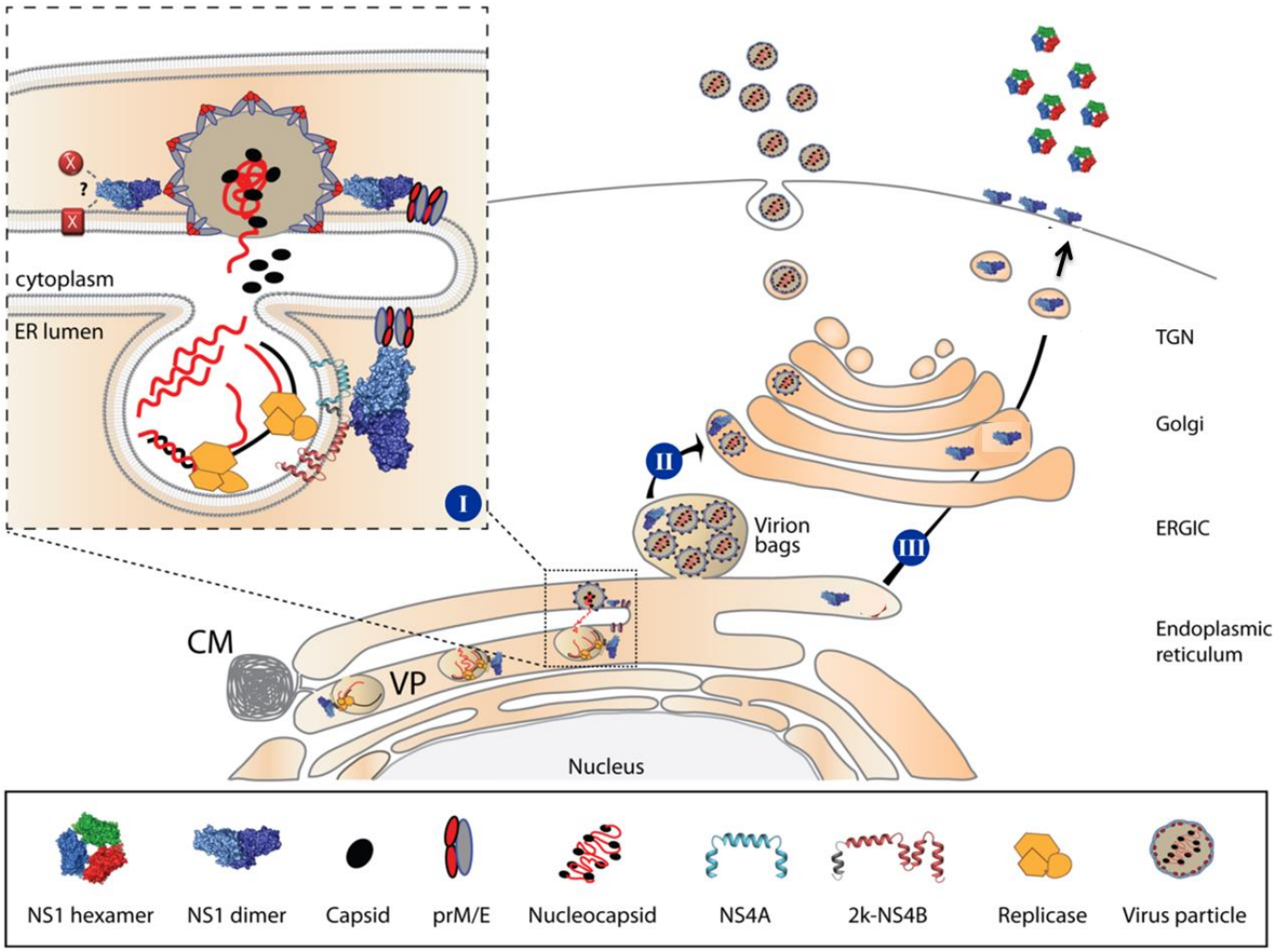


Figure 3.8. NS1 protein synthesis and secretion pathway

Dengue virus NS1 is translocated co-translationally to the endoplasmic reticulum (ER). In the ER lumen NS1 dimerizes with the addition of a mannose rich carbohydrate chain. These NS1 dimers have three possible destinations: replication site, plasma membrane and extracellular. The majority of NS1 is secretory type. On the cell surface, three NS1 dimers associate with a lipid core forming a hexameric barrel shape structure. This hexameric NS1 structure has a hydrophilic outer wall which makes it soluble and most abundant viral protein in patients' sera. VP: Vesicular pocket; TGN-Trans Golgi network; ERGIC: ER-Golgi intermediate compartments; CM: Convoluted membrane. Adapted from (Scaturro *et al.*, 2015)

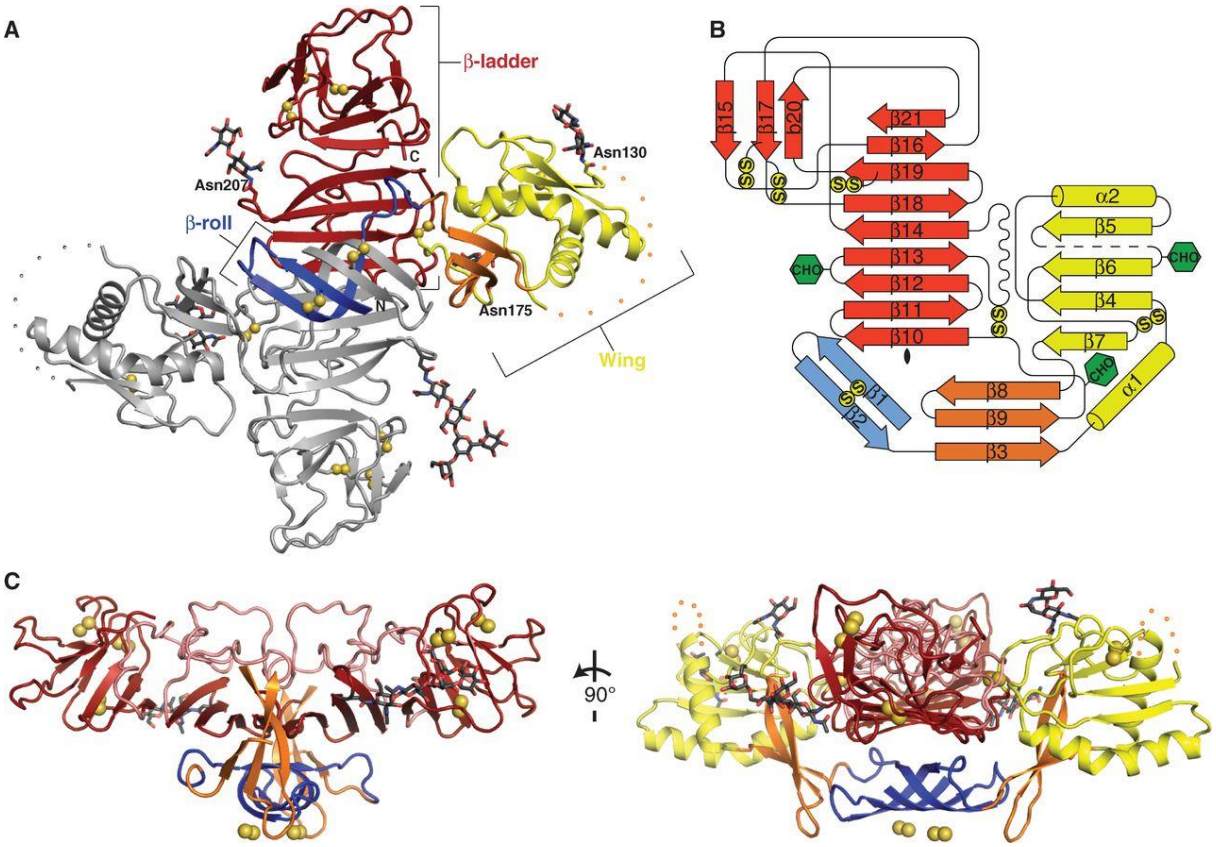


Figure 3.9. Structure of dengue virus NS1 protein

(A) NS1 dimer with one subunit as grey and other subunit is coloured as per different domains. β roll-blue; wing-yellow; orange-connector domain; central β ladder- red. Yellow sphere and black sticks represent the disulfides and N-linked glycosylation sites respectively. (B) Topology diagram of NS1 monomer. Different domains are coloured as in (A). Green hexagons are glycosylation sites and yellow circles are disulfides. (C) Perpendicular views of NS1 from the left and right edge of the β ladder. The wing domain has been omitted from left image for simplicity. Adapted from (Akey *et al.*, 2014).

3.2.2. Experimental methods

3.2.2.1. *In-silico* synthetic peptide design

The synthetic peptides were synthesised by identifying the amino acids that are interacting in two NS1 dimer structures and then inserting a flexible glycine-serine polypeptide that would attenuate the interaction between these amino acids. We docked two 4O6B PDB files in ZDOCK server. Top ten docking predictions were chosen for analysis. We analysed the predicted structures to find unique interactions (within 3.5 Å cutoff) consisting of one amino acid from each dimer. In each docking complex, a flexible polypeptide GS linker was added in between the interacting amino acids to design the synthetic polypeptides. G-S linkers of 4.5Å (GSSGSS), 4.8Å (GSSSS), and 6.2Å (SSSSSS) length were proposed to use in the designing of the synthetic peptides. Total seven peptides were prepared.

3.2.2.2. Evaluation of cellular toxicity of synthetic peptides

Synthetic peptides were dissolved in mili-Q water at 1mg/ml. stocks. Then four dilutions 0.5µg/ml, 1µg/ml, 10µg/ml, 100µg/ml were used in MTT assay to find the optimum concentration for Huh7 cells. Cell culture and MTT assay was done as described in the first part of this chapter 3.1.2.8.

3.2.2.3. Experiment with synthetic peptides

Monolayer of Huh7 cells were inoculated with DV as described before 3.1.2.2. After two hours of adsorption, inoculum was removed and washed with 1X PBS. Then DMEM 1% FBS with desired peptide concentration was added on the infected cells and kept in incubator 48 hours. After two days of incubation, cell supernatant was collected and quantitative NS1 Ag-ELISA was done to find if there is any reduction in NS1 secretion in comparison with control. ELISA was performed as described in 3.1.2.5.

3.2.3. Results

3.2.3.1. Optimal concentration of synthetic peptides

0.5µg/ml and 1µg/ml concentrations of all seven peptides showed minimum toxicity with around 90% survival rate. But in case of 10 µg/ml and 100 µg/ml cell death is quite high around 70%. So, we considered 0.5 µg/ml, 1 µg/ml and 10 µg/ml for antiviral assay.

	Survival % of Huh7 cells at different concentration of peptides			
Synthetic Peptides	0.5 µg/ml	1µg/ml	10µg/ml	100 µg/ml
SP1	96.33	89.12	72.35	68.23
SP2	95.23	91.14	70.51	69.45
SP3	96.82	91.88	74.21	70.26
SP4	94.8	87.58	70.21	66.38
SP5	96.76	92.14	77.72	65.8
SP6	97.51	90.13	76.22	64.82
SP7	94.41	90.92	74.9	67.66

Table 3.5. Huh7 cell survival rate at different concentrations of synthetic peptides.

3.2.3.2. Antiviral assay

The secreted NS1levels in the supernatant of DV infected and non-infected cells were measured. For three concentrations of synthetic peptides i.e. 0.5µg/ml, 1 µg/ml and 10µg/ml, there was some difference but it was not significant (Fig. 3.10).

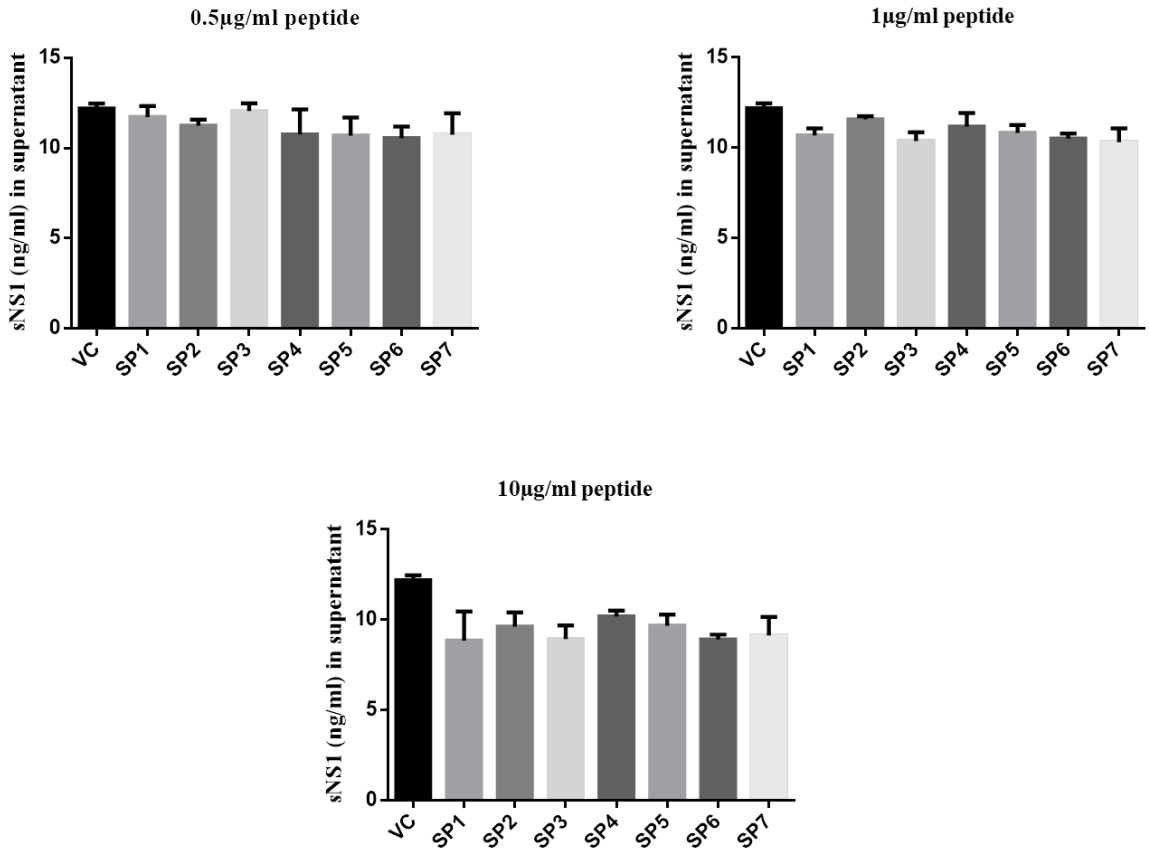


Figure 3.10. sNS1 secretion pattern in three different concentrations of synthetic peptide.

0.5µg/ml and 1µg/ml concentrations of synthetic peptides do not show significant change in sNS1 level in supernatant of DV infected Huh7 cells. At 10µg/ml concentration, there are reduction of sNS1 in comparison with virus control but not very significant.

3.2.4. Inference

In case of DV infected cells, peptides were not successful enough to reduce the secretion of sNS1. In case of 10µg/ml peptide concentration, there was a reduction in NS1 level in supernatant but it was not significant. Peptides are needed to be modified to optimize the reduction in sNS1 level.

4. Chapter 4

Serological cross-reactivity between Dengue and SARS-CoV-2

4.1. Study of dengue virus serology during COVID-19

4.1.1. A brief description of COVID-19

Since December 2019, world has experienced a devastating pandemic named COVID-19 caused by SARS-CoV-2 virus. In December 2019, the world faced the 1st wave of COVID-19. It was initially an endemic outbreak in the Hubei province, China. COVID-19 is an acute pneumonia-like respiratory illness in humans caused by a *betacoronavirus*, SARS-CoV-2. Alpha coronaviruses infect animals and beta coronaviruses e.g. NL63, HKU21, OC43, SARS-CoV-1 have history of human infections. Sequencing data confirmed that SARS-CoV-2 has considerably high sequence similarity with bat coronavirus. As the virus can spread by even droplet nuclei, person to person transmission became rampant, augmented by super-spreader events. By the end of January 2020, WHO declared the outbreak as a *Public Health Emergency of International Concern* (PHEIC) (COVID-19 Public Health Emergency of International Concern (PHEIC) Global research and innovation forum). The COVID-19 pandemic completed its 1st year at the cost of 129 million infections and 2 million deaths globally and still counting (WHO Coronavirus (COVID-19) Dashboard). India reported over 43 million confirmed cases of COVID-19 with 525 thousand deaths up to 15th July 2022 (India: WHO Coronavirus Disease (COVID-19) Dashboard with vaccination Data).

4.1.1.2. Transmission of SARS-CoV2

Respiratory droplets carrying the infectious viruses are the primary mode of transmission for SARS-CoV-2. People in close contact of the presymtomatic, asymptomatic or symptomatic individuals can get infected. Surfaces of public places have also been considered to be an important source. It has been demonstrated that infectious virus can stay up to 72 hours on stainless steel or plastic surfaces. Several case studies have reported that virus is present in

SARS-CoV-2 infected patients' feces. This also raises the possibility of fecal-oral transmission.

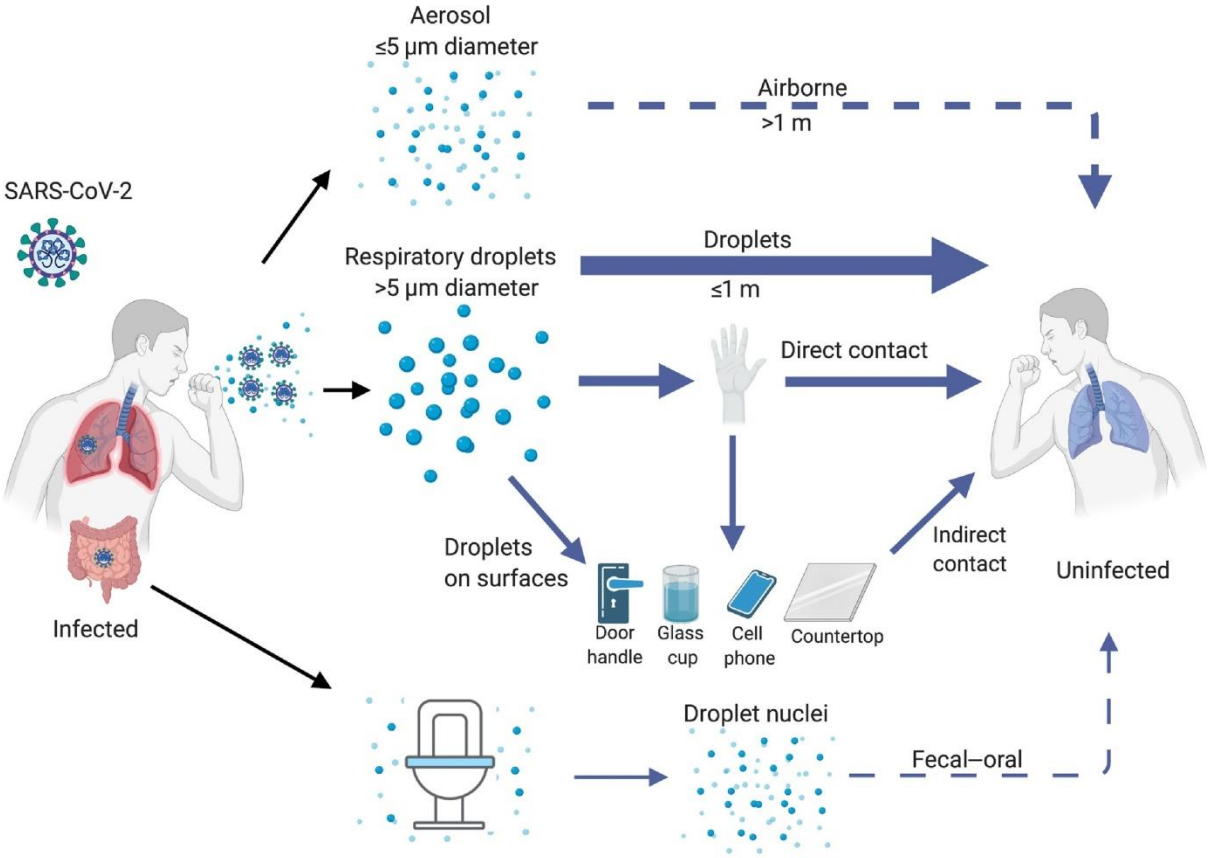


Figure 4.1. The different routes of SARS-CoV-2 transmission

The most pronounced mode of transmission is through droplet (>5μm) during the pandemic. Solid arrows represent confirmed way of viral transmission from one infected person to another. Dashed lines show the plausibility of transmission through those routes. Adapted from (Harrison, Lin and Wang, 2020)

4.1.1.3. Pathogenesis and symptoms of SARS-CoV-2

Structurally SARS-CoV-2 is quite similar with SARS-CoV and MERS-CoV and composed of four structural proteins spike, envelope glycoprotein, membrane protein and nucleocapsid. SARS-CoV-2 genome also codes for sixteen non-structural proteins along with six accessory proteins (Silvas *et al.*, 2021). The crown shaped spike glycoprotein resides at the outer surface of the virion and undergoes cleavage to form N-terminal S1 subunit which facilitates the attachment and entry inside the host cell. The S2 subunit contains a transmembrane domain, cytoplasmic domain and a fusion peptide which mediate the virus and cell membrane fusion. The S1 subunit has been divided into receptor binding domain (RBD) and N-terminal domain (NTD). The RBD domain is the fundamental of virus infection as it contains binding site (RBM-Receptor binding motif) for the human angiotensin-converting enzyme 2 (ACE2) receptors. ACE2 receptors are present abundantly on respiratory epithelium. ACE2 is also present in other organs like enterocytes from the ileum, myocardial cells, and proximal tubular cells of kidney. Anti SARS-CoV-2 immunological response is also mostly against S1 subunit of Spike protein (Hu *et al.*, 2021).

SARS-CoV-2 infection in human can manifest in mild symptoms to severe respiratory failure. After binding with the epithelial cells of the respiratory tract, it starts to replicate down in airway and reach the alveolar epithelial cells in the lungs. Rapid replication of virus in lungs initiates the strong immune response which is followed by cytokine storm syndrome. This results in acute respiratory distress and sometimes respiratory failure which has been reported to be one of the main causes of death due to COVID-19 (Hu *et al.*, 2021). Elder people (>60 years) with pre-existing health problems have a greater risk of developing respiratory distress syndrome. In some COVID-19 cases, multiple organ failure has also been reported.

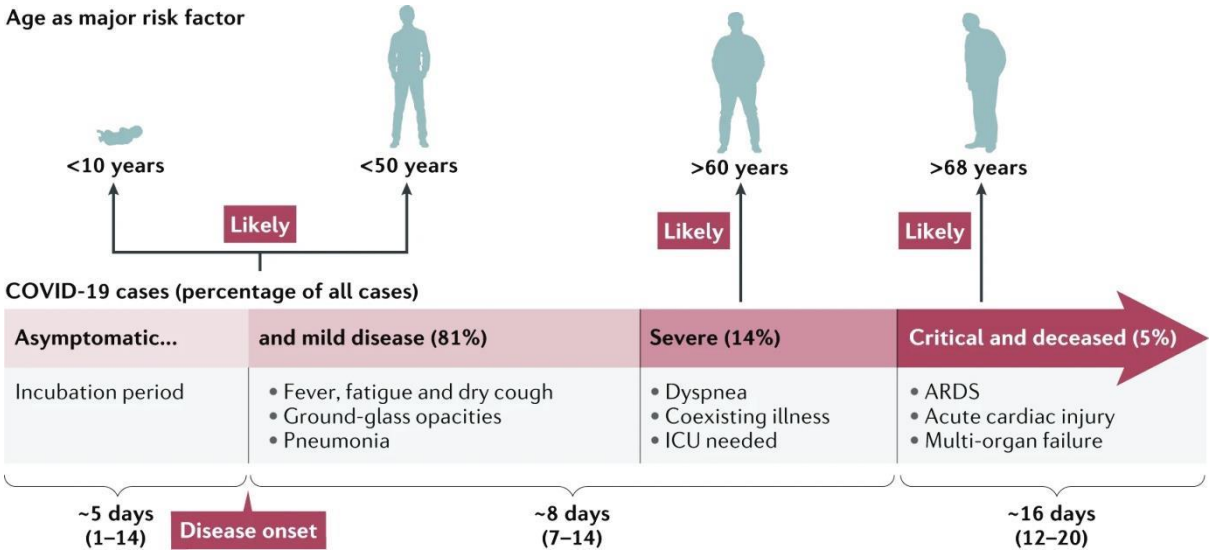


Fig 4.2. Clinical features of COVID-19 in comparison with age.

The most common symptoms of COVID-19 are fever, cough and fatigue. SARS-CoV-2 infection in young adults and children are found to be asymptomatic. But in cases of aged people with co-morbidities the risk of respiratory failure and death is quite high. Usually it takes around five days to establish the infection. Severity develops around eight days after symptom onset and critical phase may occur around sixteen days. Adapted from (Hu *et al.*, 2021)

4.1.1.4. Diagnosis

Control of COVID-19 is largely dependent on early diagnosis. Nucleic acid level detection is the gold standard for SARS-CoV-2 detection. But RT-PCR based detection is costly, time consuming, lab based and demands expertise. In early days of COVID-19, it was a challenge in India to set up appropriate laboratories for nucleic acid based detection. SARS-CoV-2 can be detected from variety of respiratory sources like throat swabs, posterior oropharyngeal saliva, nasopharyngeal swabs, sputum and bronchial fluid. The viral load is high in lower respiratory tract samples. Serological tests emerged as a faster and on site alternative for anti-SARS-CoV-2 IgG and IgM detection. Most of the serological tests were based on

immunodominant spike protein. Later nucleocapsid has also been used. Chest CT scan has also been used by the doctors to evaluate the disease pathogenesis directly.

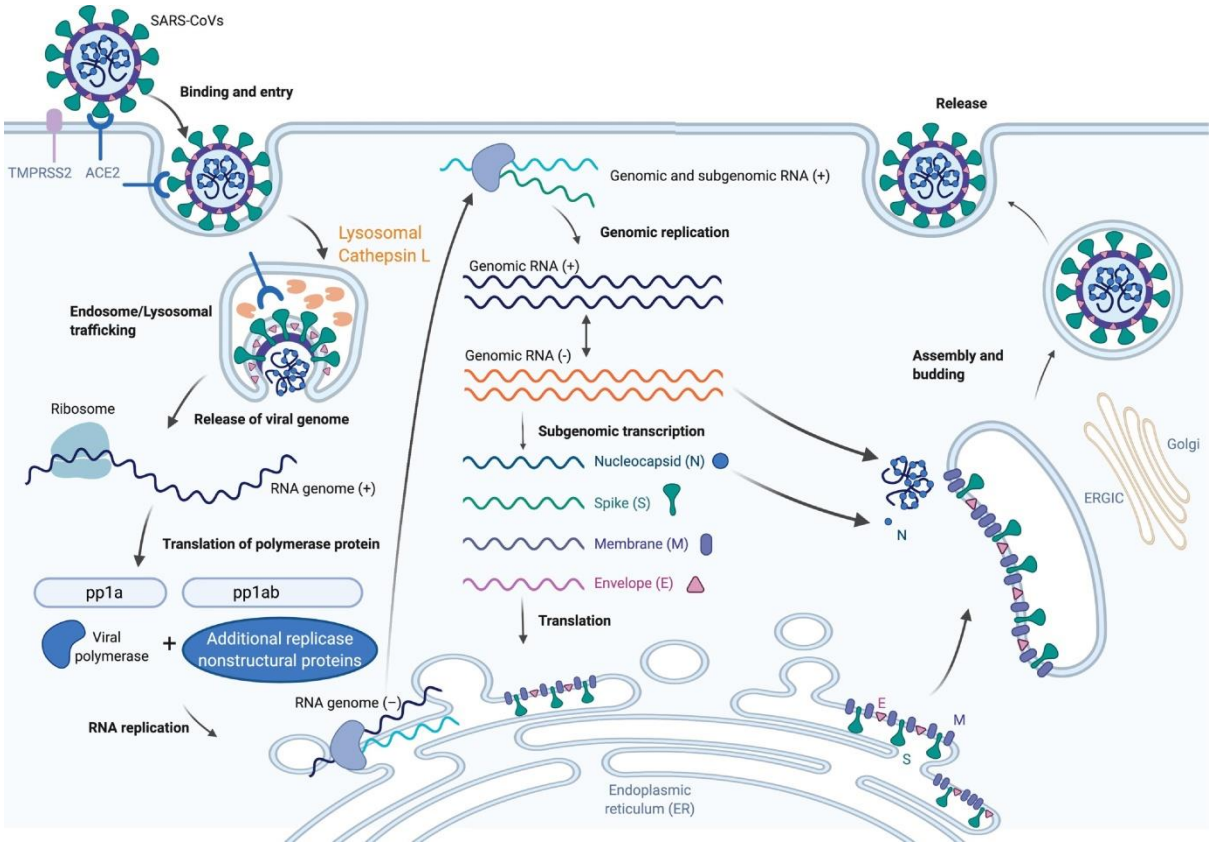


Figure 4.3. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) lifecycle.

The SARS-coronaviruses (SARS-CoV and SARS-CoV-2) binds with the host cell by interaction of spike protein to its cognate receptor, angiotensin-converting enzyme 2 (ACE2). But successful viral entry depends on few steps: (i) cleavage of the S1/S2 site by the surface transmembrane protease serine 2 (TMPRSS2); and/or (ii) endolysosomal cathepsin L mediated virus–cell membrane fusion at the cell surface and endosomal compartments, respectively. After entry viral genome is released into cytosol and translated into polyproteins viz. pp1a and pp1b. These polyproteins are then cleaved by viral genome encoded protease to form nonsytructural proteins including RNA-dependent RNA polymerase (RdRp). Replication begins in virus-induced double-membrane vesicles (DMVs) derived from the

endoplasmic reticulum (ER), which ultimately integrate to form elaborate webs of convoluted membranes. Here, the incoming positive-strand genome then serves as a template for full-length negative-strand RNA and subgenomic (sg)RNA. sgRNA translation results in both structural proteins and accessory proteins (simplified here as N, S, M, and E) that are inserted into the ER–Golgi intermediate compartment (ERGIC) for virion assembly. Then positive sense RNA genomes get packed in synthesized virions, which eventually get secreted from the plasma membrane. Adapted from (Harrison, Lin and Wang, 2020)

4.1.2. Background

Considering millions of infections every week, fast diagnosis and quarantine were the initial strategies to fight this COVID-19 pandemic. RT-PCR was the gold standard of diagnosis but it takes time, expertise and laboratory set up. In most of the cases existing facility was not ready for such diagnostic approach with large number of patients. So, an alternative method was the need of the time. Then lateral flow based rapid antibody (Ab) tests emerged as a cheaper, faster and on-site alternative to provide accurate but rapid diagnosis. So, rapid tests for SARS-CoV-2 antibodies (Abs) are being increasingly implemented to detect onset of community transmission, if any, especially the asymptomatic and convalescent cases.

In the meantime, it was anecdotally reported that antibodies (Abs) elicited against SARS-CoV-2 cross-reacted with dengue virus (DV) and gave false-positive results in DV IgG and IgM rapid tests (Yan *et al.*, 2020). At about the same time in June 2020, we monitored the onset and subsequent spread of COVID-19 over several months and observed that the highly dengue-endemic regions remained relatively less affected in terms of COVID-19 severity and mortality (Biswas and Sukla, 2020).

It is also noteworthy that early symptoms of COVID-19 can be mistaken for dengue fever including thrombocytopenia in highly dengue endemic countries like India and Brazil (Wu *et al.*, 2020). By this time, with the onset of monsoon in India, dengue infections have started increasing with COVID-19 pandemic in the background. Most cases of dengue virus (DV) infection are asymptomatic and self-limiting. One report estimated 390 million (95% CI: 284-528) infections per year globally of which 96 million (CI: 67-136) manifested clinically (Bhatt *et al.*, 2013). About 4 billion people across 129 countries are currently at the risk of DV infection, with 70% of global burden from Asia, namely the Indian subcontinent and Southeast Asia (Bhatt *et al.*, 2013). High incidence of DV infection has been regularly recorded in Kolkata, especially in 2017 (Sukla *et al.*, 2018; Murhekar *et al.*, 2019). This high

rate of infection indicates that a substantial population of Kolkata could be sero-positive for dengue. There is one study from India reported that 48% of Indian population is seropositive for dengue (Murhekar *et al.*, 2019). In this scenario, the obvious question is whether DV Abs, prevalent in people in highly dengue endemic regions like Kolkata, will cross-react in SARS-CoV-2 antibody tests. If this happens, serology-based diagnosis and sero-surveillance for these immunologically cross-reacting viruses have to be carried out with adequate precautions and other supporting information, in regions where both viruses are co-existent. Interpretation of results has to be done with caution to avoid arriving at erroneous estimates. So, we planned to test the aforesaid possibility using archived DV serum samples from 2017, long before COVID-19 emergence, to rule out the probability of pre-existing Abs.

4.1.3. Experimental methods

We have performed rapid DV IgG and IgM tests (SD Bioline, Abbott) on archived serum samples (n=33) from DV-diagnosed patients (NS1 ELISA-positive) from the 2017 dengue cases in Kolkata (pre-dating COVID-19 pandemic). Initially only DV seropositive samples were subjected towards SARS-CoV-2 Ab detection rapid tests. In context to our hypothesis we selected such samples with an incentive to monitor anti-DV Ab cross reactivity in SARS-CoV-2 Ab detection lateral flow immunoassay system.

Thirteen DV-Ab positive sera were subjected to rapid SARS-CoV-2 IgG and IgM lateral flow-based strip test (ImmunoQuick, ImmunoScience India) following manufacturers' instructions (Figure 4.1). AbCheck COVID-19 IgG and IgM test kit (NuLifecare) was also used to confirm the cross-reactivity. From the rest 20 DV-Ab negative samples, we took 10 random samples to check serological status against SARS CoV-2 to confirm background cross reactivity of the kits.

20ul of each sample was added in specified area of test strips followed by addition of two drops (~80-100ul) of kit-specific assay buffer in the designated spot, depending on the test kit. Assay buffer was added in marked region in case of dengue Ab kit and for COVID-19 rapid kits, at the same position where sample was added first. Appearance of "test line" for all strip tests was confirmed to ensure the validity of the tests performed. We have also used negative control serum samples (both dengue and COVID-19 negative) as shown (Figure 4.1C).

4.1.4. Results

Five of the thirteen DV Ab-positive samples were found to produce false-positive bands in SARS-CoV-2 IgG and IgM rapid test (Table 4.1). Same DV Ab-positive sample was found to produce false-positive result in two different COVID-19 test kits (Figure 4.1A, D). This confirms that DV Abs can, indeed, cross-react with SARS-CoV-2 antigen(s) and give false-positive results in COVID-19 rapid IgG and IgM test (Table 4.1). Four DV samples showed false-positive SARS-CoV-2 IgM bands; of these two were DV IgG and IgM both positive and two were only DV IgG positive. One DV IgM and IgG dual positive sample produced false-positive SARS-CoV-2 IgG but no IgM band (Table 4.1).

4.1.5. Discussion and inferences

Our observation confirms that DV Abs can, indeed, cross-react with SARS-CoV-2 antigen(s) and give false-positive results in COVID-19 rapid IgG and IgM test (Table 4.1). The ImmunoQuick kit insert mentions that seventy-five COVID-19-negative samples were tested for determining performance characteristics of the kit. No false positive results were observed. Cross-reactivity with dengue serum samples was also tested and results were found negative. Similarly, the product information about the AbCheck kit mentions that no cross-

reactivity was observed when twenty-four SARS-CoV-2 qRT-PCR positive and twelve virus-negative serum samples were tested.

The aforesaid antibody test results, in fact, corroborated well with our computational modelling (docking) studies that predicted with high confidence that human antibodies to DV envelope can potentially bind to "receptor-binding motif (RBM)" of SARS-CoV-2 Spike protein with some of the interactions even intercepting the ACE2 receptor binding to RBM (Nath, Mallick, Roy, Sukla and Biswas, 2021). As COVID-19 rapid Ab test kits mostly use immobilized SARS-CoV-2 surface antigen(s), our prediction is supported by the observed DV false-positivity in COVID-19 Ab rapid tests as well as by the Spike protein antibodies detecting ELISA tests (Lustig *et al.*, 2021). Our results demonstrate that in dengue endemic countries, Ab detection-based assays can result in false-positive COVID-19 IgM as well as IgG results in case of actually DV-infected patients. We were the first to detect this dengue cross-reactivity in COVID-19 antibody tests and these data was previously deposited to an open repository on June 2020 for the public awareness at the earliest possible during the pandemic (https://doi.org/10.1101/2020.07.03.20145797). Later this observation was published in Journal of Medical Microbiology (Nath, Mallick, Roy, Sukla, Basu, *et al.*, 2021).The reverse scenario had been first reported from Singapore i.e. originally two COVID-19 patients were mis-diagnosed as having dengue as antibodies to SARS-CoV-2 cross-reacted in DV antibody tests (Yan *et al.*, 2020). Both the aforesaid observations were subsequently further investigated and validated by the study from Israel, where the authors had more extensively probed and confirmed the cross-reactivity between dengue antibodies and SARS CoV-2 antigen(s) and *vice versa* via lateral flow-based rapid tests and ELISA tests in a larger number of patient samples (Lustig *et al.*, 2021). It was reported that 21 out of 95 (22%) dengue serum samples (collected before September, 2019, predating the emergence of SARS-CoV-2), showed equivocal/false-positive results in ELISA that detects antibodies

against the Spike protein of SARS-CoV-2. This was in stark contrast to the background false-positivity rate of 4%, estimated from 102 healthy subjects tested using the aforesaid ELISA (Lustig *et al.*, 2021).

From the above scenarios and our computational modelling studies (Lustig *et al.*, 2021; Nath, Mallick, Roy, Sukla and Biswas, 2021), it appears that both these viruses share antigenic similarities resulting in the observed cross-reactivity and warrants further investigation to elucidate this dengue COVID-19 conundrum. In conclusion, sero-surveillance needs to be complemented with NAT and/or virus antigen tests for definitive diagnosis of COVID-19 and dengue in regions where both the viral diseases are co-endemic now. It is also necessary to implement more specific immunoassays for accurate differential diagnosis of these cross-reacting flavivirus (dengue) and coronavirus (SARS-CoV-2). Another pertinent question is whether these two cross-reacting RNA viruses will confer some degree of cross-protection/immunity against the severity of the diseases caused by each of them? (Biswas and Sukla, 2020)

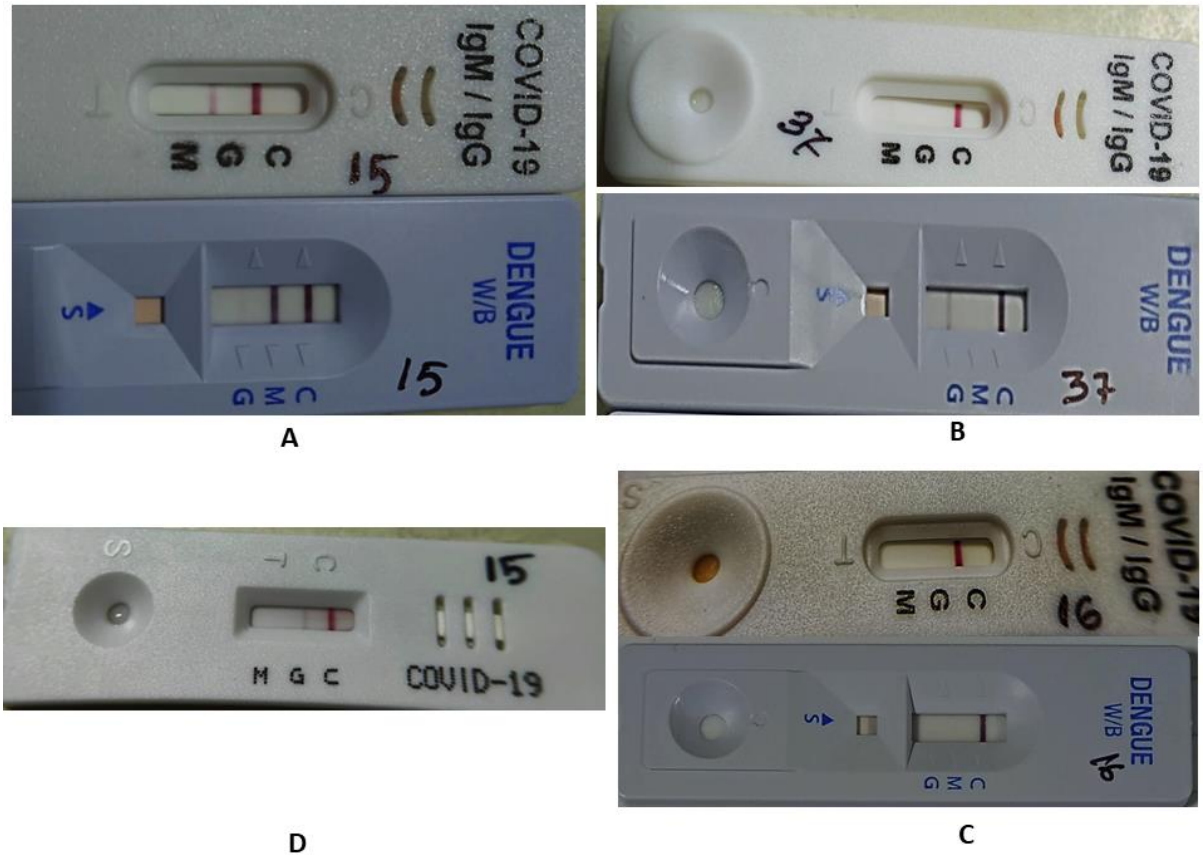


Figure 4.4. Representative images of COVID-19 and dengue rapid IgG and IgM strip tests

(A) Sample 17-D-15; (B) Sample 17-D-37; (C) Sample 17-D-16; (D) Sample 17-D-15 test using AbCheck, kit. A, D, represent a serum sample, confirmed positive for dengue but false-positive for COVID-19. B, represents a serum sample, confirmed positive for dengue but negative for COVID-19. C, represents a serum sample negative for both dengue and COVID-19 antibodies.

Sample Name	Age	Sex	Clinical Symptom(s)	SD-BIOLINE		ImmunoQuick		AbCheck	
				DV IgG	DV IgM	COVID-19 IgG	COVID-19 IgM	COVID-19 IgG	COVID-19 IgM
17-D-59	24	M	Fever, Weakness, Body ache	++	+++	-	+	-	+
17-D-68	33	M	Fever, Headache, Body ache	++	-	-	-	-	-
17-D-12	53	F	Fever, Headache, Redness of eye	+++	++	-	+	-	+
17-D-1	46	M	Fever, Body ache	+	+	-	-	-	-
17-D-7	20	M	Fever, Body ache	+++	-	-	+	-	+
17-D-11	51	F	Fever, Body ache	++	+	-	-	-	-
17-D-25	43	F	Fever, Body ache	+++	-	-	+	-	+
17-D-31	35	M	Fever, Body ache	++	-	-	-	-	-
17-D-30	34	F	Fever, Headache, Weakness	++	-	-	-	-	-
17-D-48	23	M	Fever, Headache, Rash	+++	+	-	-	-	-
17-D-37	62	M	Fever, Weakness, Body ache, Headache	+++	-	-	-	-	-
17-D-50	25	F	Fever, Headache, Weakness, Loss of appetite	-	+++	-	-	-	-
17-D-15	35	F	Fever, Body ache, Nausea	+	+++	+++	-	++	-
Healthy Control n=10	Median age 31	6F/4M	Fever and Body ache	-	-	-	-	-	-

Table 4.1. Table 1: Rapid IgG and IgM test results for COVID-19 and Dengue*

"F" denotes for Female and "M" denotes for Male.

* "+" sign denotes positive result; number of "+" sign signifies relative increase in positive band intensity in the strip tests.

"-" sign signifies negative result.

4.2. Effect of anti-SARS-CoV-2 Ab on Dengue virus neutralization

4.2.1. Background

It was anecdotally reported that antibodies (Abs) elicited against SARS-CoV-2 cross-reacted with dengue virus (DV) and gave false-positive results in DV IgG and IgM rapid tests (Yan *et al.*, 2020). At about the same time, we started monitoring the onset and subsequent spread of COVID-19 over several months and observed that the highly dengue-endemic regions remained relatively less affected in terms of COVID-19 severity and mortality (Biswas *et al*., 2020). Following up on this observation, we found that five of thirteen archived DV serum samples (from 2017, pre-dating the pandemic), cross-reacted with SARS-CoV-2 antigen(s) and gave false-positive IgG and IgM results in Spike protein-based COVID-19 rapid tests(Nath, Mallick, Roy, Sukla, Basu, *et al.*, 2021). However, other studies have shown that DV-infected serum did not cross-react with nucleocapsid-based SARS-CoV-2 Ab test kits (Faccini-Martínez et al., 2020).

Our computational docking studies detected a potential role of convalescent DV Abs in interacting with key ACE2 receptor (ACE2R)-binding regions of SARS-CoV-2 Spike antigen and strongly supported our previous cross-reactivity results (Nath, Mallick, Roy, Sukla and Biswas, 2021). Finally, scientists from Israel extensively probed and confirmed the cross-reactivity between DV Abs and SARS-CoV-2 antigen(s) and *vice versa* by means of lateral flow-based rapid tests (LFIAs) and ELISAs (Lustig *et al.*, 2021). They reported about 22% serological cross-reactivity both ways. Thus, there was ample evidence of serological cross-reactivity between the two distinct families of viruses (*Flaviviridae* and *Coronaviridae*) and this aroused the question of whether they are also cross-protective (Biswas *et al*., 2020).

4.2.2. Experimental methods

4.2.2.1. Study subjects

Serum samples from fifty-two clinically and laboratory confirmed (swab RT-PCR-positive) COVID-19 patients (Patient 1-52, Table 4.6) were collected from Behala Balananda Brahmachari Hospital and Research Centre in Kolkata from September 2020 to January 2021. All patients showed mild to severe COVID-19 symptoms but were discharged from the hospital eventually on recovery. The study was approved by the respective Institutional Ethical Committees of the aforesaid hospital and CSIR-IICB, Kolkata. Written informed consent (in their native language) was obtained from all individual participants included in this study. All experiments were carried out in accordance with the relevant guidelines and regulations.

4.2.2.2. Dengue and COVID-19 lateral flow immune-assays (LFIAs)

DV-specific IgG, IgM and NS1-Ag detection was done using the SD-Bioline Dengue Duo rapid test kit. COVID-19-specific IgG and IgM detection was carried out using the Abcheck kit. All tests were done as per manufacturer's instructions. In brief, each serum sample (20 µl) was added in a specified area of each test strip followed by the addition of two drops (~80-100 µl) of kit-specific assay buffer in the designated spot, depending on the test kit. Appearances of "test line/ Control line" for all strip tests were confirmed to ensure the validity of the assay. In the case of NS1-Ag detection strip, 100 µl of serum was added in the specified area. After 10-15 minutes IgG, IgM-specific lines were observed confirming seropositive results.

4.2.2.3. Dengue virus IgG ELISA

The ELISA was performed according to the protocol provided in the Kit (Euroimmun, Cat-EI 266b-9601G). Each patient serum was diluted (1:101) in sample buffer of the kit. The quantitative evaluation of reactive unit per ml (RU/ml) for each sample well of microplate was calculated by plotting the calibrator extinction co-efficient with calibrator's RU/ml. If the Samples with cut-off value > 22 RU/ml were considered dengue IgG ELISA-positive.

4.2.2.4. DV NS1-Ab ELISA

Dengue virus NS1 Ab ELISA was performed as per manufacturer's protocol (R&D Systems, Cat-DENG00). Recombinant NS1 antigens of DV type 1,2,3 and 4 were pre-coated onto microplate wells. This kit involves treatment of the samples to minimize false-positive results due to cross-reactive Abs to related flaviviruses, such as Zika virus. Samples were 50-fold diluted before adding in treatment plate and were overall, 100-fold diluted before addition in NS1 Ab detection plate.

4.2.2.5. RT-PCR for dengue virus

RNA was extracted from 200 µl of COVID-19 serum samples using High Pure Viral Nucleic Acid Extraction Kit (Roche), as per kit manual. RT-PCR was done using primers as described by Lanciotti et al. (Lanciotti *et al.*, 1992), to detect the presence of DV RNA. The details are given in previous chapters.

4.2.2.6. Cell Line

Huh7 cells were obtained from NCCS, India. Cells were cultured in DMEM (D5796, Sigma) supplemented with 10% FBS (Gibco) and Pen-Strep and L-Glutamine mix (Sigma) and Amphotericin B-2.5µg/ml (Gibco). Cell monolayers were grown at 37^0C with 5%CO_2. During passage, cells were washed with PBS (1X) and detached with Trypsin-EDTA (1X) (Gibco).

4.2.2.7. Virus

Dengue virus type 1 was cultured from serum sample collected during 2017 dengue outbreak in Kolkata as described before (Nath, Ghosh, *et al.*, 2021). Briefly, the serum sample was filtered using 0.22 µm PES syringe filter and inoculated in monolayer C6/36 cells and incubated for five days. After three passages in C6/36 cells, DV1 titer in supernatant was determined and stored in aliquots as stocks. NS1 gene of this virus was sequenced and deposited in GenBank (MT072226). We passaged the virus only three times to keep it close to the clinical scenario. This virus is not plaque-forming as reported before in case of other low passage clinical isolates (Raut *et al.*, 2019).

4.2.2.8. Dengue virus neutralization assay (VNT)

Serum samples were selected based on the results of LFIA and ELISA. Samples which were negative for DV-Abs, SARS-CoV-2Ab and NS1 were considered as DV negative serum controls for neutralization. Predated-COVID-19 pandemic samples which were DV-Ab+, SARS-CoV-2Ab- and NS1+, were considered as positive serum controls for DV neutralization. Both cross reacting and non-cross reacting COVID-19 serum samples were tested for DV-neutralizing activity (Table 4.2). Serum samples were inactivated at 56^0C for 30mins and diluted with equal volume of DMEM (supplemented with Pen-Strep, L-Glutamine mix, Sigma and Amphotericin-B, 2.5µg/ml). In each well of 96-well plates, 300 µl of diluted (1:1) serum was added. This was followed by addition of 300 µl DV type 1 inoculum (100 X TCID50) (Terrestrial Manual Online Access - OIE - World Organisation for Animal Health,). Diluted serum samples along with DV1 inoculum were incubated at 37^0C with 5%CO_2 for one hour. Then 300 µl of Huh7 cell suspension was added to each well and plate was kept in incubator. After 12hrs of incubation 500 µl of DMEM was added in each well. At 48hrs post-treatment, 600 µl of DMEM was added to maintain optimum pH. At 72hrs post-incubation, the supernatant was aspirated from each well and cells were washed

with 1ml of 1X PBS twice. After washing, 200 µl of fresh DMEM was added in each well and cells were harvested; stored at -80^{0}C until RNA was extracted.

4.2.2.9. RNA extraction and intracellular virus quantification

RNA was isolated from 200 µl of Huh7 cell lysate of each well as described before. RNA quantity was determined using Nanodrop one (Thermo). Virus titer was determined using SYBR-based one step qRT-PCR with Luna Universal One Step qRT-PCR reagent (NEB). qRT-PCR was done using equal quantity of RNA from each experimental condition. Quantstudio 5 (AB, Thermo) was used to run the qPCRs. Primers for qRT-PCRs were same as described for DV1 serotyping by Lanciotti et al. (Lanciotti *et al.*, 1992).

DV-Ab+, CoV-Ab-, NS1+ (Archived pre-pandemic serums of 2017)	DV-Ab-, CoV-Ab-, NS1- (Archived pre-pandemic serums)	DV-Ab-, CoV-Ab+, NS1- (COVID-19 serums)	DV-Ab+, CoV-Ab+, NS1- (COVID-19 serums)
17-D-37 **17-D-50** **17-D-30** **17-D-68**	N-2 N-4 N-8 N-10	Sample No. 18 Sample No. 19 Sample No. 41	Sample Nos. 1-16; 20-38; 42-50

Table 4.2: List of the serum samples which were used for virus neutralization test.

4.2.3. Results

4.2.3.1. COVID-19 patients' serum samples were highly false-positive in DV LFIAs

Serum samples from fifty-two clinically and laboratory confirmed (swab RT-PCR-positive) COVID-19 patients (Patient 1 – 52, Table 4.6) were collected from Behala Balananda Brahmachari Hospital and Research Centre in Kolkata from September 2020 to January 2021. All patients showed mild to severe COVID-19 symptoms but were discharged from the hospital eventually on recovery.

All the serum samples were tested for the presence of SARS-CoV-2 Abs (IgG and IgM) using the Abcheck COVID-19 rapid strip test. It was found that forty-seven of fifty-two samples were positive for IgG, IgM or both. So, 90% of serum samples contained detectable amount of SARS-CoV-2 Abs (Table 4.3).

These serum samples were tested on lateral flow-based SD BIOLINE Dengue Duo rapid strip test detecting DV IgG, IgM, and NS1 antigen as per manufacturer's instructions. Thirty-four of forty-seven COVID-19 patients' samples were only DV IgG-positive; seven were IgG and IgM dual positive and one was only IgM-positive. All the samples tested negative for NS1-Ag (Table 4.3). It is interesting to observe that overall, forty-two samples (34 IgG + 7 IgG/IgM+ 1 IgM = 42) constituted about 89% (42/47) of the COVID-19 seropositive cases that "cross-reacted" in DV serological strip tests. Serum samples from patients 33 and 52 were DV IgM and IgG positive respectively in rapid Ab tests but negative in DV IgG ELISA.

One sample (patient 40) was all negative, while some COVID-19 samples (patients 17, 39, 51 and 52) were SARS-CoV-2 Ab-negative but DV IgG positive by LFIA (patient 52) or ELISA (patient 17) or both (patient 39, 51) (Table 4.3).

SL no.	Sample Name	SD-BIOLINE Dengue IgG	SD- BIOLINE Dengue IgM	SD- BIOLINE Dengue NS1 Ag	AbCheck COVID-19 IgG	AbCheck COVID-19 IgM	EUROIMMUN DV IgG ELISA	NS1-Ab ELISA
1	Patient-1	+	+	-	+	+	+	+
2	Patient-2	+	-	-	+	+	+	-
3	Patient-3	+	+	-	+	+	+	+
4	Patient-4	+	-	-	+	+	+	-
5	Patient-5	+	-	-	-	+	+	-
6	Patient-6	+	-	-	+	+	+	-
7	Patient-7	+	-	-	+	+	+	+
8	Patient-8	+	-	-	+	+	+	-
9	Patient-9	+	-	-	+	+	+	+
10	Patient-10	+	-	-	+	+	+	+
11	Patient-11	+	+	-	+	-	+	+
12	Patient-12	+	-	-	+	+	+	-
13	Patient-13	+	-	-	+	-	+	-
14	Patient-14	+	-	-	+	+	+	-
15	Patient-15	+	-	-	+	+	+	-
16	Patient-16	+	+	-	+	+	+	+
17	Patient-17	-	-	-	-	-	+	-
18	Patient-18	-	-	-	+	+	-	-
19	Patient-19	-	-	-	+	+	-	-
20	Patient-20	+	+	-	+	-	+	+

21	Patient-21	+	-	-	+	+	+	-
22	Patient-22	+	-	-	+	+	+	-
23	Patient-23	+	-	-	+	+	+	+
24	Patient-24	+	-	-	+	+	+	-
25	Patient-25	+	-	-	-	+	+	-
26	Patient-26	+	-	-	+	+	+	-
27	Patient-27	+	-	-	+	+	+	+
28	Patient-28	+	+	-	+	+	+	+
29	Patient-29	+	-	-	+	+	+	-
30	Patient-30	+	-	-	+	+	+	-
31	Patient-31	+	+	-	+	+	+	+
32	Patient-32	+	-	-	+	+	+	-
33	Patient-33	-	+	-	+	+	-	-
34	Patient-34	+	-	-	+	+	+	-
35	Patient-35	+	-	-	-	+	+	-
36	Patient-36	-	-	-	+	+	+	-
37	Patient-37	+	-	-	+	+	+	+
38	Patient-38	+	-	-	+	+	+	+
39	Patient-39	+	-	-	-	-	+	-
40	Patient-40	-	-	-	-	-	-	-
41	Patient-41	-	-	-	+	+	-	-
42	Patient-42	+	-	-	+	+	+	-

43	Patient-43	+	-	-	+	-	+	+
44	Patient-44	+	-	-	+	+	+	-
45	Patient-45	+	-	-	-	+	+	+
46	Patient-46	+	-	-	+	+	+	-
47	Patient-47	-	-	-	+	+	+	-
48	Patient-48	+	-	-	+	+	+	+
49	Patient-49	+	-	-	+	+	+	-
50	Patient-50	+	-	-	+	+	+	-
51	Patient-51	+	-	-	-	-	+	+
52	Patient-52	+	-	-	-	-	-	+
		n=34/47	**n=8/47**	**n=0/52**	**n=43/52**	**n=43/52**	**n=43/47**	**n=19/52**
		IgG/ IgM n= 42/47			**IgG/IgM n= 47/52**			

Table 4.3: List of 52 COVID-19 NAT-positive serum samples along with the test results in SD-BIOLINE Dengue Duo (IgG, IgM and NS1), AbCheck COVID-19 rapid Ab test; Euroimmun DV IgG ELISA and R&D DV NS1 Ab ELISA. The highlighted samples were negative in SARS-CoV-2 Ab tests. The cross-reactivity with DV Ab tests were considered only for the SARS-CoV-2 Ab-positive samples. The "+" sign signifies that the test result was positive; the "-" sign signifies that test result was negative.

4.2.3.2. Dengue IgG ELISA on COVID-19 patients' serum samples confirmed the high degree of cross-reactivity

Forty-three of forty-seven SARS-CoV-2 Ab-positive samples were found DV IgG ELISA-positive which constituted about 91% of all the samples having SARS-CoV-2 (Table 4.3). This result coincided well with the strip test results (89%). Serum samples from patients 17, 36 and 47 were negative in DV rapid tests but positive in DV IgG ELISA.

4.2.3.3. COVID-19 serum samples did not contain dengue virus RNA

RT-PCR of extracted RNA from sera revealed that all samples were negative for dengue virus RNA.

4.2.3.4. DV lateral flow-based strip test of COVID-19 predated healthy control samples indicated lower DV seroprevalence

Thirty-two healthy sera (P-H-1 to 32), predating COVID-19 outbreak, collected from October 2016 to July 2017, from Kolkata were tested using the SD Bioline Dengue Duo rapid test. Ten samples gave positive results in only dengue IgG, which is about 31.25 % of the sample size (Table 4.5). No serum tested positive for dengue IgM or NS1 Ag.

4.2.3.5. NS1 Ab ELISA of COVID-19 samples to determine previous dengue-exposure

All fifty-two COVID-19 samples were tested in NS1 Ab Capture ELISA and 19 samples came out positive for DV NS1 Ab (Table 4.3). Among SARS-CoV-2 Ab-positive samples, seventeen tested positive for NS1 Ab (17/47=36.2%).

4.2.3.6. SARS-CoV-2 infected patients' serum samples can neutralize DV1 clinical isolate (VNT)

NS1 and DV Ab-positive serum samples (predating COVID-19), were considered as positive controls. Healthy serum samples (predating COVID-19 pandemic), negative for DV Abs, SARS-CoV-2 Ab and DV NS1 were used as negative serum controls. Samples that were positive for SARS-CoV-2 and DV Abs but NS1 negative, were tested for neutralization

potential in this study. Samples that were not cross-reacting i.e. SARS-CoV-2 Ab-positive but DV Ab-negative, were also tested (Table 4.2).

Intracellular DV gE (genome equivalents) was highest for the negative serum controls. In the case of positive serum controls, DV1 yield was much reduced (p=<0.0001) than negative serum controls (NSC), as expected. The COVID-19 serum samples (those were also DV Ab-positive but NS1-negative) effectively neutralized DV (p=0.0001) (Figure 4.2, Table 4.4). There were only three serum samples which were SARS-CoV-2 Ab+ but DV Ab- (Table 4.3). All three samples (Nos. 18, 19, 41) were unable to neutralize DV1 (Figure 4.5, Table 4.4).

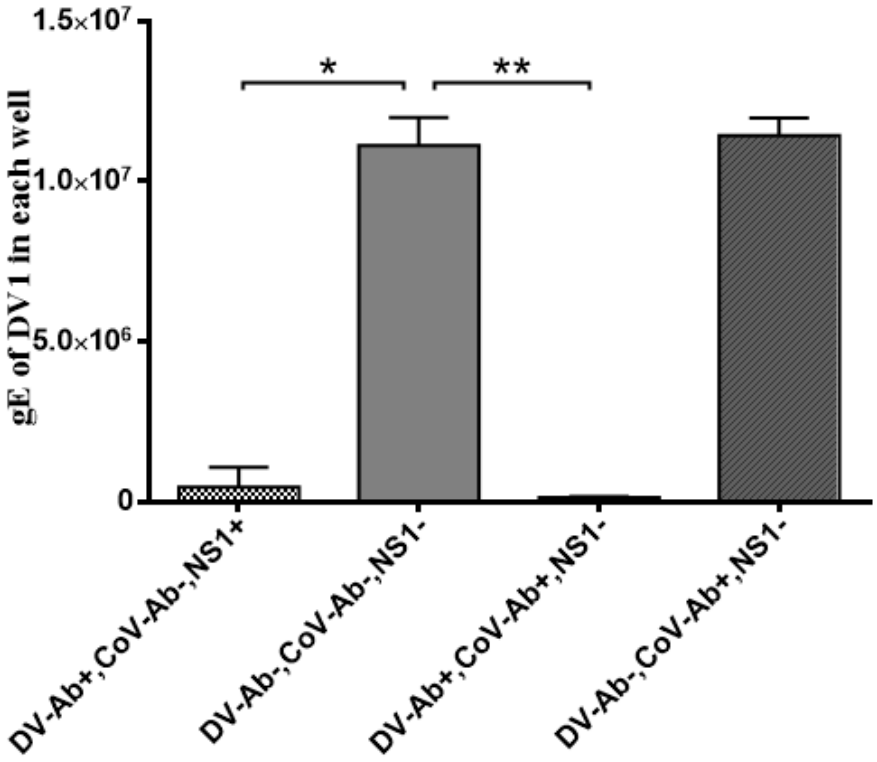


Figure 4.5: DV neutralization assay result using SARS-CoV-2 infected patients' serum samples.

DV Ab+, SARS-CoV-2 Ab-, NS1+ serum samples were considered as positive controls. They significantly reduced (*p=<0.0001) the virus yield with respect of negative controls (DV Ab-, SARS-CoV-2 Ab-, NS1-). Mean DV yield (gE) for all the DV Ab+, SARS-CoV Ab+, NS1- serum samples, was low (**p=0.0001) compared to that of the negative serum controls. In case of these samples, there was no significant difference in DV neutralization capacity between the NS1 Ab positive or negative COVID-19 samples. But for the three DV Ab-, SARS-CoV-2 Ab+, NS1- samples (Nos. 18, 19, 41), DV1 titer was quite similar to negative controls. Error bars indicate SD. DV-Ab: dengue virus antibody; CoV-Ab: SARS-CoV-2 antibody; NS1: Dengue virus non-structural protein 1.

SL No.	Sample Name	gE of DV1 (Mean)	SD
1	Patient-1	1.46 X 10^5	1756.23
2	Patient-2	8.24 X 10^4	1252.14
3	Patient-3	9.1 X 10^4	2387.96
4	Patient-4	7.96 X 10^4	3568.58
5	Patient-5	8.96 X 10^4	1946.87
6	Patient-6	1.13 X 10^5	1364.86
7	Patient-7	9.46 X 10^4	4723.91
8	Patient-8	1.22 X 10^5	4159.36
9	Patient-9	1.32 X 10^5	2391.57
10	Patient-10	9.87 X 10^4	6127.13
11	Patient-11	1.32 X 10^5	2496.1
12	Patient-12	1.3 X 10^5	3476.28
13	Patient-13	1.19 X 10^5	5219.41
14	Patient-14	1.34 X 10^5	1964.32
15	Patient-15	1.08 X 10^5	2976.31
16	Patient-16	9.75 X 10^4	4961.25
17	Patient-17	ND	
18	Patient-18	1.11 X 10^7	49167.27
19	Patient-19	1.21 X 10^7	37652.19
20	Patient-20	1.09 X 10^5	2499.86
21	Patient-21	1.45 X 10^5	3984.2
22	Patient-22	1.5 X 10^5	5219.61
23	Patient-23	1.26 X 10^5	3796.69
24	Patient-24	1.4 X 10^5	5951.31
25	Patient-25	1.08 X 10^5	7814.62
26	Patient-26	1.44 X 10^5	3382.2
27	Patient-27	1.16 X 10^5	1694.11
28	Patient-28	1.33 X 10^5	2931.29
29	Patient-29	1.36 X 10^5	4168.96
30	Patient-30	9.24 X 10^4	6791.62
31	Patient-31	1.26 X 10^5	3713.82
32	Patient-32	1.52 X 10^5	2313.61
33	Patient-33	1.46 X 10^5	4210.51
34	Patient-34	1.58 X 10^5	2247.16
35	Patient-35	1.62 X 10^5	1569.17
36	Patient-36	1.23 X 10^5	2971.65
37	Patient-37	1.3 X 10^5	7421.66
38	Patient-38	1.5 X 10^5	4691.28
39	Patient-39	ND	
40	Patient-40	ND	
41	Patient-41	1.09 X 10^7	52146.21
42	Patient-42	1.72 X 10^5	3369.91
43	Patient-43	1.48 X 10^5	2451.62
44	Patient-44	1.67 X 10^5	3497.41
45	Patient-45	1.56 X 10^5	1792.26

46	Patient-46	1.78 X 10^5	3891.51
47	Patient-47	1.75 X 10^5	4319.1
48	Patient-48	1.32 X 10^5	8479.37
49	Patient-49	1.7 X 10^5	2594.39
50	Patient-50	1.73 X 10^5	3172.41
51	Patient-51	ND	
52	Patient-52	ND	

Table 4.4. DV1 gE from virus neutralization assays with SARS-CoV-2 serum samples.

Mean is calculated from total DV1 yield (in gE) obtained from cells of one well (96 well plate) of three replicates for each sample.

SL No.	Sample Name	SD-BIOLINE Dengue IgG	SD- BIOLINE Dengue IgM	SD- BIOLINE Dengue NS1 Ag
1	P-H-1	+	-	-
2	P-H-2	+	-	-
3	P-H-3	-	-	-
4	P-H-4	-	-	-
5	P-H-5	+	-	-
6	P-H-6	-	-	-
7	P-H-7	-	-	-
8	P-H-8	+	-	-
9	P-H-9	-	-	-
10	P-H-10	-	-	-
11	P-H-11	-	-	-
12	P-H-12	-	-	-
13	P-H-13	+	-	-
14	P-H-14	+	-	-
15	P-H-15	-	-	-
16	P-H-16	-	-	-
17	P-H-17	-	-	-
18	P-H-18	-	-	-
19	P-H-19	-	-	-
20	P-H-20	+	-	-
21	P-H-21	-	-	-
22	P-H-22	-	-	-
23	P-H-23	-	-	-
24	P-H-24	-	-	-
25	P-H-25	+	-	-
26	P-H-26	-	-	-
27	P-H-27	-	-	-
28	P-H-28	+	-	-
29	P-H-29	+	-	-
30	P-H-30	-	-	-
31	P-H-31	-	-	-
32	P-H-32	-	-	-

Table 4.5. List of COVID-19-predated healthy control serum samples tested using the Abcheck COVID-19 IgG/IgM and SD BIOLINE Dengue Duo rapid test kits. Total 10 samples were positive out of 32. The "+" sign signifies a positive result; the "-" sign signifies a negative result.

Sl. No.	Isolate name	Age	Sex	Approximate date of admission of the patient to the Hospital	Sample procurement date	Symptoms
1	Patient-1	46	M	03.09.2020	08.09.2020	Severe fever, breathing problem
2	Patient-2	59	M	28.08.2020	08.09.2020	Mild fever, no breathing problem
3	Patient-3	62	M	03,09.2020	08.09.2020	Severe fever, breathing problem
4	Patient-4	52	M	21.08.2020	08.09.2020	Mild fever, no breathing problem
5	Patient-5	56	M	04.09.2020	08.09.2020	Mild fever, no breathing problem
6	Patient-6	53	M	01.09.2020	08.09.2020	Moderate fever breathing problem
7	Patient-7	50+	M	4.09.2020	08.09.2020	Moderate fever breathing problem
8	Patient-8	55	M	05.09.2020	08.09.2020	Moderate fever and breathing problem
9	Patient-9	53	M	01.09.2020	08.09.2020	Severe fever, breathing problem
10	Patient-10	49	M	05.09.2020	08.09.2020	Moderate fever and breathing problem
11	Patient-11	60	M	05.09.2020	08.09.2020	Moderate fever and breathing problem
12	Patient-12	72	M	26.08.2020	08.09.2020	Moderate fever and breathing problem
13	Patient-13	60	M	01.09.2020	08.09.2020	Mild fever, no breathing problem
14	Patient-14	56	M	03.09.2020	08.09.2020	Mild fever, no breathing problem
15	Patient-15	52	M	30.08.2020	08.09.2020	Mild fever, no breathing problem
16	Patient-16	64	M	1st admission in 1st week of August 2020; re infection and 2nd time admitted in end week of August 2020	08.09.2020	Mild fever, no breathing problem

17	Patient-17	53	M	01.09.2020	08.09.2020	Severe fever, breathing problem
18	Patient-18	23	M	29.08.2020	08.09.2020	Severe fever, breathing problem
19	Patient-19	42	M	30.08.2020	08.09.2020	Mild fever, no breathing problem
20	Patient-20	59	M	28.08.2020	08.09.2020	Severe fever, breathing problem
21	Patient-21	74	M	12.09.2020	22.09.2020	Mild fever no breathing problem
22	Patient-22	35	M	14.09.2020	22.09.2020	Severe fever, breathing problem
23	Patient-23	70	M	19.09.2020	22.09.2020	Severe fever, breathing problem
24	Patient-24	60	M	11.09.2020	22.09.2020	Severe fever, breathing problem
25	Patient-25	55	M	13.09.2020	22.09.2020	Severe fever, breathing problem
26	Patient-26	53	M	22.09.2020	22.09.2020	Mild fever no breathing problem
27	Patient-27	71	M	14.09.2020	22.09.2020	Severe fever, breathing problem
28	Patient-28	44	M	17.09.2020	22.09.2020	Mild fever, no breathing problem
29	Patient-29	32	M	18.09.2020	22.09.2020	Mild fever, no breathing problem
30	Patient-30	67	M	20.09.2020	22.09.2020	Mild fever, no breathing problem
31	Patient-31	55	M	10.09.2020	22.09.2020	Mild fever, no breathing problem
32	Patient-32	48	M	17.09.2020	22.09.2020	Moderate fever, breathing problem
33	Patient-33	71	M	20.09.2020	22.09.2020	Moderate fever, breathing problem
34	Patient-34	74	M	12.09.2020	22.09.2020	Mild fever, no breathing problem
35	Patient-35	55	M	15.09.2020	22.09.2020	Moderate fever, breathing problem
36	Patient-36	52	M	20.09.2020	22.09.2020	Moderate fever, breathing problem
37	Patient-37	64	M	12.09.2020	22.09.2020	Moderate fever, breathing problem
38	Patient-38	83	F	06.01.2021	12.01.2021	Moderate fever, breathing problem

39	Patient-39	72	F	02.01.2021	12.01.2021	Moderate fever, breathing problem
40	Patient-40	83	F	06.01.2021	12.01.2021	Moderate fever, breathing problem
41	Patient-41	65	F	07.01.2021	12.01.2021	Mild fever no breathing problem
42	Patient-42	16	F	07.01.2021	12.01.2021	Moderate fever, breathing problem
43	Patient-43	80	F	11.01.2021	12.01.2021	Moderate fever, breathing problem
44	Patient-44	78	F	09.01.2021	12.01.2021	Severe fever, breathing problem
45	Patient-45	74	F	24.12.2020	12.01.2021	Mild fever, no breathing problem
46	Patient-46	50	F	09.01.2021	12.01.2021	Moderate fever, breathing problem
47	Patient-47	48	F	31.12.2020	12.01.2021	Moderate fever, breathing problem
48	Patient-48	60	F	04.01.2021	12.01.2021	Moderate fever, breathing problem
49	Patient-49	59	F	29.12.2020	12.01.2021	Severe fever, breathing problem
50	Patient-50	73	F	05.01.2021	12.01.2021	Mild fever, no breathing problem
51	Patient-51	60	F	04.01.2021	12.01.2021	Severe fever, breathing problem
52	Patient-52	72	F	30.12.2020	12.01.2021	Mild fever, no breathing problem

Table 4.6. Patient information of 52 COVID-19 serum samples.

4.2.4. Discussion and inferences

Approximately, 89-91% COVID-19 Ab-positive serum samples cross-reacted with DV in LFIA (42/47) or ELISA test (43/47) (Table 4.3). Overall, 44 of 47 COVID-19 Ab-positive samples (93%) gave evidences of DV seropositivity (Table 4.3). This is in stark contrast to the COVID-19 samples from Israel showing 22% cross-reactivity (Lustig *et al.*, 2021) with DV, Israel being a dengue non-endemic region. The observed DV seropositivity was notably higher compared to the pre-pandemic (2017) seroprevalence of dengue in India (Murhekar *et al.*, 2019). The latter was estimated at 48.7%.

Serum samples from thirty-two apparently healthy patients (without any history of dengue), collected during 2016-17 from Kolkata, showed 32% seropositivity for DV IgG (Table 3). Thus, the serological cross-reaction of over 93% of SARS-CoV-2 infected patients' serum samples could not be explained by the background DV seroprevalence of 32% for Kolkata or 49% for India (Murhekar *et al.*, 2019). Only 36.2% samples tested positive for NS1 Ab. So, the remaining (93-36) =57% DV cross-reacting COVID-19 Ab-positive serum samples had no evidence of previous DV exposure.

The high percentage of DV cross-reactivity of the COVID-19 serum samples also did not corroborate with the fact that India has experienced a much smaller number of dengue cases in 2020 (Dengue/DHF situation in India, NVBDCP). Interestingly, none of the cross-reacting COVID-19 serum samples were DV NS1 or RNA-positive. It is also notable in this regard that many other highly dengue endemic regions (eg. Guangzhou in China, Sri Lanka) had also reported significantly reduced dengue outbreaks in 2020 during the pandemic (Jiang *et al.*, 2021; Liyanage, Rocklöv and Tissera, 2021). These observations serve as further circumstantial/epidemiological evidence of our observations.

We thought of two possible explanations for our above-mentioned observations. First, SARS-CoV-2, indeed, has some antigenic similarity with DV (Lustig *et al.*, 2021). This led to cross-reaction of Abs elicited against one another (Lustig *et al.*, 2021; Nath, Mallick, Roy, Sukla, Basu, *et al.*, 2021). This is possibly the case for the 57% COVID-19 Ab-positive serum samples that were cross-reactive in DV Ab tests but showed no evidence of DV exposure (DV RNA negative; DV NS1 negative and DV NS1 Ab-negative).

Secondly, due to antigenic similarity, SARS-CoV-2 infection may stimulate existing DV memory cells (from previously DV infected individuals, including DV asymptomatic cases) which then resulted in boost in production of DV Abs (Nath, Mallick, Roy, Sukla and Biswas, 2021). This is supported by the fact that all DV seropositive samples were DV RT-PCR and NS1 Ag negative, suggesting that none were from recently DV-infected subjects. Eight samples showed DV IgM but, as mentioned earlier, this could be non-specific. This explanation holds true for the 17 cross-reacting COVID-19 Ab-positive samples that were DV NS1 Ab-positive but DV RNA and DV NS1 antigen negative. Either way, the COVID-19 pandemic appears to immunologically stimulate a large part of the population against DV, as evident from our findings.

A study from India included 44 DV-infected children who were grouped based on clinical severity and mortality. The same children were then screened for SARS-CoV-2 infection and serological evidence. It was found that a previous exposure of SARS-CoV-2 had resulted in a less severe outcome with no death (Ravikumar *et al.*, 2021). These observations serve as further circumstantial/epidemiological evidence of our observations that SARS-CoV-2 serum samples can, indeed, neutralize and protect against dengue. All the seropositive/cross-reactive COVID-19 patients, though hospitalized, finally recovered.

Conversely, high DV Ab prevalence also appears to reduce severity and mortality due to COVID-19 as the mortality per million populations in India (July-August, 2021), despite strong second wave, was about 300 compared to around 2000 in many dengue non-endemic countries (Worldometer, 2021). A study, comprising 2351 participants from the Brazilian Amazon basin reported that COVID-19 was associated with higher risk of death in those who didn't have previous DV exposure (Silvestre *et al.*, 2021).

In order to functionally assess the impact of COVID-19 pandemic on DV host-pathogen interaction, we performed VNT of DV1 clinical strain using COVID-19 serum samples. In support of our hypothesis (that COVID-19 is protective against dengue), SARS-CoV-2 infected patients' serum samples that cross-reacted with DV envelope, had been found to significantly neutralize and restrict DV1 entry into host cells (Figure 4.5). Samples which were negative for NS1 Ab (i.e. without traceable history of DV infection) also successfully neutralized DV1. There was no significant difference in the degree of virus neutralization between the NS1 Ab-positive/negative COVID-19 serum samples, as evident form low standard deviation among the samples (Figure 4.5; Table 4.4). We used DV serum samples from 2017 i.e. predating the COVID-19 pandemic, as positive controls for DV neutralization. These serum samples significantly reduced the intracellular virus yield, confirming the approach and VNT assay conditions. Three COVID-19 serum samples (No. 18, 19, 41) that were not cross-reactive with DV were also considered for neutralization test. All these samples were RT-PCR positive for SARS-CoV-2 but negative for DV in all respects (Table 4.3). All three samples were unable to neutralize DV1 (Figure 4.5). Perhaps, these antibodies were generated against different emerging strains of SARS-CoV-2 (variants) and the Spike protein of such variants may not share enough antigenic similarity with DV to elicit cross-reactivity/DV neutralization.

From this study, it is clear that existing DV serological tests will no longer be conclusive for DV diagnosis in highly dengue-endemic countries where both the viruses are co-existent now. The same will apply to Spike protein-based sero-diagnostic tools for SARS-CoV-2.

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Publications and conferences

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V. Ghosh A, Sukla S, **Nath H**, Saha R, De A, Biswas S. Non-structural protein 1 (NS1) variants from dengue virus clinical samples revealed mutations that influence NS1 production and secretion. Eur J Clin Microbiol Infect Dis 2022;41:803–14. https://doi.org/10.1007/s10096-022-04441-4. **IF-2.83 (Not included in this thesis)**

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Presentation in Conferences

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Archived dengue serum samples produced false-positive results in SARS-CoV-2 lateral flow-based rapid antibody tests

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Abstract

Co-endemicity of SARS-CoV-2 and dengue virus (DV) infection is becoming a matter of serious concern as it has been already reported that antibodies (Ab) elicited by SARS-CoV-2 infection can produce false-positive results in dengue IgG and IgM rapid tests and *vice versa*. Here we communicate that five of thirteen DV antibody-positive serum samples from Kolkata, archived in 2017 (predating the COVID-19 outbreak), produced false-positive results in SARS-CoV-2 IgG/IgM lateral flow-based rapid tests. Our results emphasize the importance of implementing tests with higher specificity to conduct sero-surveillance for accurate estimation of SARS-CoV-2/DV prevalence in regions where both viruses now co-exist.

The world is experiencing the coronavirus disease 2019 (COVID-19) pandemic, with 21 294 845 confirmed cases and 761 779 deaths up to 16 August 2020 [1]. SARS-CoV-2 infection is increasing in India, with ~50–60 000 confirmed cases being reported daily for the last several days [1]. Due to this high daily infection rate, rapid tests for SARS-CoV-2 antibodies (Abs) are being increasingly implemented to detect the onset of community transmission, if any, especially asymptomatic and convalescent cases.

It has been previously reported anecdotally from Singapore that the Abs elicited by SARS-CoV-2 infection can produce false-positive results in dengue IgG and IgM rapid tests [2]. Recently, a report from Israel stated that 55 COVID-19 patients' sera produced 12 false-positive results (21.8%) in dengue lateral flow-based rapid tests [3]. It is also noteworthy that the early symptoms of COVID-19 can be mistaken for those of dengue fever, including thrombocytopenia, in highly dengue-endemic countries such as India and Brazil [4].

By this time, with the onset of monsoon in India, dengue infections have started increasing with the COVID-19 pandemic in the background. Most cases of dengue virus (DV) infection are asymptomatic and self-limiting. One report estimated 390 million (95% CI: 284–528) infections per year globally, of which 96 million (CI: 67–136) manifested clinically. About 4 billion people across 129 countries are currently at risk of DV infection, with 70% of the global burden in Asia, namely the Indian subcontinent and Southeast Asia [5].

In this scenario, the obvious question is whether DV Abs, prevalent in people in highly dengue-endemic regions like Kolkata, will cross-react in SARS-CoV-2 rapid antibody detection tests. If this happens, serology-based diagnosis and sero-surveillance for these immunologically cross-reacting viruses have to be carried out with adequate precautions/background and other supporting information, in regions where both viruses are co-existent. Interpretation of results has to be done with caution to avoid arriving at erroneous estimates.

We performed rapid DV IgG and IgM detection tests (SD Bioline, Abbott) on archived serum samples (n=33) from DV-diagnosed patients (NS1 ELISA-positive) from the 2017 dengue cases in Kolkata (pre-dating the COVID-19 pandemic). Initially, only DV seropositive samples were subjected to SARS-CoV-2 Ab detection rapid tests. The primary objective was to investigate DV Ab cross-reactivity

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Keywords: dengue virus; SARS-CoV-2; COVID-19; false-positive; antibody test; cross-reactive; strip test.

Abbreviations: ACE2, angiotensin-converting enzyme 2; ELISA, enzyme-linked immunosorbant assay; HCoV, human corona virus; MERS, middle east respiratory syndrome; NAT, nucleic acid test; NS1, non-structural protein 1; qRT-PCR, real time quantitative polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.

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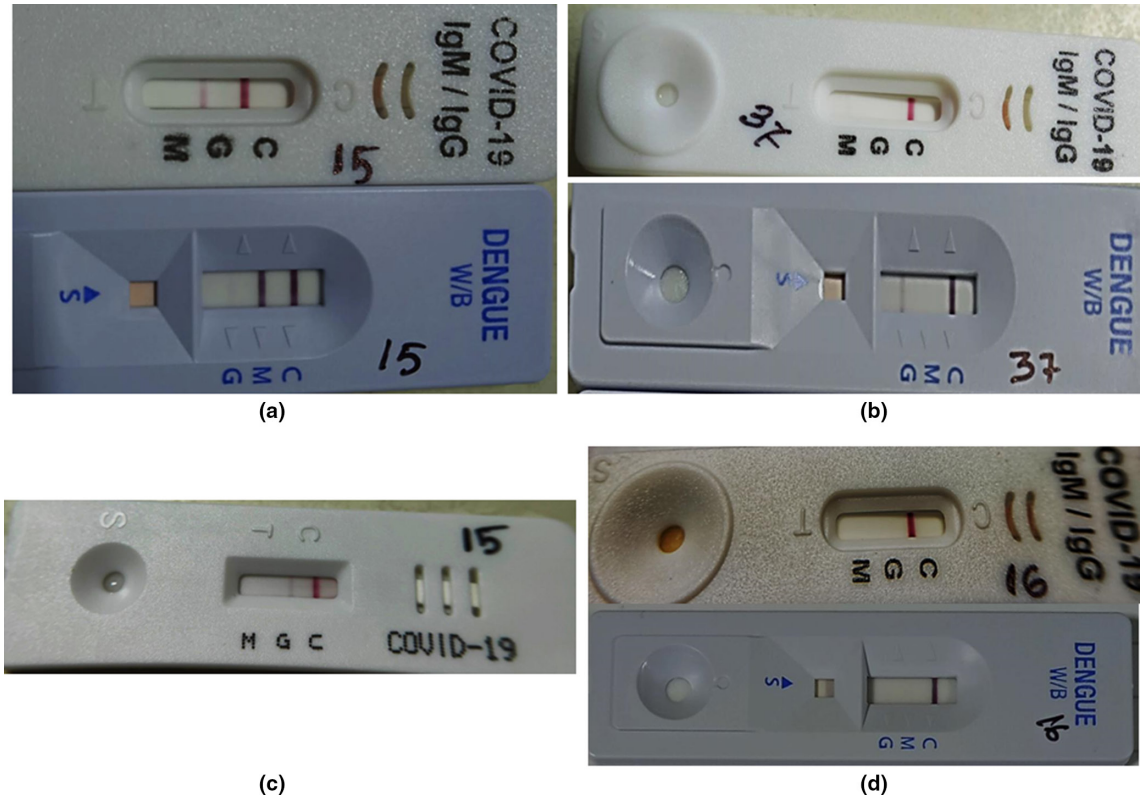

Fig. 1. Representative images of COVID-19 and dengue rapid IgG and IgM strip tests. (a) Sample 17-D-15; (b) sample 17-D-37; (c) sample 17-D-15 test using AbCheck kit; (d) sample 17-D-16. (a, c) A serum sample confirmed positive for dengue but false-positive for COVID-19. (b) A serum sample confirmed positive for dengue but negative for COVID-19. (d) A serum sample negative for both dengue and COVID-19 antibodies.

in lateral flow-based immunoassay system for SARS-CoV-2 Ab detection.

High incidence of DV infection has been regularly recorded in Kolkata [6] (from where the archived serum samples were collected). This was especially true for the year 2017 [7] and we therefore, envisaged that a substantial population of Kolkata could be seropositive for dengue. More importantly, we selected the DV serum samples from 2017, archived long before the COVID-19 emergence, in order to rule out the probability of pre-existing SARS-CoV-2 Abs in them, that would otherwise, react in the COVID-19 Ab tests.

Thirteen DV Ab rapid test positive sera (Table 1) were subjected to rapid SARS-CoV-2 IgG and IgM detection lateral flow-based strip test (ImmunoQuick, ImmunoScience India) following manufacturers' instructions (Figure 1). AbCheck COVID-19 test kit (IgG and IgM) (NuLifecare) was also used to confirm the cross-reactivity. Each COVID-19 rapid test strip was coated with SARS-CoV-2 antigen(s) as mentioned in the manufacturers' manuals. Ten out of the remaining twenty DV Ab-negative sera were randomly selected to check serological status against SARS-CoV-2 and to assess background cross-reactivity of the DV Ab-negative sera in the COVID-19 Ab kits, if any.

In brief, 20 µl of each sample was added to a specified area of the test strips, followed by the addition of two drops (~80–100 µl) of kit-specific assay buffer to the designated spot, depending on the test kit. The appearance of a 'test line' for all strip tests was confirmed to ensure the validity of the assay. We also used negative control serum samples (both DV and COVID-19 Ab-negative) as shown in Fig. 1d.

Five of the thirteen DV Ab-positive samples were found to produce false-positive bands in SARS-CoV-2 IgG and IgM detection rapid tests (Table 1). The same DV Ab-positive samples were found to produce a false-positive result in two different COVID-19 test kits (Fig. 1a,c). This confirms that DV Abs can, indeed, cross-react with SARS-CoV-2 antigen(s) and give false-positive results in COVID-19 rapid IgG and IgM tests (Table 1). The ImmunoQuick kit insert mentions that seventy-five COVID-19 negative samples were tested for determining the performance characteristics of the kit. No false-positive results were observed. Cross-reactivity with dengue sera was also tested and the results were found negative. Similarly, the product information of the AbCheck kit mentions that no cross-reactivity was observed when twenty-four SARS-CoV-2 qRT-PCR positive and twelve virus-negative sera were tested.

Table 1. Rapid IgG and IgM test results for COVID-19 and Dengue

Sample name	Age (years)	Sex	Clinical symptom(s)	SD-Bioline		ImmunoQuick		AbCheck	
				DV IgG	DV IgM	COVID-19 IgG	COVID-19 IgM	COVID-19 IgG	COVID-19 IgM
17-D-59	24	M	P, W, BA	f+	+++	–	++	–	++
17-D-68	33	M	P, H, BA	+	–	–	–	–	–
17-D-12	53	F	P, H, RE	++	+	–	+	–	+
17-D-1	46	M	P, BA	+	+	–	–	–	–
17-D-7	20	M	P, BA	++	–	–	+	–	+
17-D-11	51	F	P, BA	+	+	–	–	–	–
17-D-25	43	F	P, BA	++	–	–	+	–	+
17-D-31	35	M	P, BA	+	–	–	–	–	–
17-D-30	34	F	P, H, W	+	–	–	–	–	–
17-D-48	23	M	P, H, R	++	+	–	–	–	–
17-D-37	62	M	P, W, BA, H	++	–	–	–	–	–
17-D-50	25	F	P, H, W, LA	–	++	–	–	–	–
17-D-15	35	F	P, BA, N	+	++	++	–	++	–
DV Ab- negative controls $n=10^{\#}$	31 (Median age)	6F/4M	P, BA	–	–	–	–	–	–

"F" denotes female and "M" denotes male.
"P" denotes pyrexia; "W" denotes weakness; "BA" denotes body-ache; "H" denotes headache; "RE" denotes redness of the eye; "N" denotes nausea; "LA" denotes loss of appetite; "R"denotes rash.
"+" signifies positive result; "++" and "+++" signify relative increase in positive band intensity; "f+" stands for faint-positive band in the strip tests
"-" sign signifies a negative result.
"#": randomly selected serum samples from twenty DV Ab-negative (but NS1 ELISA-positive) serum samples.

Four DV serum samples showed false-positive SARS-CoV-2 IgM bands; of these two were DV IgG and IgM both positive and two were only DV IgG positive. One DV IgM and IgG dual positive sample produced a false-positive SARS-CoV-2 IgG but no IgM band (Table 1).

The aforesaid antibody test results corroborated well with our computational modelling (docking) studies that supported with high confidence that human antibodies to DV envelope can potentially bind to "receptor-binding motif (RBM)" of the SARS-CoV-2 Spike protein, with some of the interactions even intercepting the ACE2 receptor binding to RBM[8]. As COVID-19 rapid Ab test kits mostly use immobilized SARS-CoV-2 surface antigen(s), our prediction is supported by the observed DV false-positivity in COVID-19 Ab rapid tests as well as by the Spike protein antibodies detecting ELISA tests [3].

Our results demonstrate that in dengue-endemic countries, COVID-19 Ab detection-based assays can result in false-positive COVID-19 IgM as well as IgG results in case of DV-infected patients. We were the first to detect this dengue cross-reactivity in COVID-19 antibody tests, globally, and these data were previously deposited in an open-access repository as a preprint in July, 2020 for public awareness at the earliest possible during the pandemic [9]. The reverse scenario had been first reported from Singapore, i.e. originally two COVID-19 patients were misdiagnosed for having dengue as antibodies against SARS-CoV-2 cross-reacted in DV antibody tests [2]. Both the aforesaid observations were subsequently further investigated and validated by a study from Israel, where the authors had more extensively probed and confirmed the cross-reactivity between dengue antibodies and SARS-CoV-2 antigen(s) and *vice versa* via lateral flow-based rapid tests and ELISA tests in a larger number of patient samples [3]. It was reported that 21 out of 95 (22%) dengue serum samples (collected before September, 2019, predating the emergence of SARS-CoV-2), showed equivocal/false-positive results in ELISA, that detects antibodies against the Spike protein of SARS-CoV-2. This was in stark contrast to the background false-positivity rate of 4%, estimated from 102 healthy subjects tested using the aforesaid ELISA [3].

From the above scenarios and our computational modelling studies [3, 8], it appears that both these viruses share antigenic similarities resulting in the observed cross-reactivity and warrants further investigation to elucidate this dengue/COVID-19 conundrum.

Since the onset of the pandemic, several reports suggested potential serological cross-reactivity of SARS-CoV-2 virus with other seasonal HCoVs (NL63, HKU1, OC43 and 229E) and endemic coronaviruses (SARS-CoV-1 and MERS) [10–12]. Nevertheless, extremely low/sporadic incidences of SARS-CoV-1 and almost no incidence of MERS and the other four HCoVs had been observed in the Indian subcontinent, so far [13]. Surveying the epidemiological graph of SARS-CoV-1, it was observed that there were only three reported cases from India during the period of 25th April to 6th May, 2003 [14]. The MERS epidemiological situation report stated that there were no confirmed cases in India from 2012-2019 [9]. The above evidences suggest that there is much less probability of existing seroprevalence against circulating seasonal HCoVs and endemic coronaviruses in the Indian population. Thus, the serological cross-reactivity between SARS-CoV-2 and other human coronaviruses is less likely in the Indian sub-continent.

In conclusion, sero-surveillance needs to be complemented with NAT and/or virus antigen tests for definitive diagnosis of COVID-19 and dengue in regions where both the viral diseases are co-endemic now. It is also necessary to implement more specific immunoassays for accurate differential diagnosis of these cross-reacting flavivirus (dengue) and coronavirus (SARS-CoV-2). One open question that remains to be solved is whether there is a DV serotype specificity to cross-react with the SARS-CoV-2 Spike antigen(s) as approximately 22-38% and not all dengue serum samples produced false-positive results in COVID-19 antibody tests. This may be the reason why only one of the forty-four dengue serum samples collected from travellers before the COVID-19 emergence gave false-positive results in two different COVID-19 rapid antibody tests in a study from Italy [15]. Another pertinent question is whether these two cross-reacting RNA viruses will confer some degree of cross-protection/immunity against the severity of the diseases caused by each of them [8, 16].

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Ethical approval for the research was granted by the respective Institutional Ethical Committees of CSIR-IICB and Calcutta National Medical College, Kolkata. All experiments were carried out in accordance with the relevant guidelines and regulations. Written informed consent was obtained from all included patients.

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COVID-19 serum can be cross-reactive and neutralizing against the dengue virus, as observed by the dengue virus neutralization test

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ABSTRACT

Objectives: : Observing the serological cross-reactivity between SARS-CoV-2 and dengue virus (DV), we aimed to elucidate its effect on dengue serodiagnosis and infectivity in a highly dengue-endemic city in India.
Methods: : A total of 52 COVID-19 (reverse transcription-polymerase chain reaction [RT-PCR] positive) serum samples were tested in rapid lateral flow immunoassays and DV immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) to detect DV or SARS-CoV-2 IgG/immunoglobulin M. The COVID-19 antibody (Ab) positive samples were subjected to a virus neutralization test (Huh7 cells) using DV type 1 (DV1) clinical isolate.
Results: : Most (93%) of the SARS-CoV-2 Ab-positive serum samples cross-reacted with DV in rapid or ELISA tests. All were DV RNA and nonstructural protein 1 (NS1) antigen-negative. COVID-19 serum samples that were DV cross-reactive neutralized DV1. Of these, 57% had no evidence of DV pre-exposure (DV NS1 Ab-negative). The computational study also supported potential interactions between SARS-CoV-2 Ab and DV1.
Conclusion: : DV serodiagnosis will be inconclusive in areas co-endemic for both viruses. The COVID-19 pandemic appears to impart a protective response against DV in DV-endemic populations.

Introduction

In December 2019, the world faced the first wave of COVID-19. It was initially an endemic outbreak in the Hubei province, China. COVID-19 is an acute pneumonia-like respiratory illness in humans caused by a *betacoronavirus*, SARS-CoV-2. As even droplet nuclei can spread the virus, person-to-person transmission became rampant, augmented by super-spreader events. By the end of January 2020, the World Health Organization (WHO) declared the outbreak a Public Health Emergency of International Concern (World Health Organization, 2020). The COVID-19 pandemic completed its first year at the cost of 129 million infections and 2 million deaths globally and still counting (World Health Organization, 2022a). India reported over 34 million confirmed cases of COVID-19, with 461,000 deaths up to November 8, 2021 (World Health Organization, 2022b).

It was anecdotally reported that antibodies (Abs) elicited against SARS-CoV-2 cross-reacted with dengue virus (DV) and gave false-positive results in DV immunoglobulin G (IgG) and immunoglobulin M (IgM) rapid tests (Yan *et al.*, 2020). At about the same time, we started monitoring the onset and subsequent spread of COVID-19 over several months and observed that the highly dengue-endemic regions remained relatively less affected in terms of COVID-19 severity and mortality (Biswas *et al.*, 2020). Following up on this observation, we found that five of 13 archived DV serum samples (from 2017, pre-dating the pandemic) cross-reacted with SARS-CoV-2 antigen (Ags) and gave false-positive IgG and IgM results in Spike protein-based COVID-19 rapid tests (Nath et al., 2021b). However, other studies have shown that DV-infected serum did not cross-react with nucleocapsid-based SARS-CoV-2 Ab test kits (Faccini-Martínez *et al.*, 2020). Several publications by Lau *et al.* on SARS-CoV-2 Ab detection by highly sensitive chemiluminescent immunoassays stated that cross-reactivity with DV was not observed (Lau *et al.*, 2021, 2020a, 2020b).

Again, our computational docking studies detected a potential role of convalescent DV Abs in interacting with key angiotensin-converting

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enzyme 2 receptor-binding regions of SARS-CoV-2 Spike antigen and strongly supported our previous cross-reactivity results (Nath et al., 2021a). Finally, scientists from Israel extensively probed and confirmed the cross-reactivity between DV Abs and SARS-CoV-2 antigen(s) and *vice versa* using lateral flow immunoassay (LFIA)-based rapid tests and enzyme-linked immunosorbent assays (ELISAs) (Lustig *et al.*, 2021). They reported about 22% serological cross-reactivity both ways. Thus, there was ample evidence of serological cross-reactivity between the two distinct families of viruses (*Flaviviridae* and *Coronaviridae*). This aroused the question of whether they are also cross-protective (Biswas *et al.*, 2020).

Therefore, we investigated the reverse scenario, i.e., whether the increasing seropositivity for SARS-CoV-2 resulting from the ongoing pandemic has any impact on the dengue occurrences in highly dengue-endemic regions like Kolkata, India. Initially, we performed docking studies with SARS-CoV-2 Ab and DV envelope antigen. This was followed by checking COVID-19 Ab cross-reactivity in DV serological tests. Finally, the DV type 1 (DV1) neutralization test was conducted using the serum samples of SARS-CoV-2 infected patients.

Methods

Study subjects

Serum samples from 52 clinically and laboratory-confirmed (swab reverse transcription-polymerase chain reaction [RT-PCR] positive) patients with COVID-19 (Patients 1-52, Supplementary Table S1) were collected from Behala Balananda Brahmachari Hospital and Research Center in Kolkata from September 2020 to January 2021. All patients showed mild to severe COVID-19 symptoms but were discharged from the hospital eventually on recovery. The study was approved by the respective Institutional Ethical Committees of the previously mentioned hospital and Council of Scientific & Industrial Research-Indian Institute of Chemical Biology (CSIR-IICB), Kolkata. Written informed consent (in their native language) was obtained from all individual participants included in this study. All experiments were carried out per relevant guidelines and regulations.

Dengue and COVID-19 LFIAs

DV-specific IgG, IgM, and NS1 Ag detection were done using the Standard Diagnostics-Bioline Dengue Duo rapid test kit. As mentioned in the kit insert, the test kit was tested in four different study sites to determine its sensitivity and specificity. In brief, it showed 92.4% (95% confidence interval [CI]: 86.1-95.9%) sensitivity and 98.4% (95% CI: 95.5-99.5%) specificity for dengue NS1 Ag. For detection of dengue IgG/IgM, the kit showed 94.2% (95% CI: 88.5-97.2%) sensitivity and 96.4% (95% CI: 93.0-98.2%) specificity. This kit has also been used to test four mosquito-borne pathogens, e.g., Japanese encephalitis virus, Yellow Fever virus, *Plasmodium falciparum,* and *Plasmodium vivax,* but there was no cross-reactivity.

COVID-19-specific IgG and IgM detection was carried out using the Abcheck kit. This kit detects Abs against the Spike protein of SARS-CoV-2. Per the kit brochure, the positive and negative coincident rates for SARS-CoV-2 IgM detection were 93.42% and 96%, respectively. In the case of SARS-CoV-2 IgG detection, the positive and negative coincident rates were 98.68% and 97.5%, respectively. In both cases, results were obtained considering viral RNA detection as the gold standard.

All tests were done per the manufacturer's instructions. In brief, each serum sample (20 µl) was added in a specified area of each test strip, followed by two drops (~80–100 µl) of kit-specific assay buffer in the designated spot, depending on the test kit. Appearances of "test line/control line" for all strip tests were confirmed to ensure the validity of the assay. In the case of the NS1 Ag detection strip, 100 µl of serum was added to the specified area. After 10-15 minutes, IgG- and IgM-specific lines were observed, confirming seropositive results.

SARS-CoV-2 Ab ELISA

All COVID-19 serum samples were also tested in the SARS-CoV-2 Spike protein IgG detection ELISA kit (CST #20154). The ELISA was performed per the manufacturer's protocol. In brief, serum samples were heat-inactivated at 53°C for 30 minutes and diluted (1:800) with sample diluent (provided in the kit) before running the assay. The absorbance of blank at 450 nm was subtracted from the optical density (OD) of a sample, with positive and negative controls. Diluted samples were considered positive if OD was more than 4.1 times the negative control OD. Samples were negative if OD at 450 nm was less than three times the negative control OD. Per the kit insert, this kit has 100% specificity and 95.2% sensitivity.

DV IgG ELISA

The ELISA was performed according to the protocol provided in the kit (Euroimmun, Cat-EI 266b-9601G). Each patient's serum was diluted (1:101) in the sample buffer of the kit. The quantitative evaluation of reactive unit per ml (RU/ml) for each sample well of microplate was calculated by plotting the calibrator extinction co-efficient with the calibrator's RU/ml. Samples with a cut-off value >22 RU/ml were considered dengue IgG ELISA-positive.

DV NS1 Ab ELISA

DV NS1 Ab ELISA was performed per the manufacturer's protocol (R&D Systems, Cat-DENG00). Recombinant NS1 antigens of DV types 1, 2, 3, and 4 were precoated onto microplate wells. This kit involves treatment of the samples to minimize false-positive results because of cross-reactive Abs to related flaviviruses, such as the Zika virus. Samples were diluted 50-fold before adding to the treatment plate and were, overall, diluted 100-fold before addition to the NS1 Ab detection plate.

RT-PCR for DV

RNA was extracted from 200 µl of COVID-19 serum samples using the High Pure Viral Nucleic Acid Extraction Kit (Roche), per the kit's prescribed manual. RT-PCR was done using primers, as described by Lanciotti *et al.* (Lanciotti *et al.*, 1992), to detect the presence of DV RNA.

Cell line

Huh7 cells were obtained from National Centre For Cell Science, India. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (D5796, Sigma) supplemented with 10% fetal bovine serum (Gibco) and Pen-Strep and L-Glutamine mix (Sigma) and Amphotericin B-2.5µg/ml (Gibco). Cell monolayers were grown at 37°C with 5%CO_2. During the passage, cells were washed with phosphate-buffered saline (PBS) (1X) and detached with Trypsin-ethylenediaminetetraacetic acid (1X) (Gibco).

Virus

DV1 was cultured from a serum sample collected during a 2017 dengue outbreak in Kolkata as described previously (Nath *et al.*, 2020). Briefly, the serum sample was filtered using a 0.22 µm PES syringe filter, inoculated in monolayer C6/36 cells, and incubated for five days. After three passages in C6/36 cells, the DV1 titer in the supernatant was determined and stored in aliquots as stocks. The NS1 gene of this virus was sequenced and deposited in the Genetic Codes Databank (Gen-

Bank) (MT072226). We passaged the virus only three times to keep it close to the clinical scenario. This virus is not plaque-forming, as reported previously in the case of other low passage clinical isolates (Raut *et al.*, 2019).

DV neutralization assay (VNT)

Serum samples were selected based on the results of LFIA and ELISA. Samples negative for DV Abs, SARS-CoV-2 Ab, and NS1 were considered DV-negative serum controls for neutralization. Predated COVID-19 pandemic samples, which were DV Ab-positive, SARS-CoV-2 Ab-negative, and NS1-positive were considered as positive serum controls for DV neutralization. Cross-reacting and non–cross-reacting COVID-19 serum samples were tested for DV-neutralizing activity (Table 1). Serum samples were inactivated at 56°C for 30 minutes and diluted with an equal volume of DMEM (supplemented with Pen-Strep, L-Glutamine mix, Sigma, and Amphotericin B, 2.5μg/ml). In each well of 96-well plates, 300 μl of diluted (1:1) serum was added. This was followed by adding 300 μl DV1 inoculum (100 X median tissue culture infectious dose) (World Organization for Animal Health, 2022). Diluted serum samples and DV1 inoculum were incubated at 37°C with 5%CO_2 for one hour. Then, 300 μl of Huh7 cell suspension was added to each well, and the plate was kept in an incubator. After 12 hours of incubation, 500 μl of DMEM was added to each well. At 48 hours after treatment, 600 μl of DMEM was added to maintain optimum pH. At 72 hours after incubation, the supernatant was aspirated from each well, and cells were washed with 1 ml of 1X PBS twice. After washing, 200 μl of fresh DMEM was added to each well, and cells were harvested; stored at -80°C until RNA was extracted.

RNA extraction and intracellular virus quantification

As described before, RNA was isolated from 200 μl of Huh7 cell lysate of each well. RNA quantity was determined using Nanodrop One (Thermo). Virus titer was determined using SYBR Green dye-based one-step quantitive (q) RT-PCR with Luna Universal One-Step qRT-PCR reagent (New England Biolabs). qRT-PCR was done using an equal quantity of RNA from each experimental condition. Quantstudio 5 (Applied Biosystems, Thermo) was used to run the qPCRs. Primers for qRT-PCRs were the same as described for DV1 serotyping by Lanciotti *et al.* (Lanciotti *et al.*, 1992).

In silico docking experiment

Protein Data Bank (PDB) structure files of SARS-CoV-2 Ab, DV1, and West Nile Virus (WNV) envelope antigen were retrieved from the Research Collaboratory for Structural Bioinformatics PDB; PDB ID: 7BWJ (SARS-CoV-2 receptor-binding domain [RBD] neutralizing Ab), 3G7T (DV1 envelope protein in the postfusion conformation), 2HG0 (WNV envelope glycoprotein). The SARS-CoV-2 Ab structure was in neutralizing condition with SARS-CoV-2 Spike protein RBD. A separate PDB structure file of the Ab was created consisting of only the paratope i.e. the fragment antigen-binding part, using PyMOL Molecular Graphics System Version 2.3.3. (Schrödinger, LLC software). Subsequent processing of these structure files, such as solvent deletion, deletion of alternate positions (retaining only the highest occupancy positions), hydrogen addition, and partial charge assignment, were done using the Dock Prep plugin of Chimera software (Pettersen *et al.*, 2004). Standard residues (receptor amino acids) were assigned AMBER ff14sb partial charges (Cornell *et al.*, 1995; Maier *et al.*, 2015). For the receptor cofactors with ANTECHAMBER, AM1-BCC charges were computed, which is included in Chimera (Jakalian *et al.*, 2000; Wang *et al.*, 2006). After all the modifications, the modified Ab structure was uploaded as a receptor PDB file in each of the two Fast Fourier Transform (FFT) algorithm-based docking servers, i.e., ClusPro (Kozakov *et al.*, 2017) and ZDOCK (Pierce *et al.*, 2014), along with DV or WNV envelope antigen as ligand input PDB file. For ClusPro, Ab mode was enabled for docking. A total of 10 antigen-antibody complex structures were predicted by each of the servers. All the interaction interfaces of 20 complex structures were thoroughly analyzed in PyMol using the "find any interaction within 3.5Å cut-off" plugin. Each amino acid of antigen(s) in the interaction interface was identified and marked. The marked amino acids were listed and analyzed in Microsoft Excel (Supplementary Table S2).

Table 1
List of the serum samples used for virus neutralization tests

DV-Ab+, CoV-Ab-, NS1+ (Archived pre-pandemic serums of 2017)	DV-Ab-, CoV-Ab-, NS1- (Archived pre-pandemic serums)	DV-Ab-, CoV-Ab+, NS1- (COVID-19 serums)	DV-Ab+, CoV-Ab+, NS1- (COVID-19 serums)
17-D-37	N-2	Sample No. 18	Sample Nos. 1-16;
17-D-50	N-4	Sample No. 19	20-38; 42-50
17-D-30	N-8	Sample No. 41	
17-D-68	N-10		

DV = dengue virus; Ab = antibody; No = number; Nos = numbers; NS1 = nonstructural protein 1.

Results

The patients with COVID-19 serum samples were highly false-positive in DV LFIAs

Serum samples from 52 clinically and laboratory-confirmed (swab RT-PCR-positive) patients with COVID-19 (Patients 1-52, Supplementary Table S1) were collected from Behala Balananda Brahmachari Hospital and Research Center in Kolkata from September 2020 to January 2021. All patients showed mild to severe COVID-19 symptoms but were discharged from the hospital eventually on recovery.

All the serum samples were tested for SARS-CoV-2 Abs (IgG and IgM) using the Abcheck COVID-19 rapid strip test. It was found that 47 of 52 samples were positive for IgG, IgM, or both. Therefore, 90% of serum samples contained a detectable amount of SARS-CoV-2 Abs (Table 2). The previously mentioned results were confirmed using the SARS-CoV-2 Spike protein serological IgG ELISA kit. The ELISA results corroborated precisely with the results of the LFIA diagnostic kit.

These serum samples were tested on lateral flow-based Standard Diagnostics-Bioline Dengue Duo rapid strip test detecting DV IgG, IgM, and NS1 antigen per the manufacturer's instructions. Samples from 34 of 47 patients with COVID-19 were only DV IgG-positive, seven were IgG and IgM dual positive, and one was only IgM-positive. All the samples tested negative for NS1 Ag (Table 2). It is interesting to observe that overall, 42 samples (34 IgG-positive, 7 IgG/IgM-positive, 1 IgM-positive = 42) constituted about 89% (42/47) of the COVID-19 seropositive cases that "cross-reacted" in DV serological strip tests (Supplementary Figure S1). Serum samples from patients 33 and 52 were DV IgM- and IgG-positive in rapid Ab tests but negative in DV IgG ELISA. One sample (patient 40) was all negative, whereas some COVID-19 samples (patients 17, 39, 51, and 52) were SARS-CoV-2 Ab-negative but DV IgG-positive by LFIA (patient 52) or ELISA (patient 17) or both (patient 39, 51) (Table 2).

Dengue IgG ELISA on serum samples from patients with COVID-19 confirmed the high degree of cross-reactivity

A total of 43 of 47 SARS-CoV-2 Ab-positive samples were found DV IgG ELISA-positive, constituting about 91% of all the samples having detectable SARS-CoV-2 Ab (Table 2). This result coincided well with the strip test results (89%). Serum samples from patients 17, 36, and 47 were negative in DV rapid tests but positive in DV IgG ELISA.

Table 2
List of 52 COVID-19 NAT-positive serum samples along with the test results in AbCheck COVID-19 rapid Ab test; SARS-CoV-2 Spike IgG ELISA, CST; Standard Diagnostics-Bioline Dengue Duo (IgG, IgM, and NS1), Euroimmun DV IgG ELISA, and R&D Systems DV NS1 Ab ELISA

SL no.	Sample Name	AbCheck COVID-19 IgG	AbCheck COVID-19 IgM	SARS-CoV-2 Spike IgG ELISA	SD-BIOLINE Dengue IgG	SD-BIOLINE Dengue IgM	SD-BIOLINE Dengue NS1 Ag	EUROIMMUN DV IgG ELISA	NS1 Ab ELISA
1	Patient-1	+	+	+	+	+	-	+	+
2	Patient-2	+	+	+	+	-	-	+	-
3	Patient-3	+	+	+	+	+	-	+	+
4	Patient-4	+	+	+	+	-	-	+	-
5	Patient-5	-	+	+	+	-	-	+	-
6	Patient-6	+	+	+	+	-	-	+	-
7	Patient-7	+	+	+	+	-	-	+	+
8	Patient-8	+	+	+	+	-	-	+	-
9	Patient-9	+	+	+	+	-	-	+	+
10	Patient-10	+	+	+	+	-	-	+	+
11	Patient-11	+	-	+	+	+	-	+	+
12	Patient-12	+	+	+	+	-	-	+	-
13	Patient-13	+	-	+	+	-	-	+	-
14	Patient-14	+	+	+	+	-	-	+	-
15	Patient-15	+	+	+	+	-	-	+	-
16	Patient-16	+	+	+	+	+	-	+	+
17	**Patient-17**	-	-	-	-	-	-	+	-
18	Patient-18	+	+	+	-	-	-	-	-
19	Patient-19	+	+	+	-	-	-	-	-
20	Patient-20	+	-	+	+	+	-	+	+
21	Patient-21	+	+	+	+	-	-	+	-
22	Patient-22	+	+	+	+	-	-	+	-
23	Patient-23	+	+	+	+	-	-	+	+
24	Patient-24	+	+	+	+	-	-	+	-
25	Patient-25	-	+	+	+	-	-	+	-
26	Patient-26	+	+	+	+	-	-	+	-
27	Patient-27	+	+	+	+	-	-	+	+
28	Patient-28	+	+	+	+	+	-	+	+
29	Patient-29	+	+	+	+	-	-	+	-
30	Patient-30	+	+	+	+	-	-	+	-
31	Patient-31	+	+	+	+	+	-	+	+
32	Patient-32	+	+	+	+	-	-	+	-
33	Patient-33	+	+	+	-	+	-	-	-
34	Patient-34	+	+	+	+	-	-	+	-
35	Patient-35	-	+	+	+	-	-	+	-
36	Patient-36	+	+	+	-	-	-	+	-
37	Patient-37	+	+	+	+	-	-	+	+
38	Patient-38	+	+	+	+	-	-	+	+
39	**Patient-39**	-	-	-	+	-	-	+	-
40	**Patient-40**	-	-	-	-	-	-	-	-
41	Patient-41	+	+	+	-	-	-	-	-
42	Patient-42	+	+	+	+	-	-	+	-
43	Patient-43	+	-	+	+	-	-	+	+
44	Patient-44	+	+	+	+	-	-	+	-
45	Patient-45	-	+	+	+	-	-	+	+
46	Patient-46	+	+	+	+	-	-	+	-
47	Patient-47	+	+	+	-	-	-	+	-
48	Patient-48	+	+	+	+	-	-	+	+
49	Patient-49	+	+	+	+	-	-	+	-
50	Patient-50	+	+	+	+	-	-	+	-
51	**Patient-51**	-	-	-	+	-	-	+	+
52	**Patient-52**	-	-	-	+	-	-	-	+
		n = 43/52	n = 43/52	n = 47/52	n = 34/47	n = 8/47	n = 0/52	n = 43/47	n = 19/52
		IgG/IgM (n = 47/52)			IgG/IgM (n = 42/47)				

The samples in bold, were negative in SARS-CoV-2 Ab tests. The cross-reactivity with DV Ab tests was considered only for the SARS-CoV-2 Ab-positive samples.
The "+" sign signifies that the test result was positive; the "-" sign signifies that the test result was negative.
Ab = antibody; Ag = antigen; CST = Cell Signaling Technology; DV = dengue virus; ELISA = enzyme-linked immunosorbent assay; IgG = immunoglobulin G; IgM = immunoglobulin M; NAT = Nucleic acid testing; NS1 = nonstructural protein 1; R&D = Research & Diagnostics; SD-BIOLINE = Standard Diagnostics-Bioline; SL = serial.

COVID-19 serum samples did not contain DV RNA

RT-PCR of extracted RNA from sera revealed that all samples were negative for DV RNA.

DV lateral flow-based strip test of COVID-19 predated healthy control samples indicated lower DV seroprevalence

A total of 32 healthy sera (P-H-1 to 32), predating the COVID-19 outbreak, collected from October 2016 to July 2017 from Kolkata, were tested using the Standard Diagnostics-Bioline Dengue Duo rapid test. A total of 10 samples gave positive results in only dengue IgG, which is about 31.25 % of the sample size (Table 3). No serum tested positive for dengue IgM or NS1 Ag.

NS1 Ab ELISA of COVID-19 samples to determine the previous dengue-exposure

All 52 COVID-19 samples were tested in NS1 Ab Capture ELISA, and 19 samples were positive for DV NS1 Ab. Among SARS-CoV-2 Ab-positive samples, 17 tested positive for NS1 Ab (17/47 = 36.2%).

Computational docking studies with flavivirus envelope antigen and SARS-CoV-2 antibody predicted cross-reactive neutralization potency against DV

The structural PDB file of DV1 envelope antigen (PDB ID: 3G7T) was dock-prepared in Chimera software along with the SARS-CoV-2 RBD neutralizing Ab PDB file (PDB ID: 7BWJ). After adding additional rotamers, charges, and forcefield in the structural files, the DV antigen and SARS-CoV-2 Ab PDB files were docked using two FFT-based docking servers, i.e., ClusPro and ZDOCK. Each docking server provided 10 predicted antigen-antibody complex structures. A total of 20 predicted structures were analyzed in PyMOL software, and the antigen-antibody interacting surface was mapped. Only the common interactions predicted by both ClusPro and ZDOCK were considered to increase the specificity. It was observed that among the 20 predicted structures, SARS-CoV-2 Ab interacted 46 times with the DV1 neutralization epitope region (aa 295-395) (Chen *et al.*, 2017) (Figure 1) (Supplementary Table S2). To compare the interaction frequency, we also set a control docking experiment with another flavivirus envelope antigen, i.e., WNV envelope antigen (PDB ID: 2HG0). After similar structural refinement and docking with the SARS-CoV-2 Ab, it was found that the SARS-CoV-2 Ab interacted only nine times (Supplementary Table S2) in the neutralization epitope site of WNV envelope antigen (aa 295-395) (Beasley and Barrett, 2002).

Table 3
List of COVID-19-predated healthy control serum samples tested using the Abcheck COVID-19 IgG/IgM and Standard Diagnostics-Bioline Dengue Duo rapid test kits. A total of 10 of 32 samples were positive.

SL No.	Sample Name	SD-BIOLINE Dengue IgG	SD- BIOLINE Dengue IgM	SD-BIOLINE Dengue NS1 Ag
1	P-H-1	+	-	-
2	P-H-2	+	-	-
3	P-H-3	-	-	-
4	P-H-4	-	-	-
5	P-H-5	+	-	-
6	P-H-6	-	-	-
7	P-H-7	-	-	-
8	P-H-8	+	-	-
9	P-H-9	-	-	-
10	P-H-10	-	-	-
11	P-H-11	-	-	-
12	P-H-12	-	-	-
13	P-H-13	+	-	-
14	P-H-14	+	-	-
15	P-H-15	-	-	-
16	P-H-16	-	-	-
17	P-H-17	-	-	-
18	P-H-18	-	-	-
19	P-H-19	-	-	-
20	P-H-20	+	-	-
21	P-H-21	-	-	-
22	P-H-22	-	-	-
23	P-H-23	-	-	-
24	P-H-24	-	-	-
25	P-H-25	+	-	-
26	P-H-26	-	-	-
27	P-H-27	-	-	-
28	P-H-28	+	-	-
29	P-H-29	+	-	-
30	P-H-30	-	-	-
31	P-H-31	-	-	-
32	P-H-32	-	-	-

The "+" sign signifies a positive result; the "-" sign signifies a negative result. Ag = antigen; IgG = immunoglobulin G; IgM = immunoglobulin M; SD-BIOLINE = Standard Diagnostics-Bioline; SL = serial.

SARS-CoV-2 infected patients' serum samples can neutralize DV1 clinical isolate (VNT)

NS1 and DV Ab-positive serum samples (predating COVID-19) were considered positive controls. Healthy serum samples (predating the COVID-19 pandemic), negative for DV Abs, SARS-CoV-2 Ab, and DV NS1 were used as negative serum controls. In this study, samples that were positive for SARS-CoV-2 and DV Abs but NS1-negative were tested for neutralization potential. Samples that were not cross-reacting, i.e., SARS-CoV-2 Ab-positive but DV Ab-negative, were also tested (Table 1).

Intracellular DV genome equivalents were highest for the negative serum controls. In the case of positive serum controls, DV1 yield was much reduced ($P = <0.0001$) than in negative serum controls, as expected. The COVID-19 serum samples (those were also DV Ab-positive but NS1-negative) effectively neutralized DV ($P = 0.0001$) (Figure 2, Supplementary Table S3). Only three serum samples were SARS-CoV-2 Ab-positive but DV Ab-negative (Table 2). All three samples (Nos. 18, 19, 41) were unable to neutralize DV1 (Figure 2).

Discussion

Approximately 89-91% of COVID-19 Ab-positive serum samples cross-reacted with DV in LFIA (42/47) or ELISA tests (43/47) (Table 2). Overall, 44 of 47 COVID-19 Ab-positive samples (93%) gave evidence of DV seropositivity (Table 2). This starkly contrasts with the COVID-19 samples from Israel showing 22% cross-reactivity (Lustig *et al.*, 2021) with DV, Israel being a dengue non-endemic region. The observed DV seropositivity was notably higher than the prepandemic (2017) dengue seroprevalence in India (Murhekar *et al.*, 2019). The latter was estimated at 48.7%.

Serum samples from 32 apparently healthy patients (without any history of dengue), collected from 2016 to 2017 in Kolkata, showed 32% seropositivity for DV IgG (Table 3). Thus, the serological cross-reaction of over 93% of SARS-CoV-2 infected patients' serum samples could not be explained by the background DV seroprevalence of 32% for Kolkata or 49% for India (Murhekar *et al.*, 2019). Only 36.2% of samples tested positive for NS1 Ab. Therefore, the remaining (93-36) = 57% DV cross-reacting COVID-19 Ab-positive serum samples had no evidence of previous DV exposure.

The high percentage of DV cross-reactivity in the COVID-19 serum samples also did not corroborate that India experienced a much smaller number of dengue cases in 2020 (National Vector Borne Disease Control Program, 2022). Interestingly, none of the cross-reacting COVID-19 serum samples were DV NS1-positive or RNA-positive.

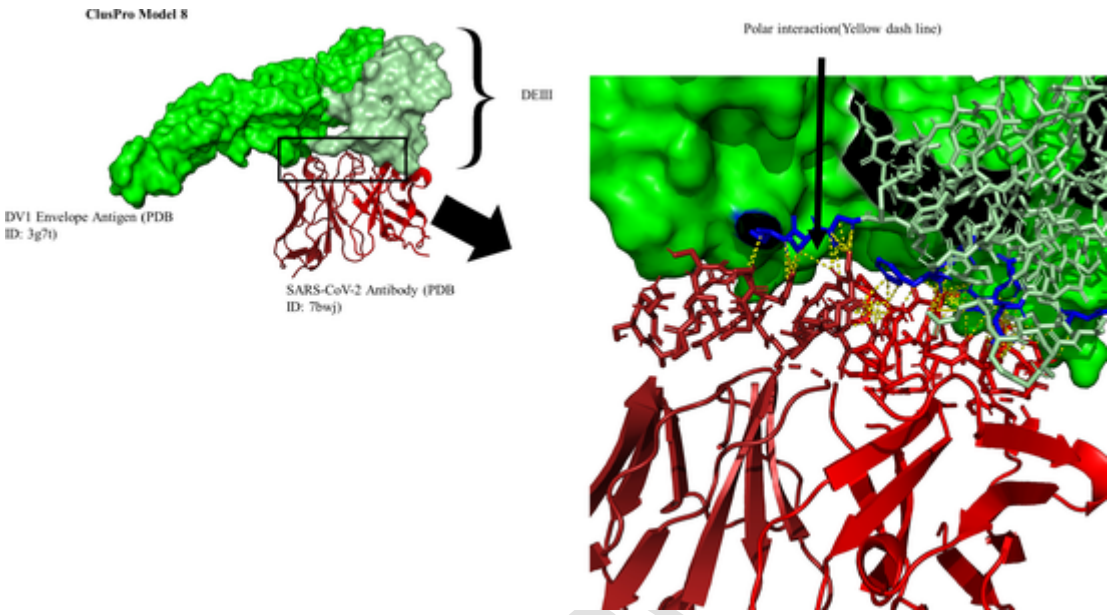

Figure 1. Representative docking complex of DV antigen and SARS-CoV-2 antibody. Among the 10 docked models of the ClusPro server, model 8 showed the highest number of interactions by SARS-CoV-2 Ab in the DEIII region of DV type 1 envelope antigen. The SARS-CoV-2 Ab is colored red and the envelope antigen is colored green. The amino acids in the epitope region of DEIII interacting with the Ab are colored blue. Polar interactions between amino acids are marked as yellow dotted lines. Ab = antibody; DEIII = dengue envelope protein domain III; DV = dengue virus.

To study this cross-reactivity further, we docked SARS-CoV-2 Ab (isolated from patients) with DV1 envelope antigen and found noticeable interactions (46) in the neutralization epitope of the dengue envelope protein domain III (DEIII) region. In addition, we analyzed docking of WNV envelope antigen and SARS-CoV-2 Ab, but the number of interactions was only nine. This indicated the potential of SARS-CoV-2 Ab to neutralize DV.

To functionally assess the impact of the COVID-19 pandemic on DV host-pathogen interaction, we performed VNT of DV1 clinical strain using COVID-19 serum samples. In support of our hypothesis (that COVID-19 is protective against dengue), SARS-CoV-2 infected patients' serum samples that cross-reacted with DV envelope had been found to neutralize and restrict DV1 entry into host cells significantly. Samples that were negative for NS1 Ab (i.e., without a traceable history of DV infection) also successfully neutralized DV1. There was no significant difference in the degree of virus neutralization between the NS1 Ab-positive/negative COVID-19 serum samples, as evident from the low SD among the samples. We used DV serum samples from 2017, i.e., predating the COVID-19 pandemic, as positive controls for DV neutralization. These serum samples significantly reduced the intracellular virus yield, confirming the approach and VNT assay conditions. During the period of sample collection (September 2020 to January 2021), four variants of concern (VOC) were reported in India and Kolkata. These were Alpha (B.1.1.7), Beta (B.1.351), Gamma (P1), and Delta (B.1.617.2). Among them, the highest number of reports were of the Delta variant, followed by Alpha, Beta, and Gamma VOC (GISAID, 2022 https://www.gisaid.org/hcov19-variants). Thus, our observations were relevant to the subjects under study and may not apply to all diagnostic tests and all SARS-CoV-2 variants identified to date.

Three COVID-19 serum samples (No. 18, 19, 41) that were not cross-reactive with DV were also considered for the neutralization test. All these samples were RT-PCR positive for SARS-CoV-2 but negative for DV in all respects (Table 2). All three samples could not neutralize DV1: perhaps these antibodies were generated against different emerging variants of SARS-CoV-2. The Spike protein of such variants may not share enough antigenic similarity with DV to elicit cross-reactivity/DV neutralization.

We thought of two possible explanations for our previously mentioned observations. First, SARS-CoV-2 has some antigenic similarities with DV (Lustig *et al.*, 2021). This led to cross-reaction of Abs elicited against one another (Lustig *et al.*, 2021; Nath et al., 2021b). This is possibly the case for the 57% COVID-19 Ab-positive serum samples that were cross-reactive in DV Ab tests but showed no evidence of DV exposure (DV RNA-negative; DV NS1-negative and DV NS1 Ab-negative).

Secondly, because of antigenic similarity, SARS-CoV-2 infection may stimulate existing DV memory cells (from previously DV-infected individuals, including DV asymptomatic cases), resulting in a boost in DV Abs production (Nath *et al.*, 2021a). This is supported by the fact that all DV seropositive samples were DV RT-PCR and NS1 Ag negative, suggesting that none were from recently DV-infected subjects. Eight samples showed DV IgM, but, as mentioned earlier, this could be non-specific. This explanation holds true for the 17 cross-reacting COVID-19 Ab-positive samples that were DV NS1 Ab-positive but DV RNA and DV NS1 antigen-negative. Either way, the COVID-19 pandemic appears to immunologically stimulate a large part of the population against DV, as evident from our findings.

A study from India included 44 DV-infected children grouped based on clinical severity and mortality. The same children were then screened for SARS-CoV-2 infection and serological evidence. It was found that previous exposure to SARS-CoV-2 had resulted in a less severe outcome with no death (Ravikumar *et al.*, 2021). These observations serve as further circumstantial/epidemiological evidence of our observations that SARS-CoV-2 serum samples can neutralize and protect against dengue. In addition, all the seropositive/cross-reactive patients with COVID-19, although hospitalized, finally recovered. Notably, many other highly dengue-endemic regions (e.g., Guangzhou in China and Sri Lanka) also reported significantly reduced dengue outbreaks in 2020 during the pandemic (Jiang *et al.*, 2021; Liyanage *et al.*, 2021). These observations serve as further circumstantial /epidemiological evidence of our observations.

Conversely, high DV Ab prevalence also appears to reduce severity and mortality because of COVID-19 as the mortality per million populations in India (July-August, 2021), despite a strong second wave, was about 300 compared with around 2000 in many dengue non-endemic countries (Worldometer, 2021). A study comprising 2351 participants from the Brazilian Amazon basin reported that COVID-19 was associated with a higher risk of death in those who did not have previous DV exposure (Silvestre *et al.*, 2021).

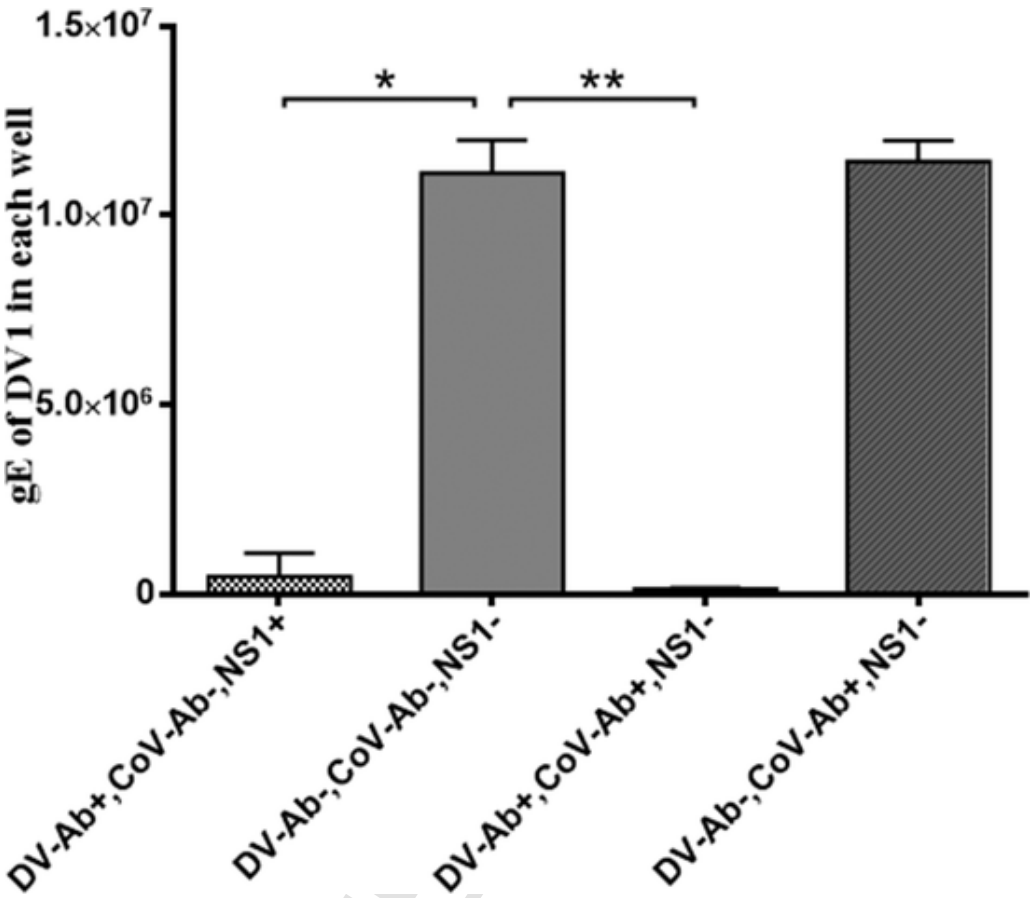

Figure 2. DV neutralization assay result using SARS-CoV-2 infected patients' serum samples. DV Ab+, SARS-CoV-2 Ab-, and NS1+ serum samples were considered positive controls. They significantly reduced (*P = <0.0001) the virus yield concerning negative controls (DV Ab-, SARS-CoV-2 Ab-, NS1-). Mean DV yield (gE) for all the DV Ab+, SARS-CoV Ab+, NS1- serum samples was low (**P = 0.0001) compared with the negative serum controls. In the case of these samples, there was no significant difference in DV neutralization capacity between the NS1 Ab + and NS1 Ab - COVID-19 samples. However, for the three DV Ab-, SARS-CoV-2 Ab+, NS1- samples (Nos. 18, 19, 41), DV1 titer was quite similar to negative controls. Error bars indicate SD. Ab = antibody; DV = dengue virus; gE = genome equivalent; Nos = numbers; NS1 = nonstructural protein 1; SD = standard deviation.

Several reports stated that SARS-CoV-2 Ab tests cross-reacted with DV; others did not. Because of these contradictory findings, we performed DV VNT using COVID-19 serum samples and discovered that COVID-19 serum samples (even with no evidence of DV pre-exposure, i.e., DV RNA-negative; DV NS1-negative, and DV NS1 Ab-negative) could, indeed, neutralize DV. The present study's findings also indicate that the existing DV serological tests may no longer be conclusive for DV diagnosis in highly dengue-endemic countries where both the viruses co-exist.

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Ethical approval

This study was performed in accordance with the ethical standards (on par with the 1964 Declaration of Helsinki and its later amendments) of the ethical committees on human subjects of relevant institutions. SSG obtained ethical approval (Ref no. BBBH/0164/2021-2022) from Behala Balananda Brahmachari Hospital and Research Center. SB obtained ethical approval (Ref No. IICB/IRB/2021/4) from CSIR-IICB. All experiments were carried out in accordance with the relevant guidelines and regulations.

Author contributions

SB, SS, and HN conceived and designed the study. HN, AM, SR, and TK performed the experiments. Clinical sample collection and management was done by SRN, SSG, HN, SR, AM, and TK. SB acquired the funding. SB, SS, HN, and AM wrote the original draft of the manuscript. SB, SS, and HN performed the critical analysis of data. All the authors did a formal analysis of the data. The final manuscript was reviewed and edited by all the authors.

Extended data

The structures of docking models, both in raw and analyzed format, are available at Mendeley Data (https://data.mendeley.com/datasets/y6xjbjcgz8/1).

Data sharing

Further information and resource requests should be directed to and will be fulfilled by the corresponding author, Dr. Subhajit Biswas (subhajit.biswas@iicb.res. in).

Competing interests

The authors have no competing interests to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2022.07.013.

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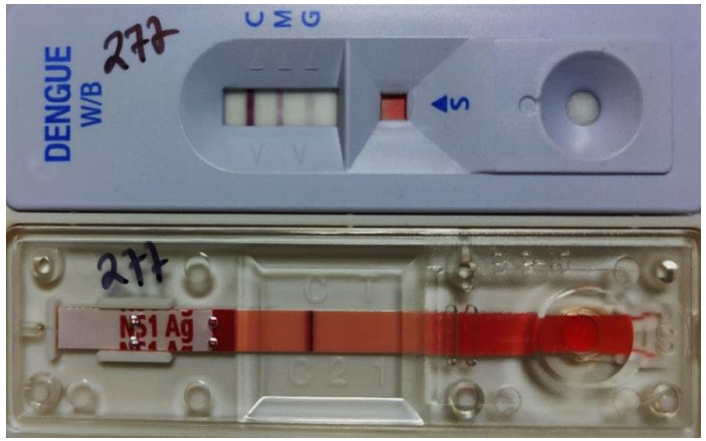
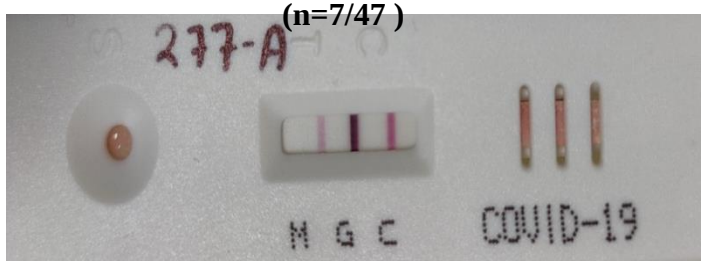
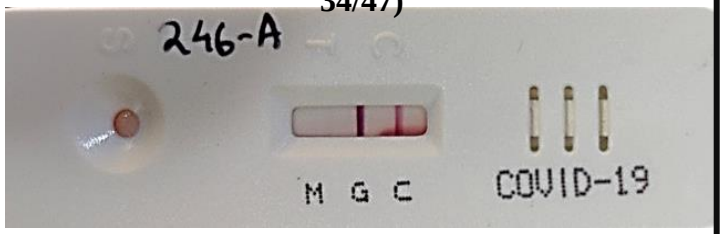


SD bioline DV strip test IgG & IgM positive (n=7/47)

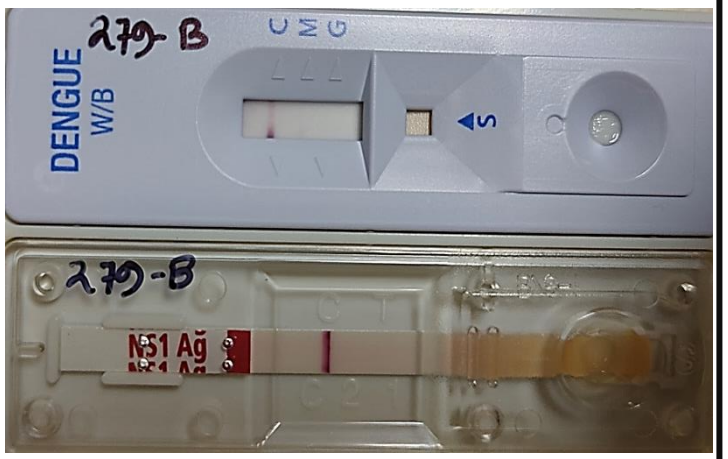


COVID-19 strip test IgG & IgM positive (n=39/52)

SD bioline DV strip test IgG positive (n= 34/47)



COVID-19 strip test IgG positive (n=4/52)



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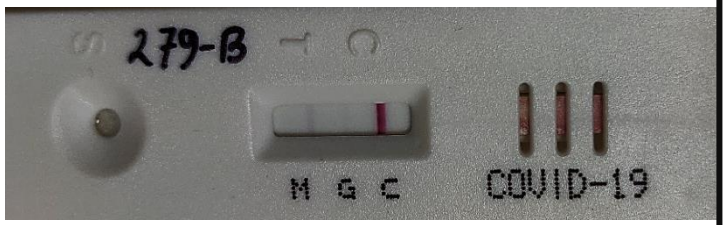
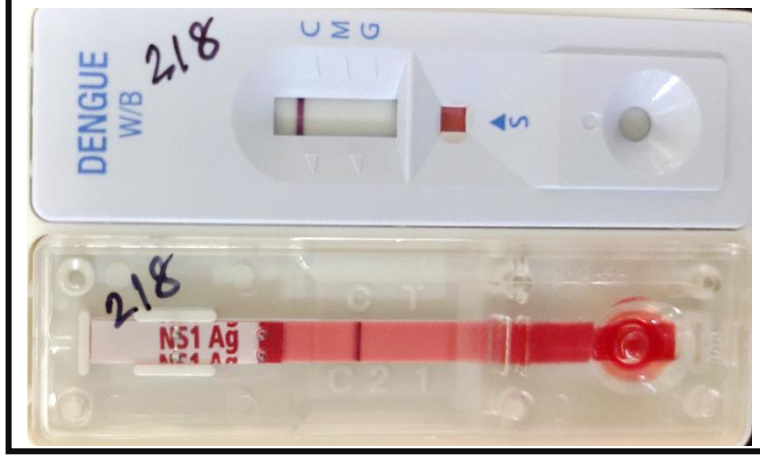
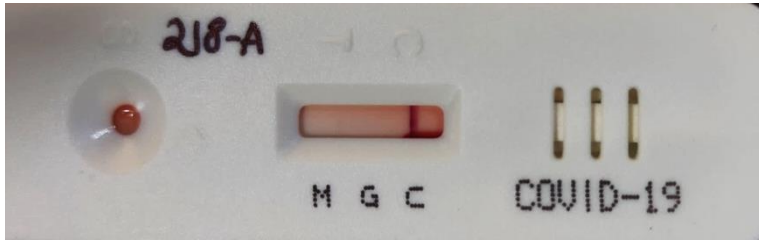


COVID-19 strip test IgM positive (n=4/52)



SD bioline DV strip test negative (n=7/52)



COVID-19 strip test negative (n=5 /52)

Figure S1. Representative images of lateral flow-based immunoassay test strips

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Computational modelling supports that dengue virus envelope antibodies can bind to SARS-CoV-2 receptor binding sites: Is pre-exposure to dengue virus protective against COVID-19 severity?

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ABSTRACT

The world is going through the scourge of the COVID-19 pandemic since January 2020. However, the pandemic appears to be less severe in highly dengue endemic countries. In this connection, several studies reported that sero-diagnostic tests for dengue virus (DV) yielded considerable false-positive results for SARS-CoV-2 and *vice versa* in dengue endemic regions, thereby indicating towards potential cross-reactivity between these two viruses. We anticipated that SARS-CoV-2 and DV might share antigenic similarity and performed computational docking studies to test this hypothesis. Our results predicted with high confidence that human DV antibodies can indeed, bind to RBD of SARS-CoV-2 Spike protein. Some of these interactions can also potentially intercept human ACE2 receptor binding to RBM. Dengue serum samples predating the COVID-19, had been found to cross-react with SARS-CoV-2 Spike and this provides direct experimental validation of our predictions. Our analysis also showed that m396 and 80R antibodies (against SARS-CoV-1) did not dock with RBM of SARS-CoV-2, a fact already proven experimentally. This confirmed reliability and robustness of our approach. So, it is highly probable that immunological memory/antibodies to DV in endemic countries may reduce the severity and spread of COVID-19. It is not known whether SARS-CoV-2 antibodies will hinder DV infections by binding to DV particles and reduce dengue incidences in the future or, augment DV infection and severity by deploying antibody-dependent enhancement.

1. Introduction

Since the beginning of 2020, people around the world are confronting the COVID-19 pandemic, caused by SARS-CoV-2, a beta coronavirus. As of 26th August 2020, 23,697,273 confirmed cases with 814,438 deaths have been reported worldwide [1]. This infection is believed to originate from Wuhan city, Hubei province, China in December 2019. The virus is highly contagious and easily transmissible from human to human. The virus caused numerous outbreaks across the globe and WHO declared a public health emergency of international concern (PHEIC) on January 30, 2020.

Initially studying the global map of the COVID-19 pandemic, it occurred to us that SARS-CoV-2 is showing less transmission, severity and overall mortality per million population in highly dengue endemic countries [2], i.e. the COVID-19 and dengue global severity maps do not tend to overlap [3]. Despite having large population size, high population density, less public health awareness, relatively poor health and hygiene conditions and inadequate healthcare facilities, the highly dengue endemic countries in Southeast Asia, the Indian subcontinent, Latin America and Africa have experienced comparatively lower degree of COVID-19 severity so far.

On the other hand, developed countries in Europe, North America and Asia (China, Iran) with insignificant or sporadic dengue virus infection history, have been worst affected by SARS-CoV-2. The COVID-19 mortality in highly DV endemic countries was estimated at 24 per million population compared to 118 in the DV non-endemic regions as of 3rd June 2020 [3]. The epidemiological weekly update (17th to 23rd August 2020) reported cumulative deaths per million population for the Americas and Europe at 65

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and 32 respectively. During the same period cumulative deaths per million population in Southeast Asia was only 12 [4].

As an exception to our proposition, Brazil, a DV-endemic country recorded 3,622,861 infections and 115,309 deaths as on 26th August 2020 [1]. It is universally accepted and recommended that preventive measures are crucial to contain the spread of COVID-19 like social distancing, quarantine and lockdown in the early phases of the pandemic. In support of our hypothesis, a recent study from Brazil revealed that states reporting higher incidences of dengue during 2019–20 recorded lower COVID-19 cases and deaths. The exponential community transmission was also delayed due to slower SARS-CoV-2 growth rates [5]. The same study also described four major factors that contributed to the COVID-19 epidemic in Brazil including "super-spreader" events [5].

Even in the face of COVID-19 pandemic, dengue remains the most important arboviral disease of global concern. In last few years incidence of dengue cases has increased rapidly although a vast majority of the cases (~80%) are mild, asymptomatic and self-limiting. One report estimated 390 million (95% CI: 284–528) infections per year globally of which 96 million (CI: 67–136) manifested clinically. About 4 billion people across 129 countries are currently at the risk of DV infection, with 70% of global burden from Asia, namely the Indian subcontinent and Southeast Asia [6]. Consequently, COVID-19 pandemic overlapped with high dengue endemicity in many tropical and sub-tropical regions of the world as mentioned above.

So far, many theories have been put forward to explain why COVID-19 is less severe in many countries and we have discussed this elsewhere [3]. One such hypothesis was that COVID-19 spread was hindered by warmer climate. This could have been an alternative theory to explain why highly dengue endemic countries, falling in hot and humid regions of the world, were less affected by COVID-19. But several published reports on impact of weather conditions on virus spread suggest COVID-19 to be equally infectious under hot and humid conditions [7]. From the above observations, it appeared that pre-exposure to DV may render partial protection against COVID-19 as may be the case in highly dengue endemic regions of the world. This epidemiological observation has now been supported by biological evidences. One report from Singapore stated that an elderly man and a woman were actually SARS-CoV-2 positive but misdiagnosed for dengue due to similarities in disease presentation and more importantly, false-positive results in DV IgM and IgG serological tests. Both the patients were confirmed qRT-PCR negative for DV-, ZIKA- and Chikungunya-RNA [8]. Another study reported from our laboratory showed that the reverse scenario is also possible. We reported that five of thirteen DV NS1-positive serum samples from 2017 (predating the COVID-19 outbreak), gave COVID-19 IgG and IgM false-positive results [9]. Subsequently, another group from Israel confirmed both the scenarios i.e. approximately 22% cross-reactivity between dengue antibodies (Abs) and SARS CoV-2 antigen(s) and *vice versa* via lateral flow-based rapid tests and ELISA tests targeting antibodies to Spike protein in a larger number of patient samples [10].

Both the aforesaid scenarios unequivocally indicate towards some degree of antigenic similarities between SARS-CoV-2 and DV. This led us to investigate the effects of human DV Abs on SARS-CoV-2 Spike protein using molecular docking studies with two FFT algorithm-based docking servers i.e. ClusPro and ZDOCK 3.0.2. We chose four DV serotype 2 envelope Abs X-ray crystallography PDB structures (4UTA, 4UTB, 4UT6 and 4UT9) and SARS CoV-2 Spike protein trimer X-ray crystallography PDB structure (6VSB) for the docking studies.

2. Results

2.1. Dengue virus antibodies are predicted to bind to RBD of SARS-CoV-2 Spike protein

Four monoclonal antibodies namely EDE2 A11, EDE2 B7, EDE1 C8 and EDE1 C10 that are elicited in response to natural DV infection in humans [11,12], have been used in this study. Each Ab has been docked with the crystal structure of SARS-CoV-2 Spike protein (PDB ID: 6VSB) [13], using two docking servers ZDOCK and ClusPro. From the output of each algorithm, top 10 predictions were considered. All the selected interactions were within a distance cut-off of 3.5 Å [14]. Available PDB files of aforesaid Abs and SARS-CoV-2 Spike proteins were processed for docking as stated in "Methods and Models" section. In ZDOCK [15,16], protein–protein interaction and in ClusPro [17,18], protein–protein interaction with antibody mode were used. Only those common interactions that are predicted by both the algorithms were considered for interpretation. During analysis of docking results, interactions predicted to involve amino acid positions 333 to 527 of SARS-CoV-2 Spike protein (RBD), were only considered, as the immunogenic epitopes of the virus fall in this region [19].

For EDE1 C8 Ab docking with SARS-CoV-2 Spike protein, there were 48 occasions in total 20 predicted models (10 predicted model from each server), when EDE1 C8 Ab was found to bind to different amino acid residues in the SARS-CoV-2 Receptor Binding Domain (RBD, 333–527 amino acid positions on Spike protein[20]) (Fig. 1A). These 48 events include repetitions of different amino acids coming once in a particular prediction. Among these 48 interactions, 13 involved Ab binding to different residues of the Receptor Binding Motif (RBM, 438–506 amino acid positions on Spike protein [20]) and 35 involved Ab binding to RBD regions outside RBM.

Of twenty predictions for EDE1 C10 Ab binding (Fig. 1B), 38 events of interactions were observed in RBD which included 19 in RBM and 19 in RBD outside RBM. Similarly, EDE2 B7 Ab was found to bind to different amino acids in RBD for 30 times in 20 predictions. There were 20 incidences when EDE2 B7 Ab interacted with RBM of S protein and 10 interactions with RBD region outside RBM (Fig. 1C). In case of EDE2 A11 Ab, only five interactions were detected and all occurred involving the RBM (Fig. 1D). Representative images of docking have been presented (Fig. 2). Overall, there were 121 events in 80 predictions where DV MAbs interacted with SARS-CoV-2 RBD, including 57 events involving RBM (Fig. 3A).

2.2. DV antibodies are predicted to bind with Spike RBD amino acid residues which are crucial for interaction with ACE2 receptor

It is notable that DV antibodies were also found to bind to RBD amino acid residues that are crucial for interaction with the human ACE2 receptors, important for SARS-CoV-2 entry into the cells. SARS-CoV-2 Spike RBD interaction with ACE2 receptor has already been elucidated through crystal structure analysis with resolution of 2.45 Å [20]. A total of 17 residues (with a distance cut-off of 4 Å) of SARS-CoV-2 RBD interact with 20 residues of ACE2 receptor [20]. In our docking study, we discovered that DV-EDE antibodies bind with several of the above-mentioned S protein residues with a distance cut-off of 3.5 Å. EDE1 C10 Ab contacts with four amino acid residues with a total frequency of 13 among 20 predictions. Similarly, EDE1 C8 Ab interacts with four amino acid residues in RBM (with a total frequency of 9) that have been predicted to interact with ACE2 receptors. Likewise, EDE2 B7 Ab and EDE2 A11 Ab bind with different receptor-engaging amino acid residues in RBD on 14 and 5 occasions respectively (Table 1). Overall, the DV Abs used in

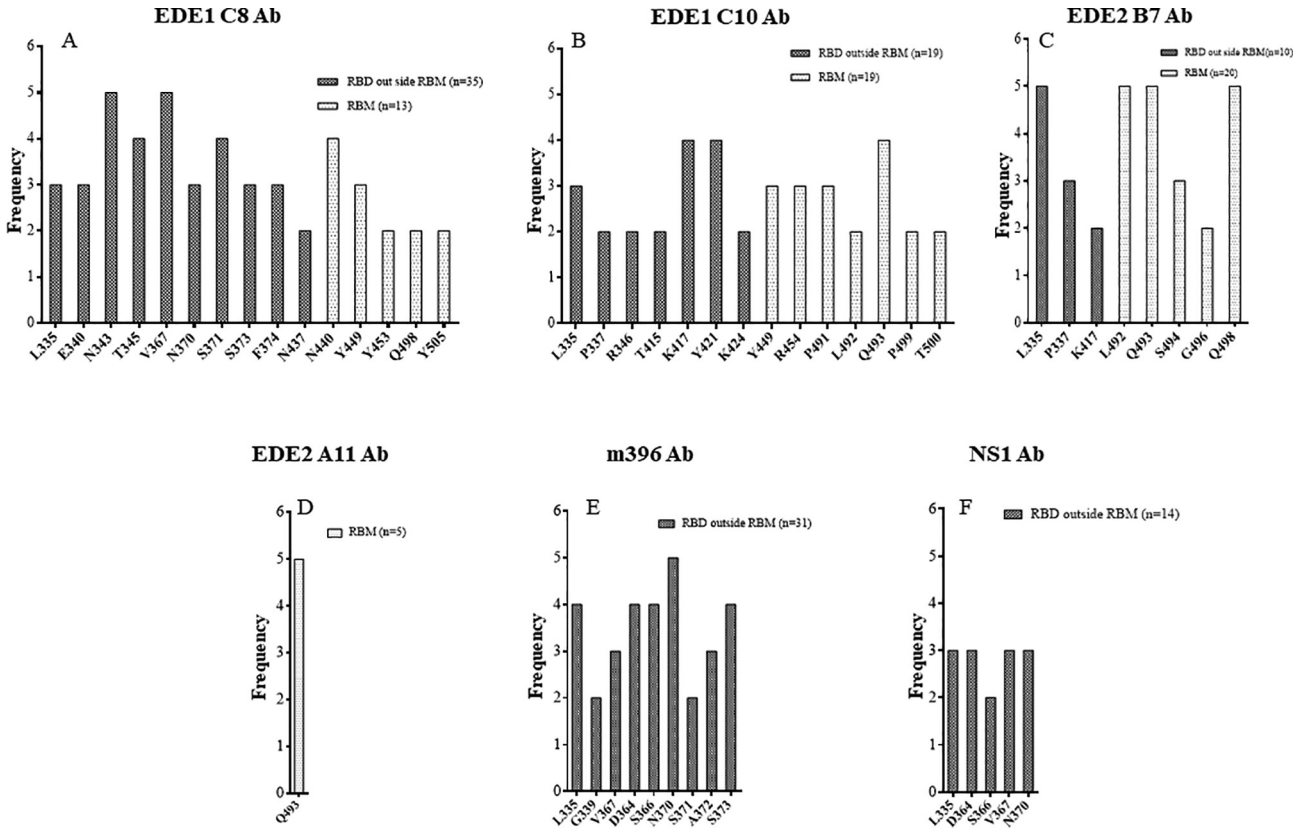

Fig. 1. Bar graphs representing frequency of each amino acid residue in the Spike protein predicted to bind to each antibody. Docking frequency of SARS-CoV-2 Spike protein amino acids with (A) EDE1 C8 Ab; (B) EDE1 C10 Ab; (C) EDE2 B7 Ab; (D) EDE2 A11 Ab; (E) m396 Ab and (F) NS1 Ab. Y axis represents frequency of specific SARS-CoV-2 Spike protein amino acid interacting with respective antibody in 20 predicted docking models. The X axis shows the positions of the Spike protein amino acids against the single letter amino acid codes. Common interactions between Abs and SARS-CoV-2 Spike protein predicted by both ZDOCK and ClusPro that fall within the distance cut-off of 3.5 Å, were considered only. "n" denotes the cumulative docking frequency for each type of interaction.

this study, docked with eight S protein amino acids that are crucial for binding to ACE2 receptor. These eight amino acid residues appeared on 41 occasions with repetitions in total 80 predictions (Table 1).

2.3. Reproducibility of this docking study with experimental data

Several neutralizing Abs against SARS-CoV-1 (like m396, 80R) are known to interact with RBD of Spike protein and compete with ACE2 receptor for binding [21]. But these antibodies do not bind with SARS-CoV-2 RBD as determined experimentally [13]. We "docked" m396 crystal structure (PDB ID: 2G75) with SARS-CoV-2 Spike in the same procedure as done before (Fig. 1E). Analysis of 10 ZDOCK and 10 ClusPro predictions revealed 31 interactions within amino acid positions L335 to S373 of SARS-CoV-2 Spike RBD. However, not a single common interaction was found to occur involving the RBM, from both algorithms. Similarly, docking of 80R (PDB ID: 2GHW) with SARS-CoV-2 Spike protein did not result in any common interaction between 10 ZDOCK and 10 ClusPro predictions. Furthermore the above 31 interaction points for m396 were far away from the ACE2 receptor interacting residues, which fall in the region spanning from K417-Y505 [20].

NS1 is an abundant viral protein in DV infected patients' serum [22] and elicits detectable antibodies [23]. So, we modeled another docking experiment to check, other than DV envelope antibodies (DV-EDE), whether or not NS1 antibodies have the potential to cross-react with SARS-CoV-2. There was no PDB structure available for DV NS1 antibody. We, therefore, used the three-dimensional structure available for NS1 of West Nile Virus (WNV), another flavivirus, in complex with the WNV NS1 Ab, known as 22NS1 (PDB ID: 4OII) for docking [24]. It has been already reported that the epitope for the 22NS1 Ab (i.e. WNV NS1 protein region 172–352) is similar but not identical to DV [24]. Thus, we chose 22NS1 as a representative flavivirus NS1 antibody to check cross-reactivity with SARS-CoV-2 Spike protein. The docking study revealed 14 interactions within RBD (333–527) but not a single interaction within RBM (438–506) (Fig. 1F). All these observations confirmed the reliability and robustness of our approach.

3. Discussion

Our computational modelling studies predicted, with high confidence, that DV Abs can interact with SARS-CoV-2 RBD (Fig. 1, Fig. 3A) and are also capable of intercepting eight key RBD interactions that are crucial for binding to ACE2 receptors (Fig. 3B, Table 1). From these findings we propose that DV Abs have the potential to compete with ACE2 receptors for access to RBD of SARS-CoV-2. So, theoretically, they can "mask" SARS-CoV-2 RBD and block its interaction with host cell receptors and thereby prevent virus entry. Our prediction is supported by the biological evidences of DV and SARS-CoV-2 cross-reactivity data as presented before [8–10] and provides a logical explanation to our previous observation that SARS-CoV-2 infections are causing less severity and mortality in the highly dengue endemic countries, where more than 80% of the population can be sero-positive for dengue [3]. First DV false-positivity report from Singapore [8]confirmed the absence of dengue infection in COVID-19 patients through DV-specific RT-PCR negativity. However, after 10 days of infection,

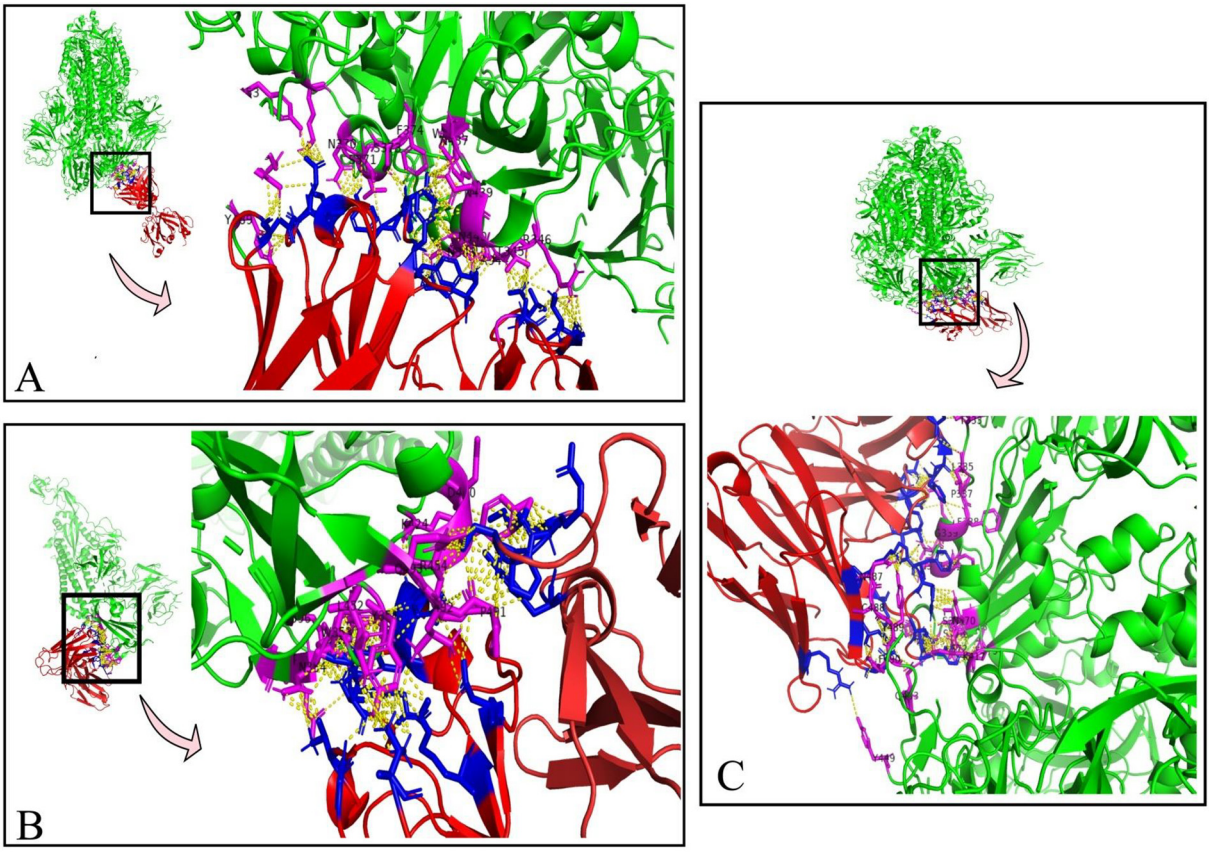

Fig. 2. Representative images of DV antibodies "docking" with SARS-CoV-2 Spike protein. (A) EDE1 C8 Ab (Red) is docked with Spike protein (green) of SARS-CoV-2 through ClusPro, Model 0. (B) EDE2 B7 Ab (Red) is docked with Spike protein (green) of SARS-CoV-2 through ZDOCK, Complex 5. (C) EDE1 C10 Ab (Red) is docked with Spike protein (green) of SARS-CoV-2 through ClusPro, Model 3. Spike protein amino acids involved in the interactions are depicted in violet. Interacting residues of respective antibodies are marked in blue. Hydrogen bonds within distance cut-off of 3.5 Å are marked as yellow dotted lines.

DV- RNA may not be detected. So, the possibility of previous infection could not be ruled out. This was taken care of in a subsequent study from Israel. The possibility of pre-existing DV antibodies (from previous infection) in COVID-19 serum samples was ruled out by anti-NS1 IgG ELISA [10] and such serum samples showed about 22% cross-reactivity in DV lateral flow-based antibody strip tests.

The reliability and robustness of our computational predictions were highlighted by the facts that m396 and 80R antibodies (against SARS-CoV-1) did not dock with RBM of SARS-CoV-2, a fact already confirmed experimentally by others [13] and that WNV NS1 antibody also did not bind satisfactorily with SARS-CoV-2 Spike protein RBD with no interaction in the RBM region. Although incapable of neutralizing SARS-CoV-2, a recent in-silico analysis suggested that suitable substitution of amino acids in the RBD region of m396 and 80R antibodies may increase computational docking efficiency [25]. The WNV NS1 Ab binds to WNV NS1 at an epitope which is similar but not identical to DV NS1 epitope. DV NS1 antibody could not be used in our docking studies as no X-ray crystallography structure for the same is available in the databases. The NS1 Ab and Spike protein docking results further support that it is the DV envelope Abs (and not NS1 Abs) in the DV diagnosed human serum samples that actually cross-reacted with the Spike antigens, immobilized in the SARS-CoV-2 rapid antibody tests and Spike Abs-detecting ELISA tests [9,10]. It has been predicted by others that some structural similarity may exist between DV envelope and the HR2 domain of the SARS-CoV-2 Spike protein [10]. However, the RBD domain is located far away from HR2 domain [20]. So, the results of our study do not correlate directly with the antigenic similarities proposed in the other study [10].

The four DV Abs used in this study, are known to neutralize DV and were identified from serum of dengue fever convalescent patients [11]. This ensures that both asymptomatic and symptomatic patients recovering from DV infection will possess immunological memory to these Abs. In highly Dengue endemic countries, where infections occur regularly, majority of the population has pre-exposure to DV and has turned DV sero-positive. In our present study only four Abs have been considered but immunological response against any pathogen comprises of a repertoire of Abs; so, it is likely there will a larger repertoire of DV antibodies which can bind to and block RBD in humans (Fig. 4). One limitation of our study is that we used only four DV antibodies to test binding to Spike protein, although there can be much higher number of antigen–antibody interactions in reality. Here, we were limited by available crystal structures for DV envelope antibodies. Nevertheless, we still believe these four antibodies were relevant and closer to real life scenario as they were originally isolated from dengue convalescent patients. As new variants of SARS-CoV-2 Spike protein emerge, it would be interesting to see how such amino acid substitution(s) impact on the interactions with DV antibodies.

Interestingly, some studies predicted pre-exposure of humans to animal coronaviruses from syananthropic animals such as bovines and dogs and it was speculated that antibodies elicited against animal coronaviruses could confer partial protection against SARS-CoV-2 [26–28]. In this One-Health approach, epitope mapping by homology modeling revealed high degree of similarity in nucleo-

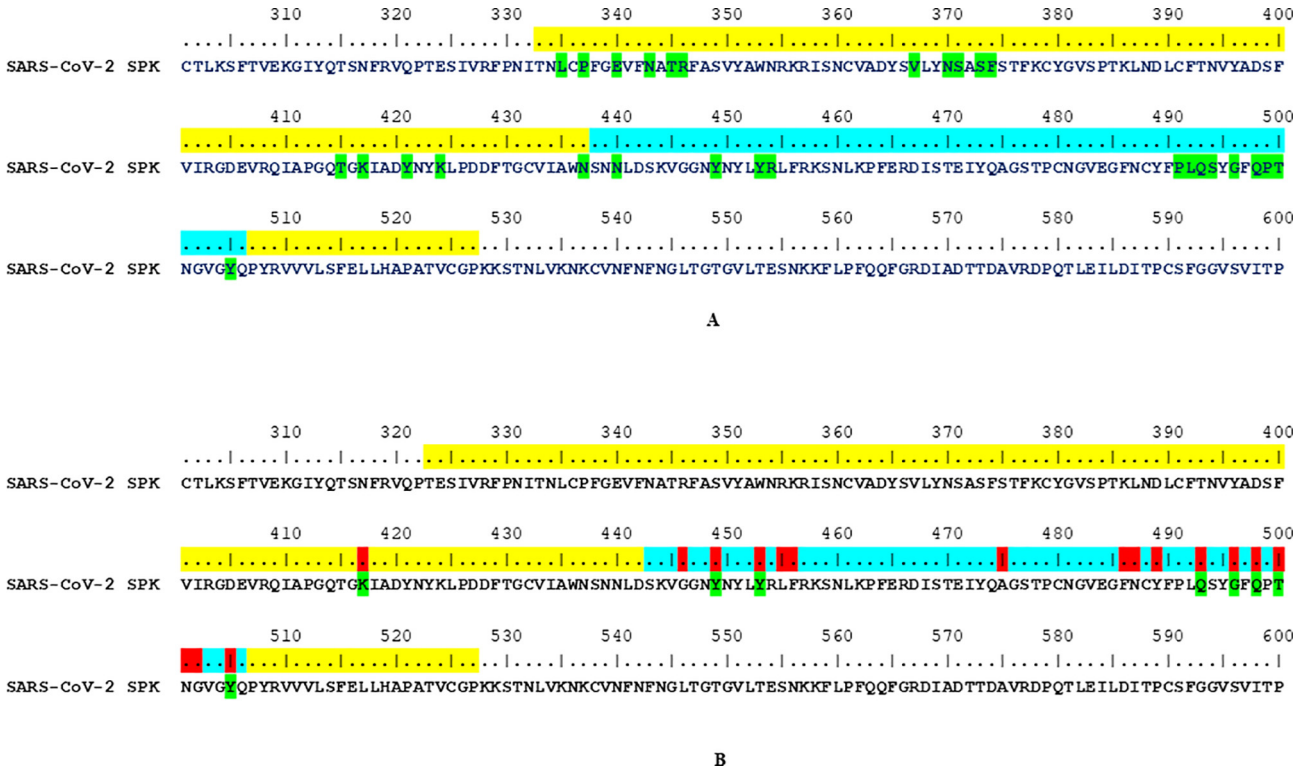

Fig. 3. Predicted interaction sites of DV antibodies with SARS-CoV-2 Spike protein. Yellow-marked region denotes RBD, spanning 333–527 amino acids. In RBD, sky blue-marked regions represent RBM, spanning 438–506 amino acids of the Spike protein. (A) All the amino acid residues in RBD, identified in overall 80 predictions to interact with DV antibodies, have been marked green. (B) Amino acids marked red are crucial for interactions between RBD and ACE2 receptor. Among these receptor binding residues of SARS-CoV-2 Spike, the green highlighted positions denote the residues of the RBD that were predicted to interact also with DV antibodies.

Table 1
Amino acid residues of SARS-CoV-2 RBD that interact with ACE2 receptor including the ones that were predicted to interact also with DV antibodies. Columns to the right show frequencies of interactions of DV Abs with some of the ACE2-engaging amino acid residues (bold, left column). These frequencies were obtained from the ZDOCK and ClusPro predictions.

	Frequency of interaction with DV antibody in ZDOCK and ClusPro models			
RBD residues that interact with ACE2 receptor [20]	EDE1 C8 Ab	EDE1 C10 Ab	EDE2 B7 Ab	EDE2 A11 Ab
K417	–	**4**	**2**	–
G446	–	–	–	–
Y449	**3**	**3**	–	–
Y453	**2**	–	–	–
L455	–	–	–	–
F456	–	–	–	–
A475	–	–	–	–
F486	–	–	–	–
N487	–	–	–	–
Y489	–	–	–	–
Q493	–	**4**	**5**	**5**
G496	–	–	**2**	–
Q498	**2**	–	**5**	–
T500	–	**2**	–	–
N501	–	–	–	–
G502	–	–	–	–
Y505	**2**	–	–	–
Total frequency	**9**	**13**	**14**	**5**

Total interaction events in 80 predictions: **41**.

capsid and envelope proteins between SARS-CoV-2 and taxonomically related coronaviruses [27,28]. In this context, it is noteworthy that human to animal transmission has been reported to be more common but the reverse is rare so far [29]. This proposition is thought-provoking and awaits experimental validation. Further research is warranted to confirm protective cross-reactivity between human and other animal coronaviruses.

One future challenge in case of COVID-19 is the yet undetermined impact of possible "Antibody Dependent Enhancement (ADE)" in already exposed populations [30,31]. ADE results from recurrent exposure to the immune stimulant within a defined time frame. This happens when antibodies to one SARS-CoV-2 strain fail to effectively neutralize another strain (s) (as often observed in case of dengue serotypes) and at the same time, enable the virus

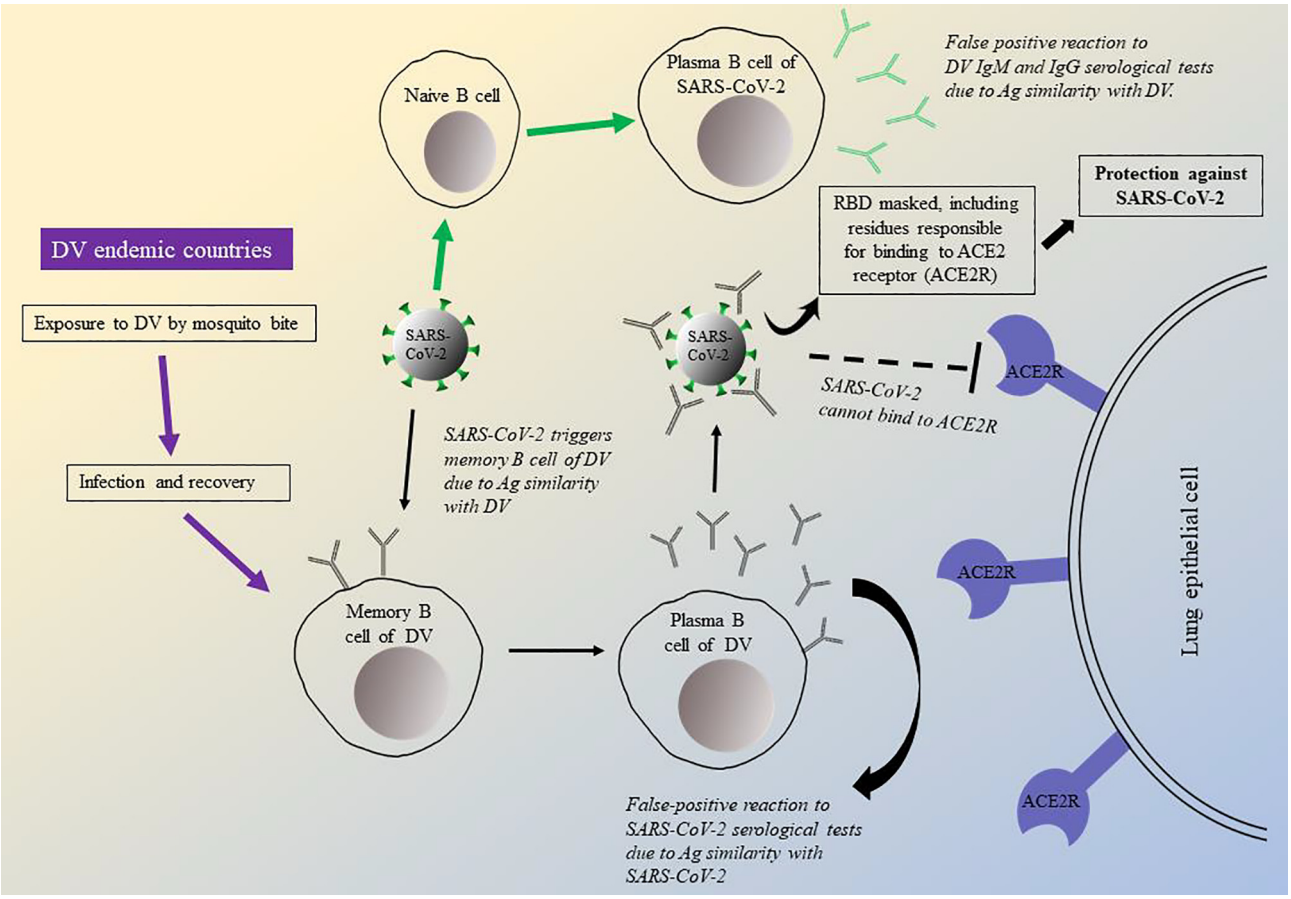

Fig. 4. Schematic diagram depicting why COVID-19 may be less severe in highly DV-endemic countries. In highly dengue-prone areas, SARS-CoV-2 infection may stimulate the immunological memory to DV in people with previous DV exposure (s), which could be asymptomatic. Due to antigenic similarity, the resultant dengue antibodies (grey "Y"-shaped) may bind to SARS-CoV-2 virus particles. DV Abs can even block Spike protein attachment to ACE2R by binding to Spike protein RBD and RBM. These are possible ways by which pre-exposure to DV infections can potentially reduce COVID-19 severity. SARS-CoV-2 Abs (green "Y-shaped") may cross-react in DV serological tests for detecting DV-specific IgM and/or IgG (top right) and *vice versa* (bottom).

of the other strain (s) to infect more cells by binding to the virus and bringing them closer to susceptible cells to which the antibodies are often attached by their Fc region.

From the results of our study, one can argue that DV Abs may also bind to SARS-CoV-2 and cause ADE for SARS-CoV-2 infections. But this does not appear to be the case, otherwise all highly dengue endemic countries would have been more hit by the COVID-19 pandemic than the dengue non-endemic countries due to pre-existing DV antibodies in the population. On the contrary, we are observing just the opposite scenario globally and therefore, DV antibodies are not involved in ADE of SARS-CoV-2 as per circumstantial evidence. Perhaps they are preventing SARS-CoV-2 severity as explained above [3]. But ADE can still represent a challenge for those individuals experiencing the first exposure to DV during this period of high SARS-CoV-2 presence/ transmission. This can happen due to the presence of SARS-CoV-2 Abs (potentially cross-reacting with DV) already in the COVID-19 affected individuals.

Our predicted computational models as well as growing experimental reports [9,10] of cross-reactivity between DV antibodies with SARS-CoV-2 and *vice versa* can affect sero-surveillance of COVID-19 in dengue endemic countries like India [32] and Brazil. Sero-diagnosis may come up with false-positive results in areas where both the viruses now co-exist. In such regions, due to antigenic similarity, SARS-CoV-2 Abs may cross-react in DV serological tests for detecting DV-specific IgM and/or IgG. Alternatively, SARS-CoV-2 infection may trigger the immunological memory to DV in people with previous DV exposure (s), which could be asymptomatic (Fig. 4). This will result in production of DV Abs, also resulting in false-positive results for COVID-19 patients in DV serological tests. It is now evident from the Spike protein ELISA results that DV Abs can, indeed, bind to SARS-CoV-2 Spike protein [10]. Further biological data to confirm the potential of DV EDE Abs to cross-react with SARS-CoV-2 are warranted. It would be also interesting to investigate whether, conversely, SARS-CoV-2 Abs can protect against DV.

4. Methods and models

4.1. Preparation of antibody for docking

The PDB files of all the antibodies were retrieved from RCSB PDB; PDB ID: 4UTA (EDE1 C8 Ab), 4UTB (EDE2 A11 Ab), 4UT6 (EDE2 B7 Ab), 4UT9 (EDE1 C10), 2G75 (m396 Ab), 2GHW (80R) and 4OII (WNV NS1-Ab). Most of the antibody structures in PDB were in neutralizing condition with their antigenic ligand molecule. From an original antibody PDB file, a separate PDB file consisting of only the antigen binding fragment, Fab (devoid of its natural antigenic counterpart) was created using PyMOL Molecular Graphics System, Version 2.3.3, Schrödinger, LLC software. These newly created PDB files were then processed in Chimera software using the Dock Prep plugin [33]. Subsequently solvent deletion, deletion of alternate positions (retaining only the highest-

occupancy positions), hydrogen addition, partial charge assignment, and output in Mol2 format were modulated through graphical interface. Standard residues (receptor amino acids) were assigned AMBER ff14sb partial charges [34,35]. AM1-BCC charges were computed for the receptor cofactors with ANTECHAMBER, which is included in Chimera [36,37]. The modified molecular structures were then used as receptor inputs in docking servers i.e. ZDOCK 3.0.2 [15,16] and ClusPro [17,18,38].

4.2. Refined protein data input in ZDOCK 3.0.2 and ClusPro web server for protein–protein docking predictions

For each antibody-antigen interaction, two FFT algorithm-based docking servers were used and the predictive results for each antibody were analyzed side by side to forage any similarity or pattern in the predictive interaction in accordance with our hypothesis. The ClusPro Server included FFT based protein–protein docking program PIPER. The simple user interface of the webserver allowed inputting PDB ID or PDB files for respective docking predictions. We used special antibody mode plugin in the server for the docking [39], where each dock- prepared antibody was uploaded as receptor input and SARS-CoV-2 trimeric spike protein (PDB ID: 6VSB) was uploaded as ligand input. Additional specification, such as automatic non-complement determining region masking of the antibody, was also enabled for the docking. The top 1000 results from the docking were then clustered using the optimal clustering algorithm in the server. The top 10 docking predictions were then downloaded as PDB files from the server for analysis.

ZDOCK 3.0.2 is also a FFT algorithm-based server for initial stage protein docking predictions. The user interface of the webpage enables uploading of PDB files or specifying PDB IDs. We put the dock-prepared antibody as Input protein 1. Due to PDB file size uploading restrain, we were unable to upload the entire trimeric Spike protein, 6VSB in the server as Input protein 2. Instead of the whole complex, we uploaded only one monomer of the Spike protein complex i.e. chain A as Input protein 2.

In the residue selection module of ZDOCK server, we blocked the SARS-CoV-2 Spike protein amino acid region 910–1146 of the monomeric chain A, as this region is unlikely to have any interaction with antibody [19] and stays mostly buried inside the trimeric Spike protein and envelope portion of the virus. After docking was done, the top 10 predictive structures were downloaded and analyzed in PyMOL. The unique interaction sites i.e. each amino acid residue and its position in the Spike protein, interacting with an antibody, and predicted by both the algorithms, have been tabulated (Supplementary data 1).

4.3. Analysis of predictions and image refinement using PyMOL

We used the PyMOL Molecular Graphics System, Version 2.3.3, Schrödinger, LLC for analyzing the predicted PDB structures obtained from both the servers. For each predicted docked complex, the interaction surface between antigen and antibody was determined through the "find any interaction within 3.5 Å cutoff" plugin. Amino acid residues of the Spike antigen within RBD region that were involved in an interaction with the target antibody, were identified and marked. All pictures were also refined and modified using the software.

4.4. Representative two-dimensional frequency bar graph generation

Two-dimensional frequency bar graph for each Spike protein amino acid interaction event with each antibody within RBD region from predicted docked complex, were created using the GraphPad Prism 6 software.

4.5. Data availability

The structures of docking models both in raw and analyzed format are available at Mendeley Data (https://data.mendeley.com/datasets/hpjyhjvrvv/1). Further information and requests for resources should be directed to and will be fulfilled by the corresponding author, Dr Subhajit Biswas (subhajit.biswas@iicb.res.in).

Author contributions

S.B., S.S., H.N. and A.M. conceived and designed the study. A.M. performed the docking experiments. H.N. and A.M. contributed equally and are joint first authors. All authors analysed and discussed the data. All authors wrote the manuscript to its final version. S.B. supervised the work and critically reviewed and edited the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2020.12.037.

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Title of the Paper presented:

Predated COVID-19 serum samples from Dengue infected patients produced falsepositive results in SARS-CoV-2 lateral flow-based rapid antibody tests

Hadida Yasmin Samikshinder — *Organising Secretary*

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6th Molecular Virology Meeting 2019

School of Bioscience, IIT Kharagpur

Certificate of Participation

This is to certify that

Mr./Ms./Dr. Himadri Nath has participated in the 6th Molecular Virology Meeting held from 28th February to 2nd March 2019 at Indian Institute of Technology, Kharagpur, India

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This is to certify that ***Himadri Nath*** *of Barasat Govt. College, has participated on* **20 & 21 November 2014** *in the National Level Seminar entitled* ***'FRONTIERS OF MICROBIOLOGY: PROSPECTS AND CHALLENGES'*** *organised by the* ***Department of Microbiology, Ramakrishna Mission Vidyamandira,*** *in collaboration with the* ***CSIR-IICB, Kolkata,*** *during* ***20-21 November, 2014.***

Belur Math
21 November 2014

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