Identification of differentially regulated proteins in dengue virus infected samples and evaluation of diagnostic tests for dengue viral infections

DOCTOR OF PHILOSOPHY

By

Srinivasarao Juvvala

August 2016

Enrollment No. 09LTPH04

Department of Biotechnology & Bioinformatics
School of Life Sciences
University of Hyderabad
Prof. C. R. Rao Road
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THESIS SUBMITTED FOR THE AWARD OF DEGREE 'DOCTOR OF PHILOSOPHY' IN BIOTECHNOLOGY AND BIOINFORMATICS

By

Srinivasarao Juvvala

To

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DECLARATION

I hereby declare that the work presented in this thesis entitled "**Identification of differentially regulated proteins in dengue virus infected samples and evaluation of diagnostic tests for dengue viral infections**" is entirely original work and was carried out by me in the Department of Biotechnology & Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, under the supervision of Dr. Musturi Venkataramana. I further declare that to the best of my knowledge this work has not formed the basis for the award of any degree or diploma of any university or institution.

Date:

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CERTIFICATE

This is to certify that this thesis entitled "**Identification of differentially regulated proteins in dengue virus infected samples and evaluation of diagnostic tests for dengue viral infections**" is a record of bonafide work done by Mr. Srinivasarao Juvvala, a research scholar for Ph.D. programme in the Department of Biotechnology & Bioinformatics, School of Life Sciences, University of Hyderabad under my guidance and supervision.

The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

(Signature of Supervisor)

(Head of the Department)

(Dean of the School)

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LIST OF ABBREVIATIONS

DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
ADE	Antibody-dependent enhancement
E	Envelope
ELISA	Enzyme-linked immunosorbent assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
WHO	World Health Organization
TBS	Tris buffered saline
TCA	Trichloroacetic acid
PBS	Phosphate buffer saline
DTT	Dithiothreitol
kDa	Kilo-daltons
BSA	Bovine Serum Albumin
HRP	Horse radish peroxidase
NVBDCP	National Vector Borne Disease Control Programme
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum glutamic-pyruvic transaminase
2D GE	Two-dimensional gel electrophoresis
OFI	Other febrile illness
CHAPS	3-[(3-cholamidopropyl) dimethylammonio] -1-propanesulphate
APS	Ammonium persulphate
TEMED	N,N',N'- tetramethylenediamine

SDS	Sodium dodecyl sulphate
Kb	kilobase
NCBI	National Center for Biotechnology Information
ACN	Acetonitrile
TFA	Trifluoroacetic acid
CHCA	α-cyano-4-hydroxycinnamic acid
H_2SO_4	Sulfuric acid
TMB	3, 3', 5, 5'-Tetramethylbenzidine
AUC	Area under curve
TP	True positive
FP	False positive
ROC	Receiver operator curve
PPV	Positive predictive value
NPV	Negative predictive value
CBB	Coomassie brilliant blue
IPG	Immobilised pH gradient
PVDF	polyvinylidene difluoride
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight

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Last but not the least; I thank the Lord Jesus for guiding me all through and for his generous blessings.

Srinivasarao Juvvala

Chapter 1

Introduction

1.1 Brief history of dengue

Dengue fever is an ancient disease with potentially fatal complications. The name "dengue" is thought to have come from Swahili through Spanish. The first probable dengue fever (DF) case was reported in Chinese medical encyclopedia during 265–420 AD from the Jin Dynasty which indicated as "water poison" identified with flying insects. The first dengue epidemic was recorded almost simultaneously in Asia, Africa, and North America in the 1780s (1). Due to shipping and growth of cities around the port in the 18th and 19th centuries, the spread of mosquito vectors and the dengue viruses to new geographic areas have increased and finally led to cause major epidemics. The ecologic disruptants occurred in Southeast Asia and Pacific theaters during World War II has created an ideal condition for increased transmission of the diseases, and it led to the global spread of dengue. Many parts of South and Central America have now reached hyperendemicity (multiple serotype co-circulating), and this has resulted in increased levels of DHF (PAHO 1994).

The dengue viruses are highly adapted to their mosquito hosts and most likely evolved as mosquito viruses (2). The geographic origin of dengue virus is less clear. The latest evidence in this direction suggests that the Malay Peninsula is the place where dengue viruses first adapted to lower primates and humans.

1.2 Global distribution of dengue

Recently one report estimated that nearly 390 million new dengue cases are arising every year across the globe (3). The World Health Organization (WHO) report indicated that there has been a 30-fold increase in DF incidence and over 2.5billion people or more than 40 % of the world population living in the tropical and sub-tropical areas is at risk of this infection (Figure 1.1) across the world.

Southeast Asia, Africa, the Eastern Mediterranean, the Americas, and the Western Pacific are the most seriously affected regions across the world (4).

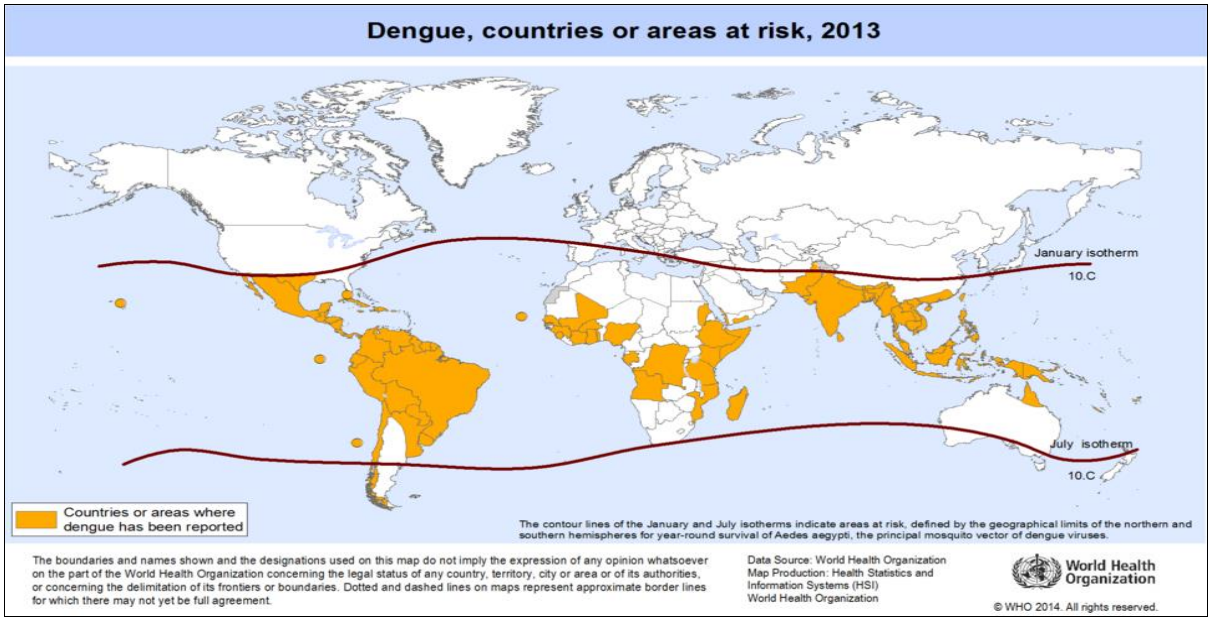

Figure 1.1: Global distribution of dengue. Areas that dengue has been reported was shown in orange and contour lines indicate geographic limits of A. *aegypti.*

The epidemics with severe dengue were from only nine countries before 1970 which is expanded to more than 100 countries at present. 5, 00,000 people are being hospitalized every year of which most of them are children and 2.5% of them reported be died (5).

1.3 Transmission

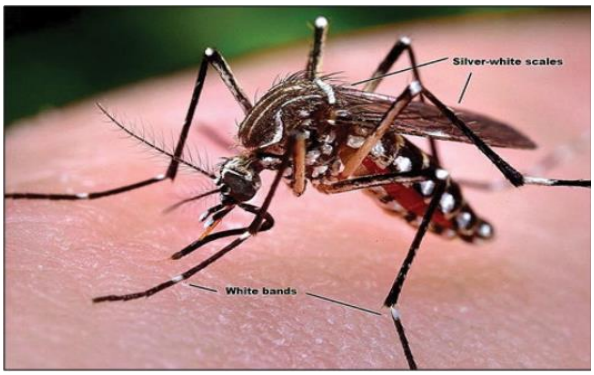

Figure 1.2: *Aedes aegypti* mosquito

http://www.nature.com/scitable/topicpage/dengue-transmission-22399758 (Accessed on 3/10/2015)**.**

The primary vector of dengue is female *Aedes aegypti* mosquito (Figure 1.2). It is seen in abundance in at-risk areas. It was found between latitudes 30° north and 20°south and 2,200 meters above the sea level at various altitudes. The vector mosquito lives indoors, mainly in

living rooms and bedrooms, and in small collections of water such as flowerpots or coconut shells (6, 7).

Dengue virus enters into humans by the bite of an infected *Aedes aegypti* mosquito. This mosquito gets infected with the dengue virus after taking blood from an infected person during the acute phase of infection (viremic phase). After 8-10 days of incubation period, this infected mosquito is capable of transmitting the virus for the rest of its life. Humans are the main reservoirs and multipliers of the virus and serve as a source of the virus for uninfected mosquitoes. The infected mosquito transmits the virus through its salivary fluid into the wound of another person. Then the infected mosquito gets the capability of vertical transmission of the dengue virus from one generation to another, which is important for virus maintenance (Figure 1.3).

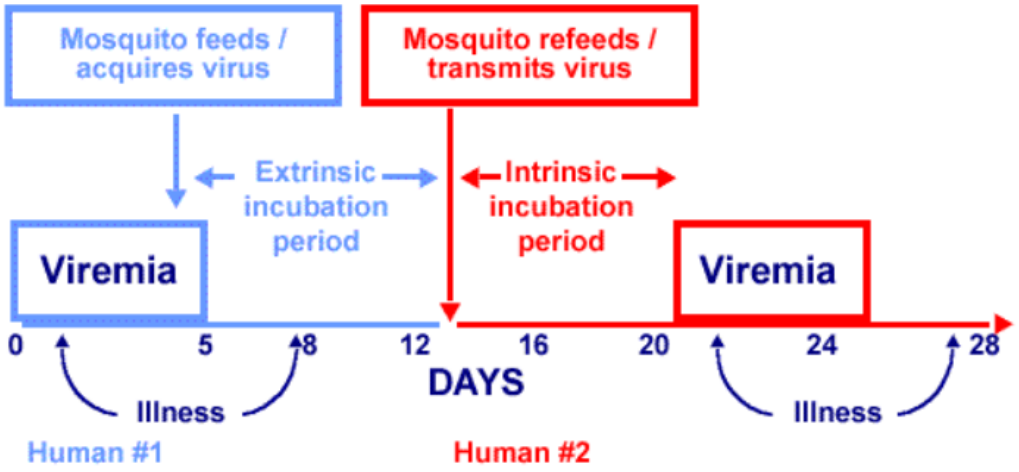

Figure 1.3: Transmission cycle of Dengue

Courtesy: CDC http://www.cdc.gov/ncidod/dvbid/dengue/slideset/set1/i/slide04.htm).

The role of environmental factors in causing infectious diseases is well known. Tropics and subtropics are frequently reporting dengue epidemics every year, usually during monsoon season where *Aedes mosquito* populations are high and are optimal for breeding (8, 9). These areas are at periodic risk for epidemic dengue where large numbers of people become infected during a short period.

1.4 Epidemiology

Dengue virus infection is a major, fastly growing public health problem with an estimated 2.5 billion people at risk of infection. Dengue viruses can cause a wide range of clinical illnesses

ranging from mild symptomatic dengue fever (DF) to more severe clinical conditions with capillary leakage syndrome such as dengue shock syndrome (DSS) and dengue hemorrhagic fever (DHF).

Worldwide, dengue virus transmission has increased in recent years, and all four dengue serotypes are co-circulating in Asia, Africa, and the Americas (10). Approximately 5, 00, 000 people with severe dengue patients are hospitalizing each year and around 2.5% of those patients affected die (11). Southeast Asian countries such as India, Indonesia, Myanmar, and Thailand are major countries at the highest risk of dengue and accounting for nearly half of the global risk (12). Globally, dengue is distributed throughout the tropics and subtropics between 30°N and 40°S and endemic in India, Pakistan and Srilanka (13). In recent years, dengue infection was found to be endemic in most parts of India for over the centuries and it is a self-limiting disease (14). In recent years, the disease has changed its clinical course into severe forms such as dengue hemorrhagic fever and dengue shock syndromes with increasing outbreaks (15). Over the past few decades, epidemiology of dengue has dramatically expanded (16, 17).

Although the first reported evidence of dengue fever in India was in 1946, there were no considerable major outbreaks in the country for almost 20 years, until a major epidemic reported in 1963–1964 in Kolkata (18–20). After that, it has gradually spread to North India in 1967–1968 and also reported in South India (21, 22). Repeated DF epidemics were reported frequently and its geographic expansion is increasing in countries like Bangladesh, India, and Maldives. The initial major outbreak of dengue was recorded in India in 1991. In 2003, another major outbreak occurred in northern and central India and by that time, co-circulation of all four serotypes was seen for the first time in Delhi (23, 24). In 2006 another major outbreak affected India and disrupted the health care system (25).

1.5 Genome organization of dengue virus

Dengue virus is an RNA virus, belongs to the family Flaviviridae and genus Flavivirus. It is spherical with 40-50 µm size and has a lipid envelope derived from host cell membranes. The genome is about 11, 000 bases that codes three structural (Capsid, Membrane protein, and Envelope glycoprotein) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) and flanking either side by untranslated regions (Figure 1.4) (26). The main biological properties of the viruses are associated with the E protein. Among seven nonstructural proteins, some are involved in viral replication (27).

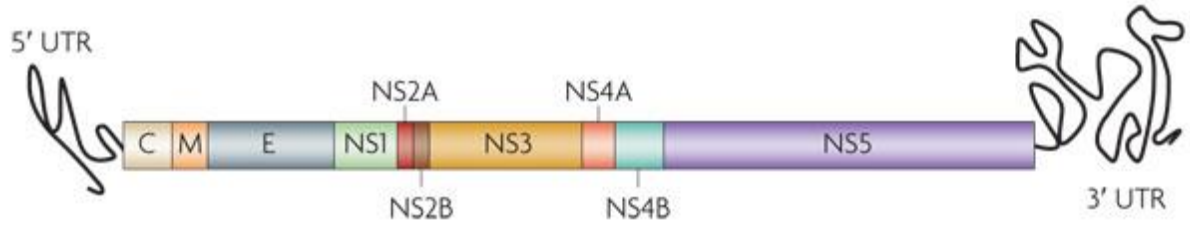

Figure 1.4: Genome organization of dengue virus. *Microbiology* 8, S7–S16 (2010)

There are four serotypes existing all over the world referred to as DENV-1, DENV-2, DENV-3, and DENV- 4. Each serotype resolved in to several genotypes. Infection with one serotype gives lifelong immunity to that specific serotype but partial immunity against the other serotypes (28).

1.6 Clinical manifestations

Dengue is the fastly growing viral disease and present as a spectrum of illnesses from mild dengue fever to most serious forms of the disease such as dengue hemorrhagic fever and dengue shock syndrome (29). The case definitions for DF, DHF and DSS are classified by the world health organization (2009 WHO).

1.6.1. Dengue fever

WHO defines the dengue as an acute onset febrile illness that lasts 2-7 days, with two or more of the following symptoms including a headache, myalgia/arthralgia, maculopapular rash, petechiae, retro-orbital pain and positive tourniquet test (30). Symptoms predominantly occur in the early phase of the disease (31). Hemorrhagic manifestations are rare but epistaxis, petechiae, and gingival bleeding will sometimes occur in DF, although they are rarely associated with severe hemorrhage leading to shock (32). A maculopapular rash will appear on the trunk after 35 days of post onset of fever (33). DF is referred as ‘break bone fever' due to myalgia and joint pain (34) or sometimes called as ‘seven-day -fever' because the symptoms usually persist for 7 days.

1.6.2 DHF

DHF is a severe complication of dengue viral infections. In Southeast Asia, the majority of patients affected by DHF are children, whereas, in the Americas, this is seen in all age groups. DHF usually starts with a sudden onset of fever and other symptoms identical to those of dengue fever and temperature remains high for 2 to 7 days. Hepatomegaly and splenomegaly are

especially seen in infants. Hemorrhagic tendency may occur in many ways, positive tourniquet test, ecchymoses or purpura, petechiae, mucosal bleeding and hematemesis or melena. Petechiae, easy bruising are the most common hemorrhagic features. Epistaxis and gingival bleeding are uncommon, and gastrointestinal bleeding may be observed in severe cases. Sometimes, the bleeding may be occult and intracranial bleeding is rare.

In DHF, bleeding may not connect with the platelet counts but usually occurs when the fever has subsided. It is characterized by vascular permeability resulting in plasma leakage, bleeding, thrombocytopenia, and haemoconcentration which finally lead to shock. Secondary dengue infection is thought to be the principal risk factor for DHF but the interaction of virus, host, and epidemiological risk factors are major elements of the occurrence of DHF epidemics (35). Although it can occur in adults, DHF or severe dengue usually affects children younger than 15 years (36). In DHF convalescence is short and uneventful. The recovery of appetite is a good indicator of recovery from shock and bradycardia is also seen in this period. During the convalescent stage, many patients also complain of severe itching, especially on the palms and soles.

1.6.3 DSS

WHO defines DSS as DHF symptoms plus signs of circulatory failure characterized by rapid and weak pulse, narrow pulse pressure, cold, clammy skin and restlessness. The onset of shock is transient and occurs at the time of defervescence, usually after 2-5 days of onset of fever. The temperature is often subnormal and skin is cold and clammy, and pulse rapid and feeble. Pleural effusion and ascites are the predictors for the development of DSS. Severe abdominal pain is a complaint that develops shortly before the onset of shock. Consciousness is usually perfect. The duration of the shock is transient. Recovery from properly treated DSS is short and uneventful. Although pleural effusion and ascites may be detected for a little longer time, survivors recover within 2 to 5 days. The course of DHF/DSS is approximately 7 to 10 days.

1.7 Dengue serotyping

The dynamics of dengue fever in India becomes complex and changed considerably for past six decades and circulation of different serotypes being changed drastically (37,38).

1.8 Dengue virus life cycle

Dengue virus belongs to the family of flaviviruses and the life cycle of flavivirus includes the following steps.

1) Binding of virions to the cell surface attachment molecules and receptors, followed by internalization through endocytosis. 2) Because of low pH of the endosome, viral and cellular membrane fusion occurs via viral glycoproteins, allowing uncoating of the virion and release of viral RNA into the cytoplasm. 3) Translation of viral RNA into a polyprotein which was cleaved by viral and cellular proteases and 4) the viral nonstructural proteins replicate the viral RNA. 5) Assembly of virus particles at the endoplasmic reticulum membrane, where nucleocapsid was enveloped by the ER membrane and glycoproteins for the formation of an immature virus.

After the bite of an infected mosquito, the viral entry into the host cell is mediated by receptor-mediated endocytosis. Various glycoprotein receptors such as heparin sulfates, mannose receptors and dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrins are required for the entry of the virus into the host cell (39 – 41)

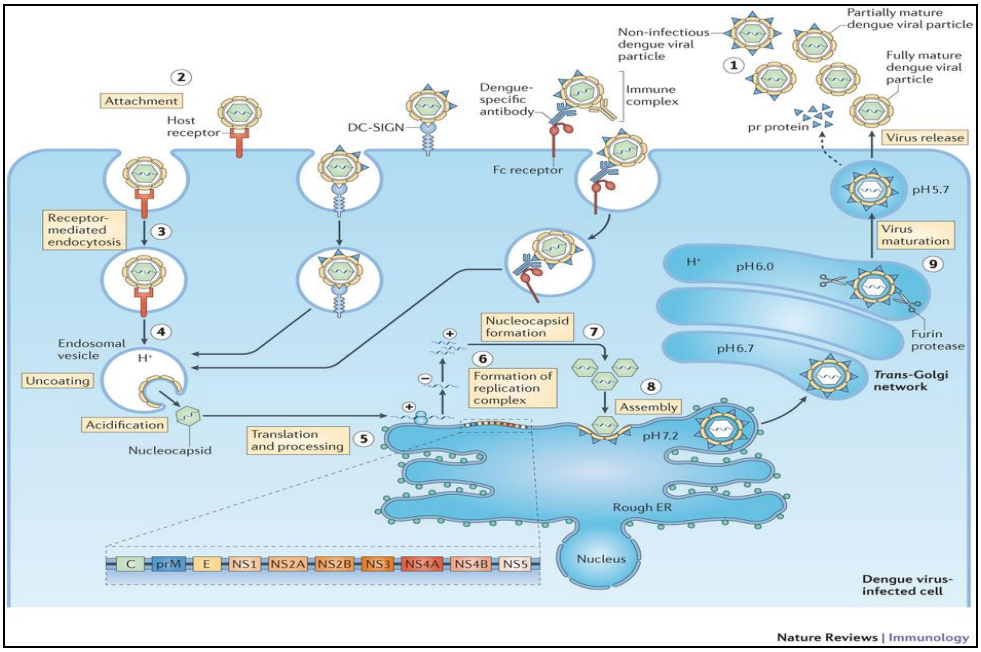

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Figure 1.5: Life cycle of dengue virus.

After internalization, due to the acidic pH in the endosome generates a conformational change in the envelope protein mediating membrane fusion. Then uncoating and release of the viral genome

into the cytoplasm takes place (42). Translation of the input strand takes place followed by switching from translation to the synthesis of a negative-strand intermediate. This negative strand serves as a template for the production of multiple copies of positive strand viral RNA. These successive rounds of translation allow the production of high levels of viral proteins such as the structural proteins, capsid or core, premembrane and envelope proteins, along with viral RNA, are assembled into progeny virions, which are secreted after transport through the Golgi compartment (43).

1.9 Pathogenesis

After the bite of an infected mosquito, there will be an incubation period of 4 to 7 days (range of 3-14 days), during which the patient may or may not show symptoms. This depends on the virus strain, age, immune status, and other factors. This is followed by viremia, which begins with sudden onset of fever and constitutional symptoms lasting for 5-6 days (range of 2 to 12 days).

The four dengue virus serotypes (DENV 1-4) shares 65-70% homology. Infection with a particular serotype for the first time is known as a primary infection, which is usually asymptomatic and results in mild clinical manifestations. Subsequent infection with other serotypes may cause severe disease which results in the form of DHF/DSS. However, only 0.18-1% of patients with primary infections and 2-9% of patients with secondary infections manifest as DHF/DSS. Therefore, all the individuals who are infected with dengue do not develop the symptomatic disease or only a few patients may develop mild clinical manifestations. A primary dengue infection is usually associated with DF. Antibody-dependent enhancement is the mechanism that has been considered to cause DHF.

In secondary infection, heterologous non-neutralizing antibodies from the primary infection may lead to severe dengue (44). The DHF occurs in regions where multiple serotypes co-circulate or when a new serotype is introduced into a dengue prevalent region. The virus, host genetic makeup, and host immune factors are thought to be playing an important role in the occurrence of DHF/DSS. The pathophysiology of dengue viral infections which results in severe disease is poorly understood. The factors which are involved in the pathogenesis of DHF continue to be one of the most active areas of dengue research. Several factors may influence disease severity, virus, host genetic makeup, virus serotype or genotype, sequential virus infection, differences in dengue cross-reactive antibody, and T-cell responses (45).

The replication of virus takes place within the cells of the mononuclear phagocyte lineage such as macrophages, monocytes, and B cells. Besides infection of mast cells, dendritic cells, and endothelial cells are also known to be infected (46). The major hallmark of dengue is thrombocytopenia which contributes to disturbed hemostasis in dengue patients (47). The virus may also infect peripheral blood leukocytes, human organs and possibly the brain, suggesting blood-brain barrier disruption (48).

1.10 Treatment

Currently, there is no curative treatment in terms of antiviral drugs for dengue. Managed with rest and aggressive supportive therapy are only the ways without hospitalization. At present, vector control is expensive and ineffective is the only method for prevention of disease. That makes it important to diagnose the infection and supportive care given to the patients as soon as possible. There are two types of treatments, home management, and hospital-based management. Hospital-based management has fluid therapy, platelet transfusion, supportive therapy.

1.11 Clinical course

The common symptoms of dengue are sudden-onset fever, headache, muscle and joint pains, and a rash. The course of infection is classified into 3 phases: febrile, critical, and recovery (49).

Febrile phase:

The febrile phase starts with high-grade fever potentially over 40°C (104°F) and is associated with a headache. This phase usually lasts 2–7 days (49, 50). Vomiting may also occur (51). A rash occurs in 50%–80% of the patients with symptoms (52). In the early phase of onset of symptoms as flushed skin, or later in the course of illness (days 4–7) as a maculopapular rash like measles (52, 53). The biphasic or saddleback fever occurs and then returns in 1 or 2 more days (53,54). Mild hemorrhagic manifestations like petechial and mucosal membrane bleeding (e.g: nose and gums) may occur. The liver is often enlarged and tender after a few days of fever. In the early febrile phase, distinguishing dengue from non-dengue is difficult and a positive tourniquet test increases the probability of dengue. Besides these, clinical features are indistinguishable between severe and non-severe dengue cases. Therefore checking for warning signs and other clinical parameters is important in recognizing progression to the critical phase.

Critical phase:

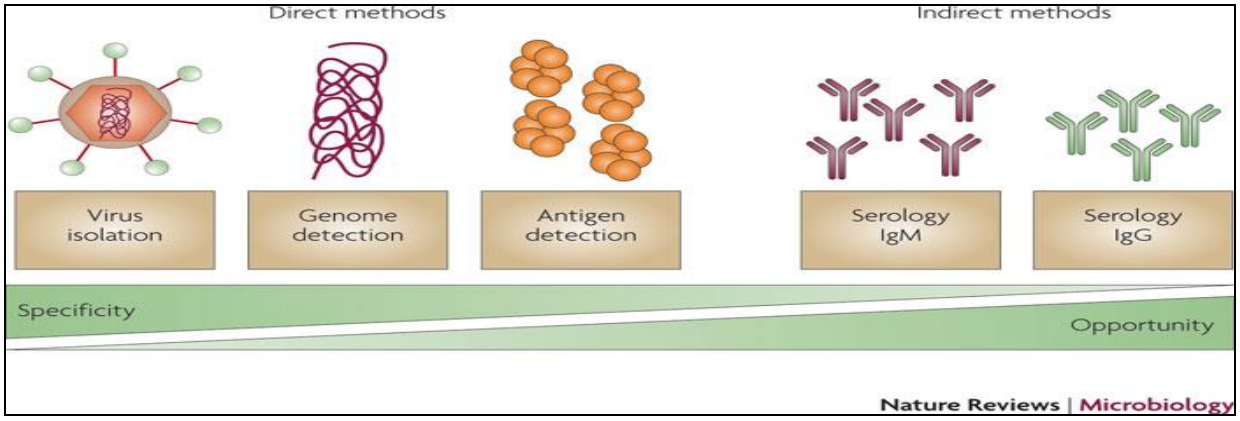
In some cases, the disease progress to a critical phase as the fever resolves (51). This phase is characterized by leakage of plasma typically lasting 1–2 days. Due to plasma leakage pulmonary edema and ascites, as well as hypovolemia and shock occur (49). Organ dysfunction and severe bleeding may also occur typically from the gastrointestinal tract (49, 55). Most serious forms of the disease such as dengue shock syndrome and hemorrhagic fever occur in less than 5% of all cases of dengue (55). However, those people who have previously been infected with heterologous serotypes of dengue virus are at increased risk (55, 56). Although critical phase is rare, it is more common among children and young adults (51).

Recovery phase:

The recovery phase occurs after the critical phase with resorption of the leaked fluid into the bloodstream (49). During this phase, a fluid overloaded state may occur and rarely cerebral edema occurs in rare instances that lead to reduced level of consciousness or seizures (56).

1.12 Dengue Diagnosis

Due to the broad spectrum of clinical manifestations, ranging from mild febrile illness to severe syndromes such as dengue hemorrhagic fever and dengue shock syndrome can make accurate diagnosis difficult. The conclusive diagnosis of DENV infection depends on virus isolation, detection of viral antigens, RNA in serum or tissues and detection of specific antibodies in the serum.



Nature Reviews Microbiology **8**, S30-S37

Figure 1.6: Comparative merits of direct and indirect laboratory methods for the diagnosis of dengue infections. Opportunity refers to the fact that antibody testing is usually the most practical diagnostic option available.

Table 1.1: Advantages and limitations of different dengue diagnostic tests.

Diagnostic tests	Advantages	Limitations
Viral isolation and identification	• Confirmed infection • Specific • Identifies serotypes	• Requires acute sample (0–5 days post onset) • Requires expertise and appropriate facilities • Takes more than 1 week • Expensive
RNA detection	• Confirmed infection • Sensitive and specific • Identifies serotype and genotype • Results in 24–48 hours	• Potential false positives owing to contamination • Requires acute sample (0–5 days post onset • Requires expertise and appropriate facilities
Antigen detection (NS1 assay)	• Confirmed infection • Easy to perform • Less expensive than virus isolation or RNA detection	• Require acute sample (0 -5 days post onset)
Serological tests IgM and IgG detection	• Least expensive • Easy to perform • Identifies probable dengue cases	• Cross-reactivity • Confirmation requires two or more serum samples •Five or more days after the onset of symptoms

*Primary infection: IgM-positive and IgG-negative (if samples are taken before day 8–10); secondary infection: IgG should be higher than 1,280 haemagglutination inhibition in convalescent serum.

The tests that are currently used in the laboratory diagnosis of dengue infections, and their advantages and limitations are shown (Table 1.1). The sensitivity and positive predictive value of all the above methods vary in epidemic and endemic regions. Among the available dengue diagnosis methods, virus isolation provides the most specific test result but virus isolation is a time consuming and requires expertise and good laboratory facilities. However, facilities that can support viral culture are not always available. Besides detection of the viral genome, viral antigens also provide evidence of infection.

Early diagnosis of dengue plays an important role in identifying the outbreaks and initiating a well-managed frontline response. This will not only reduce the number of unnecessary hospital admissions but also reduce the mortality rates due to dengue. Hence the differential diagnosis of dengue conditions that can mimic febrile phase and critical phase of dengue needs to be considered.

The relative merits of the different diagnostic tests and their optimal time frame for use are summarized (Figure 1.6). It is very important to have an accurate and speedy diagnosis of the dengue infection so that it can be differentiated from other diseases such as rubella, leptospirosis as well as other flavivirus infections. The characteristics of an 'ideal' dengue diagnostic test depend on the purpose for which the test will be used (Table 1.2).

Table 1.2: The characteristics of an Ideal dengue test

The characteristics of an 'ideal' dengue diagnostic test
• It should be able to distinguish between dengue and other diseases with similar clinical presentations (such as leptospirosis, Zika and Chikungunya) • Positive as soon as possible after onset of symptoms to provide early warning • Highly sensitive during the acute stage of infection • Inexpensive • Easy to use • Provide rapid results • Stable at temperatures greater than 30°C for field use, if necessary • Helpful in outbreak investigations • Highly specific

Virus isolation:

There are four methods of virus isolation have been routinely used for dengue viruses such as intracerebral inoculation of newborn mice, intrathoracic inoculation of adult mosquitoes, Inoculation of mammalian cell cultures, and inoculation of mosquito cell cultures (57, 58). The conventional test to identify DENV is virus isolation in cell culture or live mosquitoes, which was the approved test in the last century (59, 60). This test has always been the ‘gold standard' for any viral disease. Cell lines normally used to grow the virus include mosquito cell lines (C6/36 and AP61) or mammalian cell lines (Vero, LLC-MK2 or BHK-21) (61, 62). For virus serotype identification, immunofluorescent assays using serotype-specific monoclonal antibodies are used. Because of its higher sensitivity, the mosquito inoculation technique is still the method of choice for attempting dengue virus isolation from deceased patients with fatal cases or patients with severe cases (63, 64). A virus can also be isolated using intracerebral inoculation of suckling mice. Traditionally 75ml flasks were used but we have now adapted to using 6-well microtiter plates thus bringing the time of harvest down from 7-12 days to 5-6 days and are able to do multiple samples simultaneously in a single plate. Sometimes more than two passages may be required to isolate the virus. Following harvest, confirmation tests are carried out using dengue serotype-specific fluorescent monoclonal antibodies. A real-time TaqMan PCR can also be used for confirmation. As such this method requires skill and substantial equipment to be carried out and is not the choice for many laboratories. Techniques like virus isolation carries several disadvantages and limitations with less percent of diagnostic value. However this technique is considered as better than molecular techniques like PCR, if the samples are fresh (65-67)

Viral RNA detection:

Detection of dengue viral RNA can be done by using dengue gene specific primers on whole blood or sera taken from the acute phase of patients. There are various protocols developed for the identification of dengue viruses using gene specific primers designed to serotype-specific regions of the genome (68-70). The sensitivity of detection is improved by Nested PCR techniques because the initial amplification product is used as the template for the second round of amplification. However, it is important that laboratories performing nested PCR take every precaution to prevent false positive results that can occur due to contamination.

Antigen detection:

NS1 based assays:

NS1 Assay is used for diagnosis during the acute stage of dengue infection. Antigen detection in the acute stage of secondary infections can be limited by pre-existing virus IgG immuno-complexes. There are a few developments in ELISA and rapid immunochromatographic assays which are based on nonstructural protein 1 (NS1). These tests have shown that up to 4-9 days after the onset of illness, high concentrations of this antigen can be detected in patients (70). Nonstructural protein 1 (NS1) is synthesized by all Flaviviruses and is released from infected mammalian cells. The secreted NS1 (sNS1) in the bloodstream stimulates a strong humoral response. Earlier many reports indicates NS1 detection as a diagnostic tool during the febrile phase of a dengue infection. This test has the ability to differentiate primary infections from secondary dengue virus infections.

Immunohistochemistry:

Using labeled mAbs, dengue viral antigens can be detected in tissue sections.

Serological methods:

The acquired immune response raised against each serotype is mainly specific for the virus envelope (E) protein. The magnitude of the response varies depending on whether the individual has a primary or secondary dengue infection. In primary dengue infection, IgM response is higher when compared to the secondary dengue infection. During secondary infection, IgG levels are higher when compared to primary infection (Figure 1.7).

The detection of dengue virus serotypes using serology following a recent infection is very difficult because the antibodies produced after primary infection often demonstrate some degree of cross-reactivity with other dengue virus serotypes. Antibodies produced following secondary dengue infections are strongly cross-reactive within the dengue group and also cross-react with other Flaviviruses (71).

IgM based assays.

Dengue specific IgM is a useful diagnostic test. IgM levels were initially detectable between 3 - 5 days after post onset of fever in 50% of patients. This test has a sensitivity and specificity of 90%

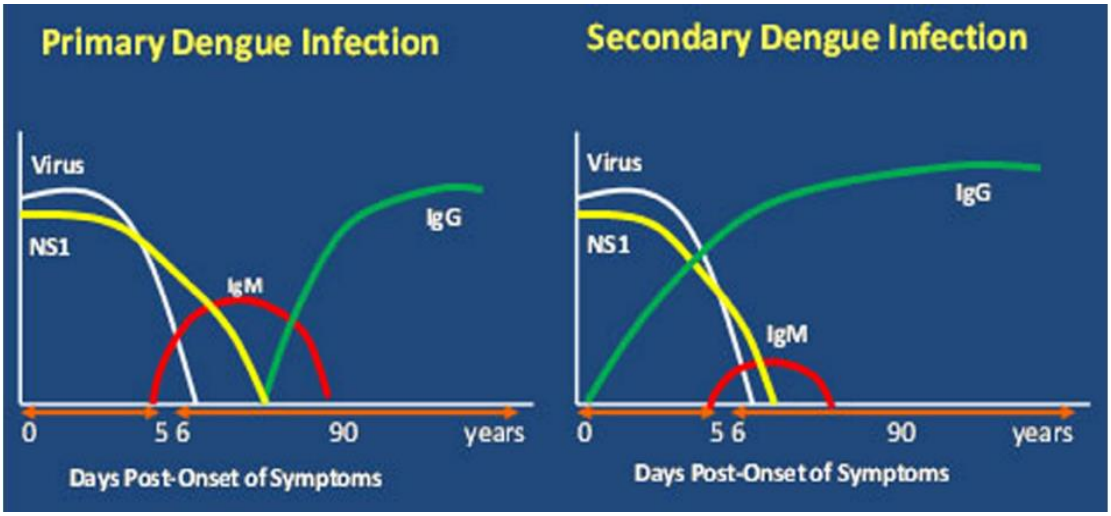
and 98%, respectively when these assays are performed after five days of onset of fever. Dengue specific IgM is detected earlier than dengue-specific IgG.

In one study conducted in Puerto Rico, at day 5 of illness, most patients (80%) with dengue infections that were confirmed by HAI on paired serum samples or by virus isolation had detectable IgM in acute phase serum. Nearly 93% patients developed detectable IgM after 6 days after onset of fever, and 99% of patients had detectable IgM between 10 and 20 days after onset of fever (72,73).

The quality of antigen used in the assay was responsible for the sensitivity and specificity of IgM-based assays and can vary between commercially available products. ELISA based IgM assays were not used for the surveillance of dengue. Most of the ELISAs use dengue E protein antigens from all four dengue virus serotypes.

IgG based assays:

These assays can be used for the detection of past dengue infections and current infections if paired sera are collected at the time of seroconversion between acute and convalescent serum samples. In this assay, multiple dilutions of each serum were tested to determine an endpoint titer. IgG avidity assays can be used to determine whether an infection is a primary or a secondary infection. It was based on the principle that the avidity of IgG is low after primary infection but reduced slowly within the weeks and months after infection. So that, these assays can be more useful than the HAI test. The IgG-based ELISAs also exhibits the cross-reactivity with other Flaviviruses as the HAI test. These assays cannot be used to identify the virus serotype. However, it has a slightly higher sensitivity than the HAI test.



Retrieved from (73); Centers for Disease Control and Prevention, USA.

Figure 1.7: Immunological response to dengue infection. The titer of the IgM and IgG response varies, depending on whether the infection is a primary or secondary infection.

1.13 Statement of the problem

Dengue fever is an expanding public health problem in tropical and sub tropical regions of the world for which there is no vaccine or specific drug available at present. In case of dengue virus, number of studies being increased for the identification of virus/host related proteins to use them either as diagnostic biomarkers, to understand the host-virus interactions or to use as targets for developing antivirals. In this direction, several techniques have been developed for rapid laboratory diagnosis of dengue virus. Current diagnostic methods includes the platelet count, virus isolation and culture, nucleic acid detection by RT-PCR, antigen detection by ELISA and other serological tests. NS1 is a non structural protein is being used as the diagnostic marker for the identification and analysis of dengue fever. The recommended confirmatory diagnostic test kits for dengue infection (isolation of the virus in cell culture, the identification of viral nucleic acid or antigens, or the detection of virus-specific antibodies by ELISA) have high specificity, but their high cost, turnover time and limited availability restricts their use in clinical practice. In addition, several rapid diagnostic tests are commercially available with different sensitivities and specificities but the performance characteristics of these tests have not been adequately evaluated. Due to the limitations of presently existing diagnostic markers, there is a need for specific, inexpensive diagnostic tests that can be used for early detection and outbreak investigations. These tests should permit early detection of the disease to treat patients and prevent or control

epidemics. Reports are indicating that platelets, dendritic cells, monocytes and macrophages are the target cells for dengue virus multiplication. So it is expected that there will be an alteration of gene expression in the virus targeted cells. Several outbreaks of dengue and other viruses took away many lives in India particularly in the states Telangana and Andhra Pradesh of South India. Hence the present investigation is being undertaken to understand the pathogenesis and mainly to identify the biomarkers in virus infected blood samples collected from patients residing in the areas where the frequent outbreaks were being recorded. The other main objective of the present study is to standardize the tests for dengue diagnosis using the identified host proteins as biomarkers and evaluation of developed tests for their use as diagnostic tests for dengue infections, if possible at early stages of the infections.

1.14 Aim and objectives:

This study mainly aims to identify differentially expressed host candidate proteins in dengue virus infected samples and validation of those candidate markers for developing diagnostic tests. In order to identify differentially expressed proteins which have diagnostic value, we followed proteomics approach. The main objectives are:

1. Analysis of current epidemiological status of dengue infections and collection of virus-infected samples
2. Identification of differentially regulated proteins in dengue virus infected samples
3. A) Standardization of DAC- ELISA and Dot-ELISA

   B) Evaluation of tests for dengue diagnosis during infection, disease progression and infections with different serotypes

1.15 Scope of the present study

It is very important to have accurate and speedy diagnosis for the dengue infection so that it can be differentiated from other diseases such as chikungunya, leptospirosis and other flavivirus infection. Early diagnosis of dengue plays an important role in identifying the outbreaks and initiating a well-managed frontline response. This will not only reduce the number of unnecessary

hospital admissions but also reduce the mortality rates due to dengue. Reports are indicating that the virus multiplication and accumulation is more in blood plate lets than in plasma. Identification of differentially regulated proteins in virus infected plate lets could leads to the development of new diagnostic kits for the early detection of the dengue virus. And also these proteins could be considered as targets for designing the antiviral drugs. Detection of the virus during early stages helps in formulating proper treatment at right time. The major detection tests for dengue virus detection at present are based on virus specific IgM/IgG, virus isolation, electron microscopy and RT-PCR. Identification of nonstructural protein-1 (NS1) emerged as an alternative for the above tests. Viremia occurs with in two days of infection but detection of virus specific antibodies is possible only after 10 days of infection and all the other above mentioned tests for dengue virus are time consuming, needs sophisticated equipment and expertise. Hence in this proposal, the aim is to identification of differentially regulated proteins (which might be useful as bio-markers of dengue virus infection) in virus infected plate lets and development of antigen based detections tests for dengue virus detection during early stages of infection.

CHAPTER 2

Analysis of current epidemiological status of dengue infections and collection of virus-infected samples

2.1 Introduction

Dengue infection is an acute viral disease and transmitted to humans by the bite of an infected female mosquito - *Aedes aegypti* (74-76). Dengue fever causes a significant public health problem, especially in endemic regions. World health organization (WHO) reported that Southeast Asia and western pacific regions are the most seriously affected and occupied 75% or of the disease burden (77-79). Asia contributed 70% or approximately 67 million of the apparent infections in disease burden and more than a billion people located in Southeast Asia are at risk of DEN infection (80). Among Southeast Asian countries, India reporting frequent outbreaks with case fatality rates as high as 3-5% (81).

Presently, the Southeast Asian countries including Bangladesh, India, Sri Lanka and Thailand are showing an increasing trend in reported cases of dengue (82). In the same way, India occupied 34% or around 33 million infections in global burden. Only a few reports have been documented from central India in past 20 years may be due to lack of proper diagnostic facilities (83-87).

The actual number of dengue infections is three times more than the WHO estimates. Because of the low level of reporting, low case fatality rate, difficulty in diagnosis and poor epidemiological surveillance the appropriate incidence and burden of dengue is significantly high when compared to reported cases (88-89). There is a limited information on epidemiology in India. Prevention and control of disease is mainly depends on the epidemiological surveillance data.

Since 2007, diagnosis and data assimilation for dengue and chikungunya in India have been facilitated by the National Vector Borne Disease Control Programme (NVBDCP). The program has 347 sentinel centers in 35 states and 14 apex referral laboratories, which are supplied with DENV and CHIKV specific IgM detection kits produced by the National Institute of Virology (NIV).

The Health Map is a global infectious disease monitoring system, collects information from different online sources like Pro MED-mail. It is one of the internet based systems for reported dengue fever cases. DENV infection is known to be endemic in many parts of world including India, and several epidemics are frequently occurring for the past six years and the year 2013 has witnessed the worst dengue outbreak in India with 75,808 dengue cases and 193 deaths were reported by National Vector Borne Disease Control Programme (NVBDCP). This study aims to know the actual disease burden compared to official data.

2.2 Materials and methods:

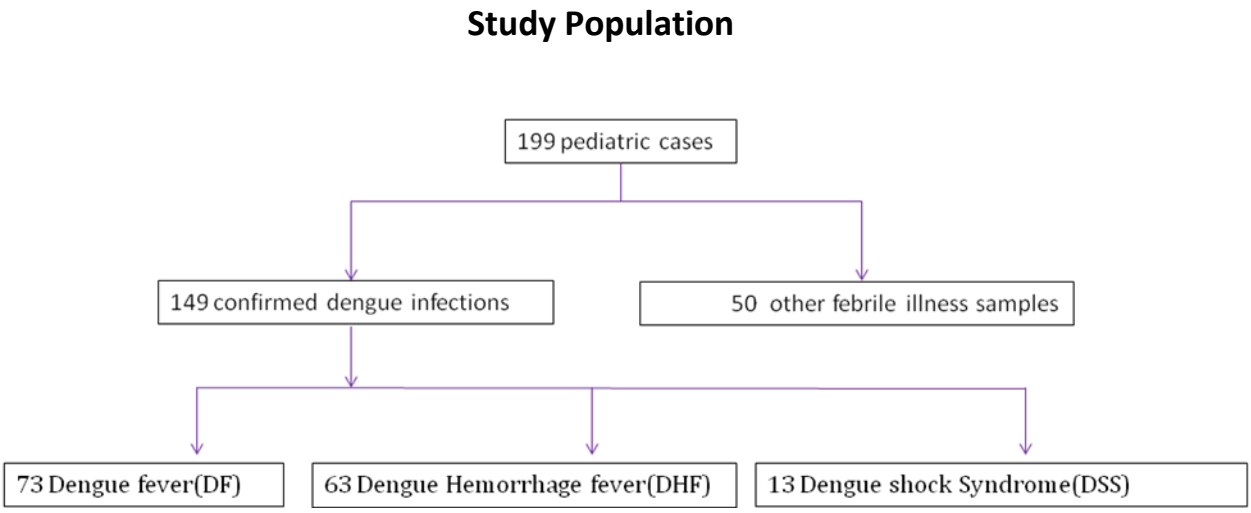
2.2.1 Data collection:

Available information about dengue from Southeast Asia was collected from Pro-MED mail (Program for monitoring Emerging diseases) as case reports. The number of cases in each report in Southeast Asia was grouped for every country from the year 2012-2015. Confirmed dengue fever and death cases information was collected from NVBDCP (National vector-borne disease control program) from 2010-2015. Chikungunya and Japanese encephalitis cases information also collected from the NVBDCP along with dengue cases information.

2.2.2 Ethical considerations:

Ethical approval for my study was obtained from the institutional ethics committee, University of Hyderabad and a written informed consent was obtained from the study patients before blood sample collection (UH/IEC/2014/24/25).

2.2.3 Patients and sample collection:



A total of 149 clinically confirmed dengue and 50 control blood samples (other febrile illness: OFI) were collected from pediatric patients at Lotus children's hospital, Hyderabad and plasma were separated instantly and kept at -80°C until use. Samples were collected during two years period (2014-2015). Clinical and demographic characters of every case were recorded in a case record form including symptoms, signs, and laboratory investigations. The detailed clinical

examination was done at the time of admission followed by serial monitoring to assess the progression of the disease.

All pediatric cases involved in this study were below 15 years of age and they came to the hospital with symptoms of dengue. All these children were monitored by doctors for vital signs including blood pressure, weight, any bleeding manifestations, edema, and consciousness. Complete blood picture including differential blood count packed cell volume and platelet count was done. The rapid decrease in platelets count is the major hallmark for dengue. PCV and APC were monitored once a day for all patients in the study group. Liver enzymes like SGOT and SGPT were measured for all children. The abdominal ultrasonography was also done by an experienced sonologist in those children who had severe abdominal pain. All pediatric patients were diagnosed as per WHO guidelines. All cases went through serological studies. All blood samples were collected after admission into the hospital. Along with these samples, some of the children samples were collected days wise from day 1(d1) to day 6 (d6). The Dengue Day1 (NS1) test kit and IgM-IgG Kit (J Mitra and Co., India) were used for screening dengue infection.

2.2.4 Serotyping analysis:

After sample collection, serotyping analysis was performed in some samples by using gene-specific primers by other colleagues.

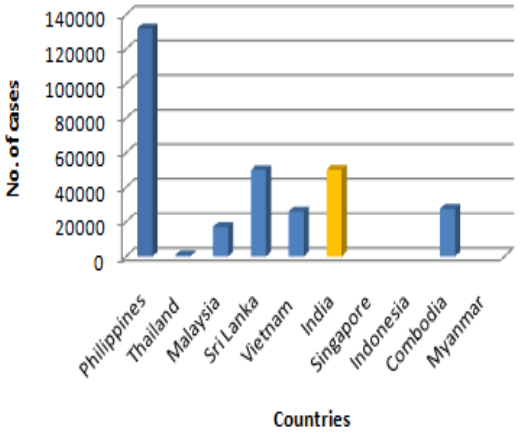
2.3 Results:

2.3.1 Disease burden in Southeast Asia

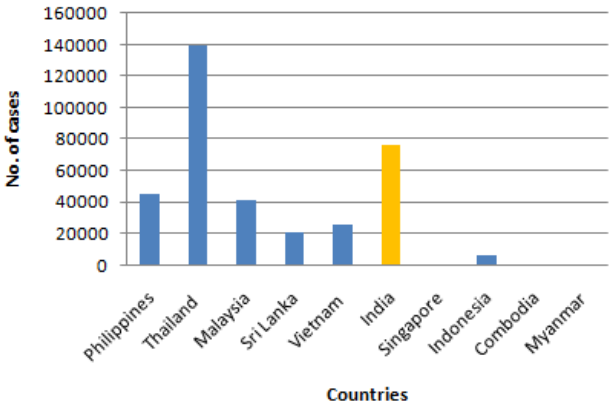
Totally ten countries in Southeast Asia are reporting dengue cases information annually. In the year 2012, among ten countries in Southeast Asia, Philippines reported 1, 32,046, Thailand (713), Malaysia (17,066), Srilanka (50,000), Vietnam (26,000), India (50,222), Cambodia (27,590) cases. Disease burden-wise, Philippines occupied first place followed by India, Srilanka, Cambodia, Vietnam, Malaysia, Thailand and India occupied second place in disease burden in 2012 (Figure: 2.1A). But in 2013 Philippines reported (45,037), Thailand (1, 39, 681), Malaysia (41, 226), Srilanka (20,216), Vietnam (25,000), India (75,808), Indonesia (5870) cases. Disease burden-wise Thailand occupied first place followed by India, Philippines, Malaysia, Vietnam, Srilanka, Indonesia and India occupied the second place (Figure 2.1B).

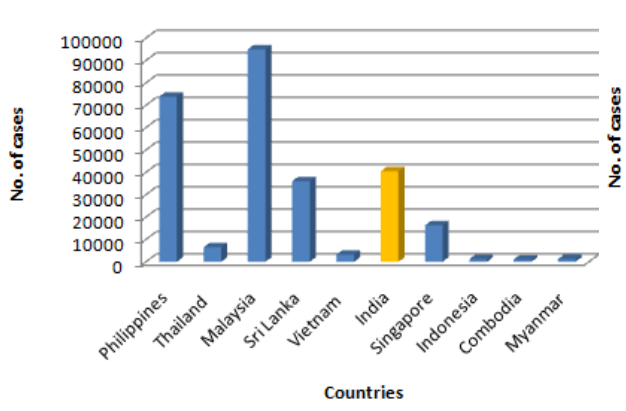
When we observed in 2014, Philippines reported (73,815), Thailand (6,420), Malaysia (95, 000), Srilanka (36, 000), Vietnam (3, 150), India (40, 471), (Singapore (16, 322), Indonesia (1, 168), Cambodia (1, 005), Myanmar (1, 200) cases. Disease burden-wise Malaysia occupied first place followed by Philippines, India, Sri Lanka, Singapore, Thailand, Vietnam, Myanmar, Indonesia and Cambodia and India occupied third place in 2014 (Figure 2.1C). When we observed in 2015, Philippines reported (1, 42, 227), Thailand (1, 31, 647), Malaysia (1, 07, 079), Sri Lanka (26, 243), Vietnam (53, 000), India (2, 77,000), (Singapore (10,142), Indonesia (3, 714), Cambodia (7, 799), Myanmar (9, 893) cases. Disease burden-wise India occupied first place followed by Philippines, Thailand, Malaysia, Vietnam, Sri Lanka, Singapore, Myanmar, Cambodia, Indonesia (Figure 2.1). In Southeast Asia disease burden is increasing in India from the year 2012- 2015.

(A)

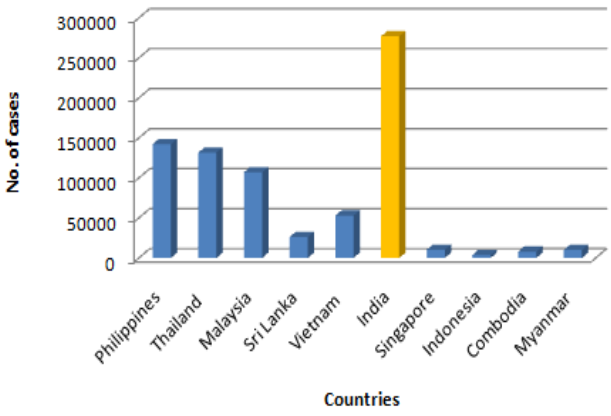


(B)





(C)



(D)

Figure 2.1: Distribution of the number of dengue cases in countries of Southeast Asia from 2012-2015. (A) 2012, (B) 2013, (C) 2014 and (D) 2015. India was shown in yellow color bar and Philippines, Thailand, Malaysia, Srilanka, Vietnam, Singapore, Indonesia, Cambodia, Myanmar countries were shown in blue color Bar.

2.3.2 NVBDCP:

In India 28,292 confirmed dengue positive cases and 110 deaths were reported to NVBDCP in 2010. In the same way in 2011, 18, 860 cases and 169 deaths, in 2012 50,222 cases and 242 deaths, in 2013 75,808 cases and 193 deaths, in 2014, 40,571 cases and 137 deaths and 99,913 cases and 220 deaths were reported in 2015 (Figure 2.2).

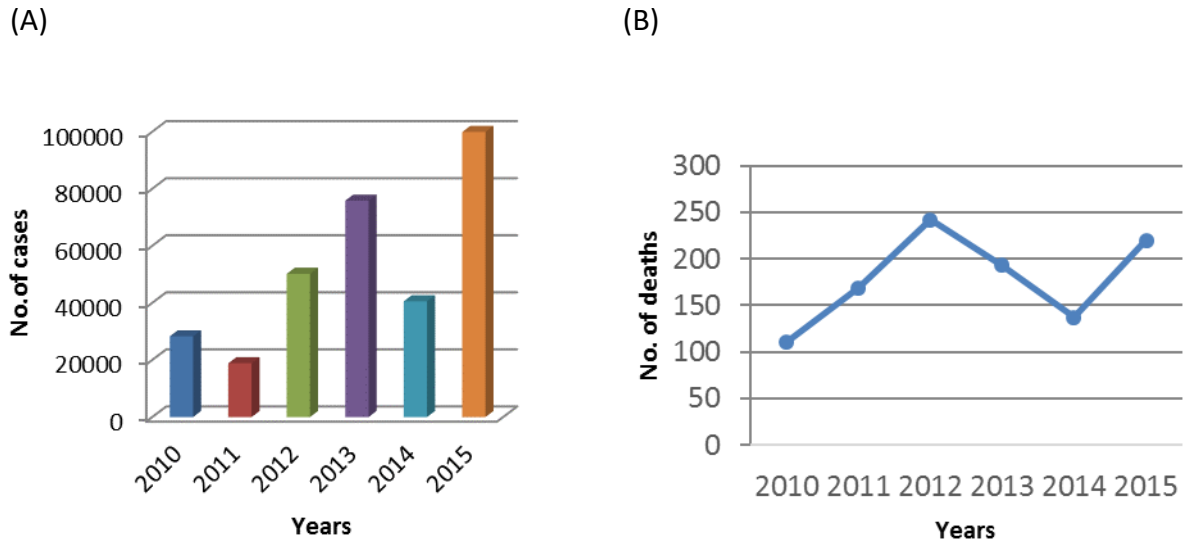


Figure 2.2: Distribution of the total number of dengue positive cases and deaths reported to NVBDCP in India (2010-2015). The total number of cases per year was shown in colored bars and annual deaths were shown in a line graph.

According to NVBDCP data, dengue positive cases and deaths were increasing from 2010 to 2015 and nearly 1 lakh cases were reported in the recent year 2015. These 99, 913 cases were distributed in different states and Union territories as shown in figure 2.3. Based on the number of cases reported from each state and Union territories dengue disease burden is shown in descending order.

Delhi occupied first place with 15,867 cases followed by Punjab (14, 128), Haryana (9, 951), West Bengal (8, 516), Gujarat(5, 590), Karnataka (5, 077), Andhra Pradesh (4, 990), Maharashtra (4, 936), Tamilnadu (4, 535), Kerala (4, 075), Rajasthan (4, 043), Uttar Pradesh (2,

892), Orissa (2,450), Madhya Pradesh (2, 108), Arunachal Pradesh (1, 933), Bihar (1, 771), Uttarakhand (1, 655), D&N Haveli (1, 154) and Assam (1, 076). Among Indian states, disease burden-wise Andhra Pradesh occupied 7th position and shown in the red bar.

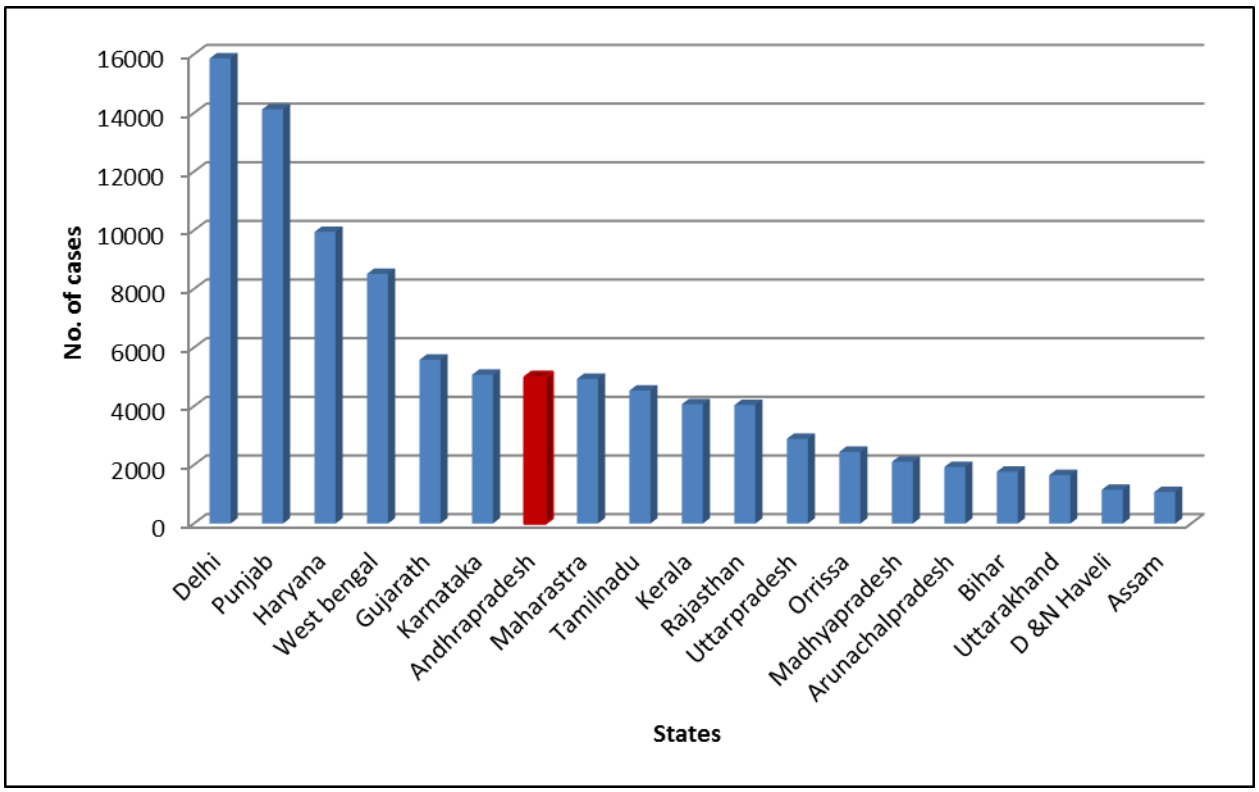


Figure 2.3: Distribution of the total number of dengue positive cases reported to NVBDCP from different states and Union Territories in 2015. The total number of dengue positive cases in Andhra Pradesh was shown in red color.

In Andhra Pradesh, 776 dengue positive cases were reported to NVBDCP in 2010 and 1, 209 cases in 2011, 2, 299 cases in 2012, 910 cases in 2013, 1966 cases in 2014 and 4,990 cases were reported in 2015 (Figure 2.4)

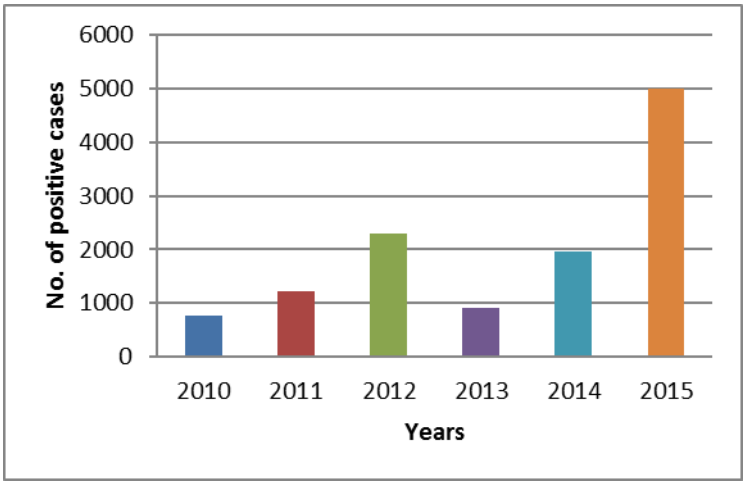


Figure 2.4: Total number of dengue positive cases reported to NVBDCP in Andhra Pradesh (2010-2015). The total number of dengue positive cases per each year were shown in colored bars.

According to NVBDCP data, nearly five thousand cases were reported from Andhra Pradesh in 2015. Distribution of cases from districts of Andhra Pradesh from 2013 to 2015 were shown in figure 2.5 and disease burden-wise Khammam occupied first place followed by Hyderabad in 2015. So Hyderabad district may be one of the hot spot regions for dengue viral infections.

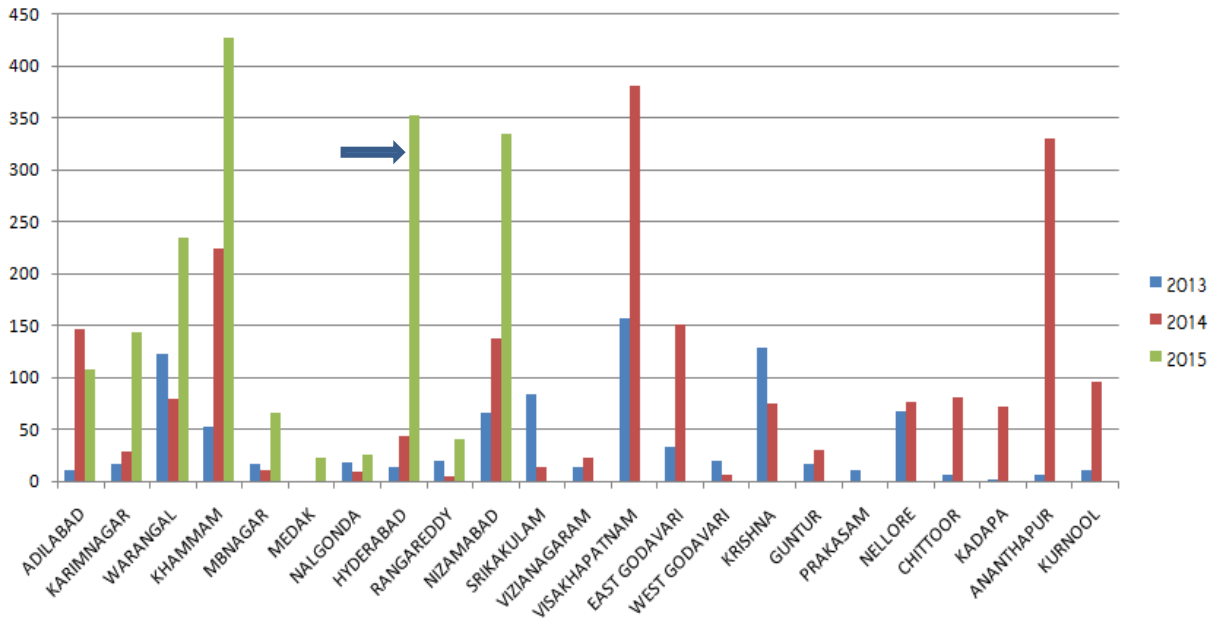


Figure 2.5: District wise distribution of dengue positive cases in Andhra Pradesh from 2013-2015. Arrow indicates Hyderabad.

Along with dengue, other viral infections such as Japanese encephalitis and chikungunya infections are also prevalent in Telangana state. So we have collected Japanese encephalitis and chikungunya cases information from NVBDCP, Hyderabad from 2011-2015. Dengue disease

burden is more when we compared with Japanese encephalitis and chikungunya cases (Figure 2.6). The total number of positive cases reported to NVBDCP were shown on X-axis and each year shown on Y–axis. Here blue line indicates dengue positive samples, red and green colored lines indicate chikungunya and Japanese encephalitis.

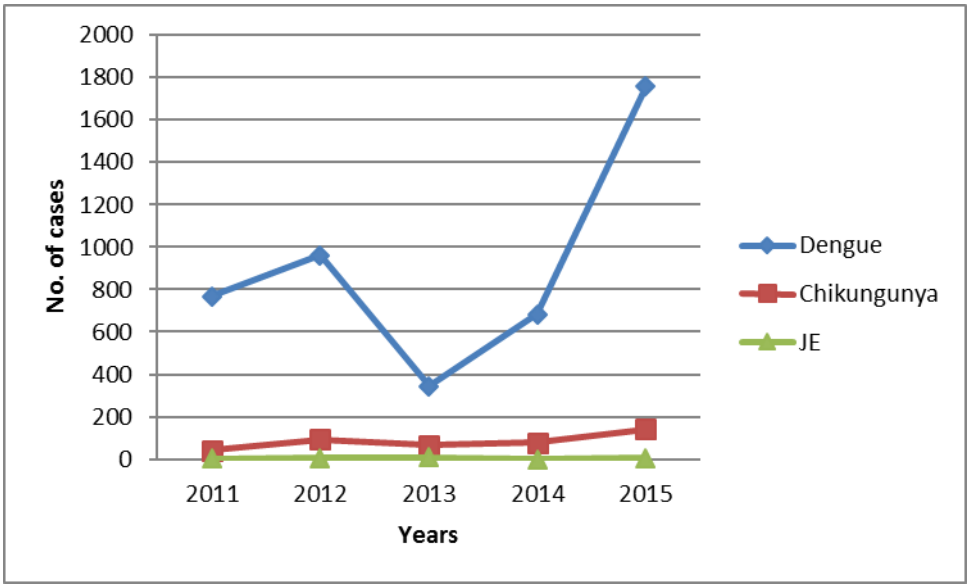


Figure 2.6: Total number of dengue, chikungunya and Japanese encephalitis cases reported to NVBDCP in Telangana state (2011-2015). Dengue cases (Blue line), chikungunya (Red line) and Japanese encephalitis (Green line).

2.3.3 Patient characteristics

A total of 199 pediatric cases were admitted with clinical symptoms of dengue infection during the period 2014-2015. 40 samples in 2014 and in 2015, 109 samples were collected. Total, 149 dengue positive samples were collected in two years. Samples were collected every week in a month for the two years. The total number of positive cases were shown on X – axis and weekly collected samples were shown on Y - axis. When we compare 2014 samples with 2015 samples, 2015 samples were increases two fold (Figure 2.7).

(A)

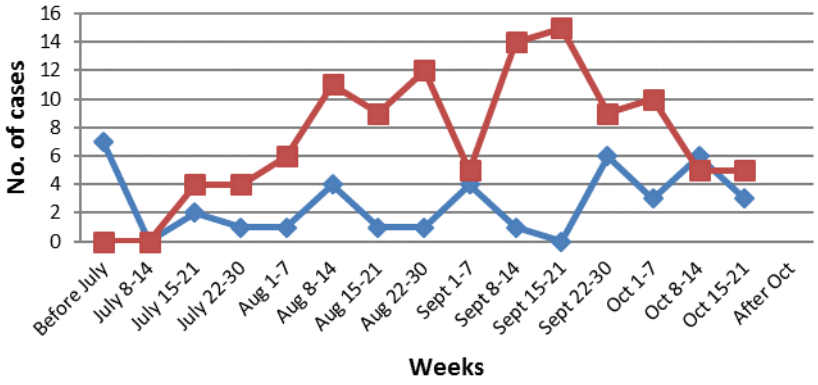


Figure 2.7: Weekly collected dengue positive samples during 2014-2015.

Among them, 100 cases were positive for NS1, 23 cases for IgG, 5 cases for IgM, 5 cases (Three tests), and 7 cases for both NS1 and IgM tests (Figure 2.8). The total number of dengue positive cases were shown on X –axis and different laboratory tests for dengue were shown on Y –axis. 50 cases negative for dengue (other febrile illness; OFI). Here other febrile illness cases were treated as controls. Among 149 cases, 73 had dengue fever, 63 had dengue hemorrhagic fever and 13 had dengue shock syndrome (Figure 2.9). All the children were below 15 years of age.

(A)

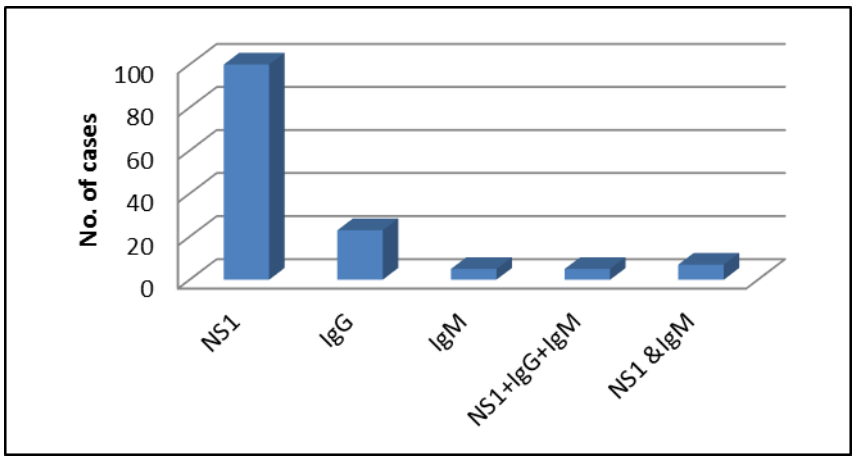


Figure 2.8: Dengue positive cases based on the laboratory diagnosis

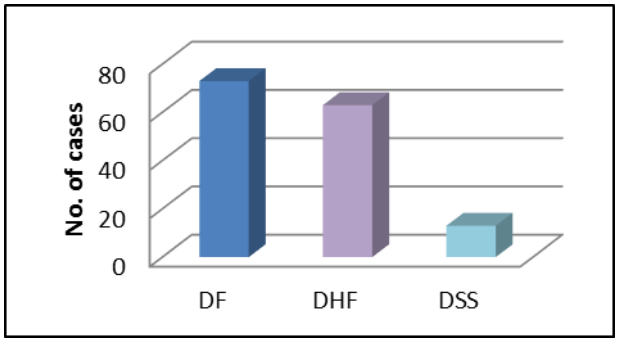


Figure 2.9: Based on the disease severity, total number of dengue positive cases. DF: Dengue fever; DHF: Dengue hemorrhagic fever; DSS: Dengue shock syndrome.

Along with laboratory diagnostic tests, clinical laboratory features such as platelet count, SGOT, SGPT, APTT, and PT were also recorded (Figure 2.10). The normal range of platelet count for control samples was between 1,40,000 – 4,00,000/ cumm and below 1,00,000 / cumm indicate thrombocytopenia. Here all the control samples were within the normal range and three subtypes of dengue fever were below 1,00,000 / cumm. Liver enzymes such as SGOT, SGPT were 15 – 40 U/L, 5 – 45 U/L (Figure 2.7).These two enzyme levels were also above the normal range in dengue fever samples. APTT and PT tests are related to blood clotting and these are also abnormal in dengue patients.

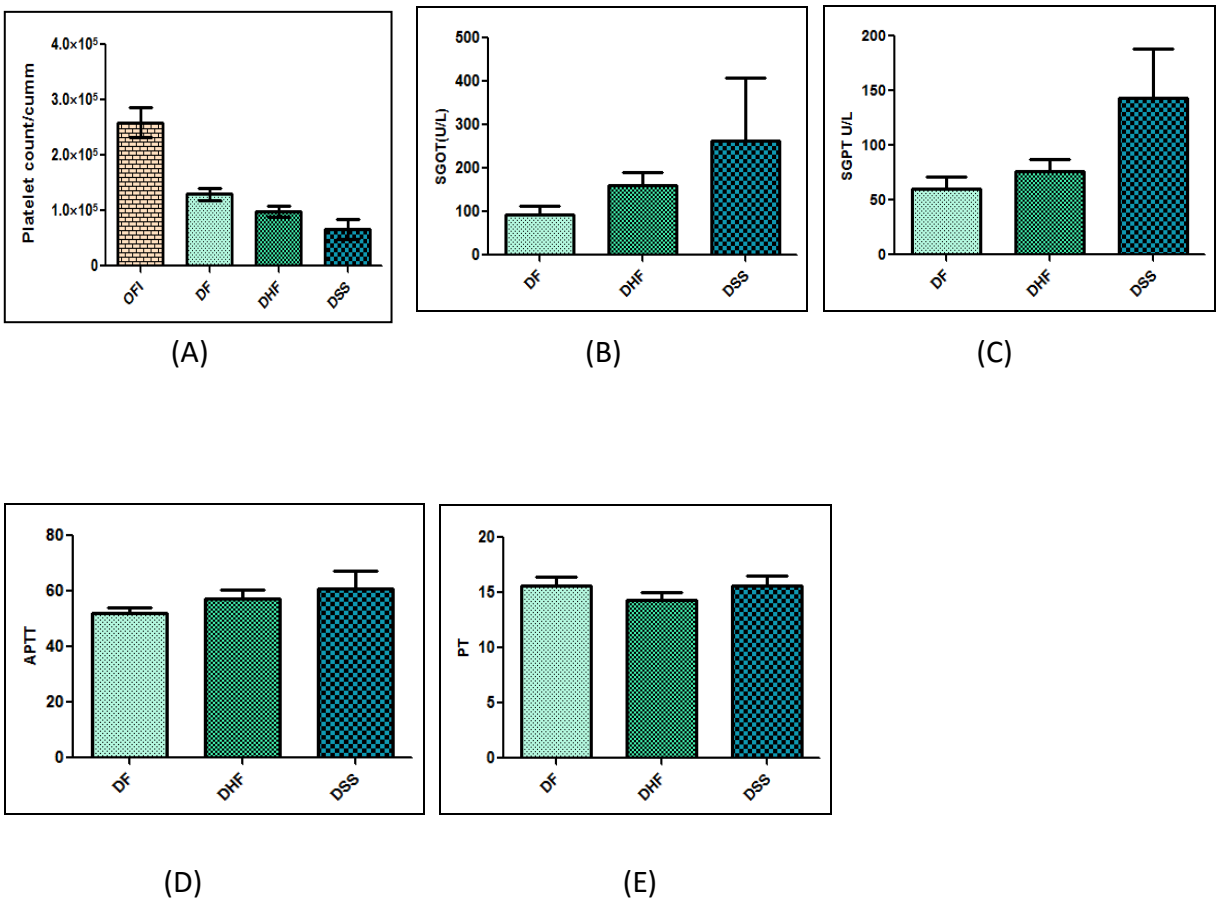
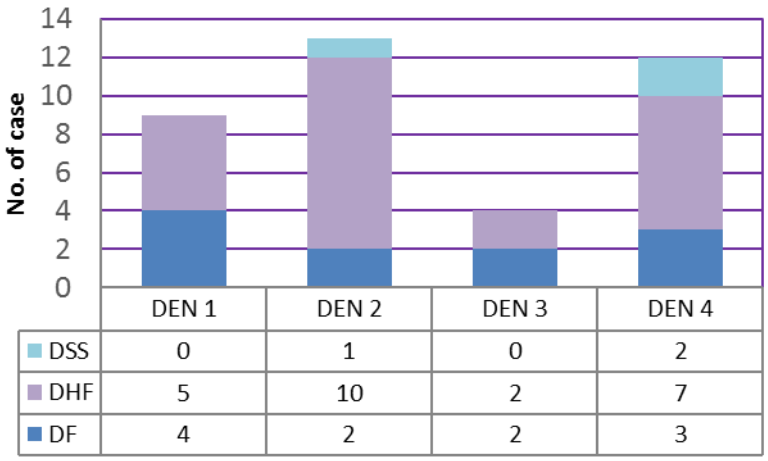


Figure 2.10: Clinical laboratory features of dengue patients. (A) Platelet count, (B) SGOT, (C) SGPT, (D) APTT and (E) PT.

2.3.4 Serotyping

In the present report, serotyping of dengue strains in clinical samples which were collected from Hyderabad, India was also carried out by our laboratory members. All four dengue serotypes and co-infections were found to be circulated in Hyderabad in 2014 (Figure 2.11).



	DEN 1	DEN 2	DEN 3	DEN 4
DSS	0	1	0	2
DHF	5	10	2	7
DF	4	2	2	3

Figure 2.11: Serotyping analysis of dengue fever samples.

2.4 Discussion

Dengue is one of the major re-emerging viral infections. The global incidence of dengue has shown a 30 fold increase over the past 50 years and the disease has become one of the most rapidly spreading mosquito-borne viral diseases (90). In India, cyclical epidemics are becoming more frequent. All the patients in the present study were children of <15 years age. This study is quite in accordance to the studies reported from Southeast Asian and Latin American countries (91-95).

Reports are indicating that this disease is changing from epidemic to endemic in India. Frequent out breaks of dengue virus infection in India are reported time to time. In South India particularly in Telangana and Andhra Pradesh, this disease severity is increasing every year and the reasons for severe form of the disease are not yet clear and complete molecular characterization of dengue virus serotypes at nucleotide level is lacking. In addition to that, there is no regular follow up of the diseases dynamics during the recent years. Although regular reports are available about the studies of dengue virus serotypes from New Delhi and its surroundings, very few reports are representing from Southern parts (Andhra Pradesh, Tamil Nadu, Karnataka and Kerala) of India. Determination of dengue virus serotypes of current and previous epidemics helps in predicting the severity of the current epidemics and switching of serotypes which help in suggesting the proper precautionary/control measures.

In the present study, the data showed that 2,27,000 cases were recorded as per the Pro-MED mail data information where as the number of cases were 1 lakh as per NVBDCP. It is interesting

to note that Pro-MED is unofficial data provider and the NVBDCP is an official data provider and indicated a huge difference between them. Many patients in the study were affected by severe dengue (DHF/DSS) which indicates the increased disease severity in this geographical location. Out of 149 samples, more than 100 samples were positive for NS1 antigen detection, which indicates the high percent of primary infections.

Alteration in the levels of serum transaminases may leads to the damage of the liver parenchyma. Studies reported a higher percentage of dengue cases with abnormal transaminases (96). Most of the patients in our study had abnormal SGOT levels recorded.These biomarkers have been proposed as indicators of severity in dengue patients. In the present report, we have done detection and serotyping of dengue strains in clinical samples which were collected from Hyderabad, India. The data suggested the circulation of all four dengue serotypes along with co-infections with more than one serotype.

CHAPTER 3

Identification of differentially regulated proteins in dengue virus infected Samples

3.1 Introduction

Since no protective vaccine or specific treatments are available for dengue fever, accurate diagnosis is critical for the early initiation of specific preventive health measures to curtail epidemic spread and reduce economic loss as well as the timely monitoring of patients to prevent fatalities. At the current time, dengue treatment is only supportive, including analgesics and antipyretics administration and careful fluid management (97, 98). Unfortunately, clinical findings alone are often not very helpful in distinguishing dengue from other febrile illnesses (OFI) such as malaria and yellow fever (infections often endemic in the same regions as DV), making diagnosis difficult (98-100). It is also known that generally viral and host factors contribute to the pathogenesis and progression of DHF/DSS (101, 102).

As per the existing literature, it is known that during dengue infection the expression of several plasma proteins will be altered which may be related to the pathogenesis of the disease, but the detailed profiles associated with dengue fever and prognosis are not well-established. Two-dimension gel electrophoresis (2-DE) offers a powerful approach to identify disease-associated proteins that can be used as biomarkers for diagnosis and which also can be considered in identifying drug targets or vaccine design. The technique has the ability to display, quantify and identify thousands of proteins in a single gel and subsequently can be used to detect differences in cell states between the healthy and the infected or diseased (103). This method was introduced by O'Farrell (104).

In this direction, Thayan et al., in 2009 identified two up-regulated proteins (α1-antitrypsin and NS1) in the serum of dengue fever patients compared with healthy individual samples (105). Some differentially-expressed proteins such as apolipoprotein A-IV, complement C3, clusterin, and α1-antichymotrypsin were found to be consistent with an earlier plasma proteomic study performed on severe DF patients from an endemic region of Brazil (106). Ray et al., 2012 reported that hemopexin, haptoglobin, serum amyloid P, and kininogen precursor, are altered in DF (107).

Many studies using 2D GE have also focused on finding biomarkers for disease conditions such as leptospirosis (108), *falciparum* and *vivax* malaria (109), severe acute respiratory syndrome (110), and swine fever virus infection (111).

The present study aims to identify the differentially regulated proteins during dengue fever, dengue hemorrhagic fever and dengue shock syndrome comparing with control plasma samples by 2 Dimensional electrophoresis.

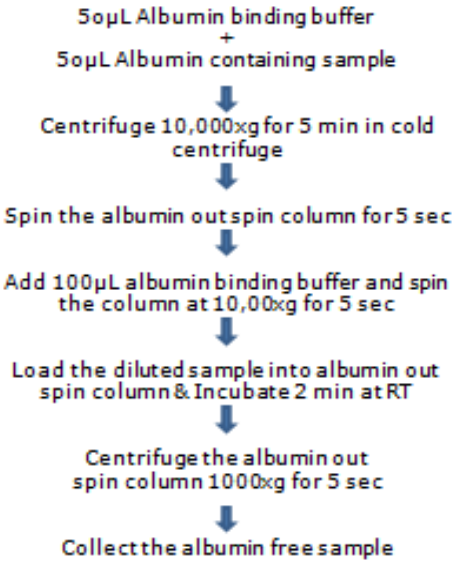
3.2 Materials and methods

3.2.1 Reagents

3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphate (CHAPS), IPG strips (7 cm, pH 3-10 and 4-7 NL), dithiothreitol (DTT), iodoacetamide, biolyte and mineral oil were procured from Biorad and acrylamide, N,N′ methylene bisacrylamide, ammonium persulphate (APS), N,N,N′,N′- tetramethylene diamine (TEMED), bromophenol blue, bovine serum albumin (BSA), ammonium bicarbonate, sodium dodecyl sulphate (SDS), urea, thiourea, glycerol, acetic acid, methanol and other chemicals required for study were of analytical grade and procured locally from SISCO Research laboratories, India.

3.2.2 High abundant plasma protein depletion

Albumin out kit (G-Biosciences) was used to remove high abundant protein from plasma samples. 50 µL of albumin-containing plasma samples were diluted in 50 µL Albumin binding buffer. High abundant protein was depleted according to manufacturer instructions. The non-specifically bound proteins were removed by wash and the protein bound to the column was eluted as albumin free fraction. The protocol used for Albumin out was shown in below flowchart.



3.2.3 Quantification of total proteins by Bradford's method

Total protein concentration of dengue virus infected samples was quantified using Bradford method by using (1mg/mL) BSA standard (112). The standard graph of BSA was shown in figure 3.1. Briefly, 5µL of the sample was diluted with 25 µL of PBS and added to 960 µL of reagent and incubated at room temperature for 10 minutes. All samples were read at 595 nm using a spectrophotometer.

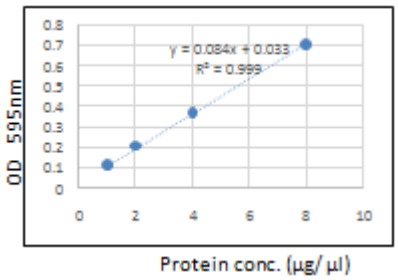


Figure 3.1: BSA standard graph

3.2.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

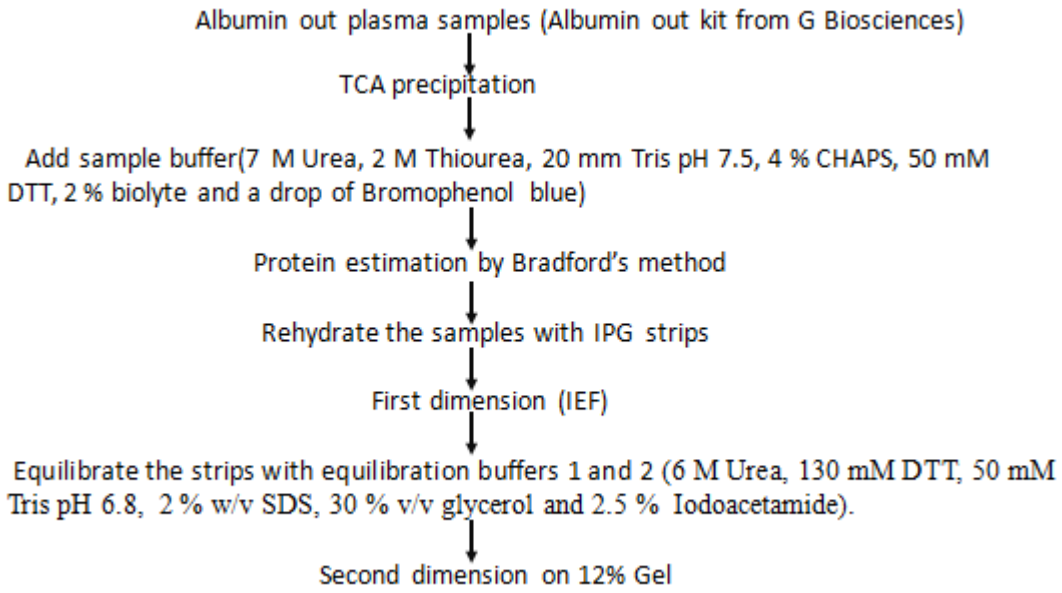
Plasma proteins were denatured by adding SDS and beta-mercaptoethanol in sample buffer and heating the samples in the water bath at 95^0 C for 5 min. Then the samples were spun briefly and loaded onto the SDS-PAGE wells containing 5% stacking and 12% resolving gels. The electrophoresis was performed at 150 volts. After the SDS-PAGE, the gels were stained with Coomassie brilliant blue and destained the gels with a destaining solution containing methanol, acetic acid, and distilled water.

3.2.5 Sample preparation for 2DE

The plasma samples stored at -20^0 C were thawed before use. Added 60 % TCA to the plasma samples and the samples were kept on ice overnight. The samples were centrifuged at 10,000 g, 4°C for 30 min. After centrifugation of the samples, the supernatant was removed and added 100 µL of ice-cold acetone. Then samples were kept on ice for 15 min and again centrifuged at 10,000 g, 4°C for 30 min. The supernatant was removed from the sample and air dried the protein pellet.

3.2.6 2-Dimensional gel electrophoresis

To above obtained protein pellet, 2d sample buffer (7 M Urea, 2 M Thiourea, 20 mM Tris pH 7.5, 4 % CHAPS, 50mM DTT, 2 % biolyte and a drop of Bromophenol blue) was added and vortexed. Then estimated the protein concentration by Bradford's method. This protocol was standardized by slight modification of the experiment (113). The protocol standardized for this experiment was shown in the below flow chart. The Immobilized pH gradient strips were rehydrated overnight in 130 µL of sample containing 300 µg of protein and ran the first dimension (Isoelectric focusing) using the following program (50 volt for 10:00 hours, 500 volts for 30 min, 5000 volts for 2:30 hour, 5000 -25000 Vhr and 500 volts for 20 hours). After isoelectric focusing, the strips were equilibrated with equilibration buffers 1 and 2, 15min each (6M Urea, 130mM DTT, 50mM Tris pH 6.8, 2 % w/v SDS, 30 % v/v glycerol and 2.5 % Iodoacetamide). In the second dimension, the strips were kept parallel on the top of the 12 % gel and sealed the strip with 0.5 % melted agarose. After the second dimension, the strips were stained with Coomassie brilliant blue and destained the gels.



3.2.7 Preparation of the protein samples for proteomic analysis

In this proteomic analysis, the Coomassie brilliant blue stained two-dimensional gels were destained with a destaining solution. The desired spots were excised manually from the gels and

stored the gel spots in 1.5 ml eppendorf containing 1 ml of 10 % acetic acid and matrix-assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF MS) analysis was conducted with MALDI-TOF/TOF mass spectrometer (Bruker Auto-flex III smart beam, Bruker Daltonics, Bremen, Germany) according to the method described briefly with slight modifications (114). After the completion of Coomassie staining, the gel was washed with Milli-Q water for 10 min. The band was excised with a scalpel, cut into small pieces and kept in microfuge tube. The gel pieces were destained with 100 µL of 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate for five times. Thereafter, the gel pieces were treated with 10 mM DTT in 25 mM ammonium bicarbonate and incubated at 56^0C for 1 h. This is followed by treatment with 55 mM iodoacetamide in 25 mM NH_4HCO_3 for 45 min at room temperature , washed with 25 mM NH_4HCO_3 and ACN, dried in a speed vac .The dried gel pieces were rehydrated and digested with of trypsin (Promega, Wisconsin, USA)(12.5 ng μL^{-1} in ammonium bicarobonate buffer) and incubated at 37^0C for overnight. Resulting tryptic digested peptides were collected in a clean microfuge tube. The peptides were extracted from gel pieces by using acetonitrile-1%TFA and pooled with previously extracted peptides. The extracted peptide were concentrated (5-10 µL) suitable for MALIDI analysis

3.2.8 Protein identification: peptide mass fingerprinting and MS/MS analysis

For MALIDI-TOF analysis peptides were spotted on the sample plate. Cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% ACN and 1% TFA (1:1) was applied to each spot, and the spots were air dried at room temperature prior to acquiring mass spectra. For MALIDI peptide mapping Mascot (www.matrixscience.com) search engine was employed.According to the MASCOT probability analysis ($P < 0.05$), only significant hits were accepted for protein identification.

3.3 Results

Further analysis of albumin removed plasma samples by SDS-PAGE shown that some of the protein bands were differentially regulated in infected samples when compared to control samples and differentially expressed protein bands were shown by black arrows (Figure 3.2).

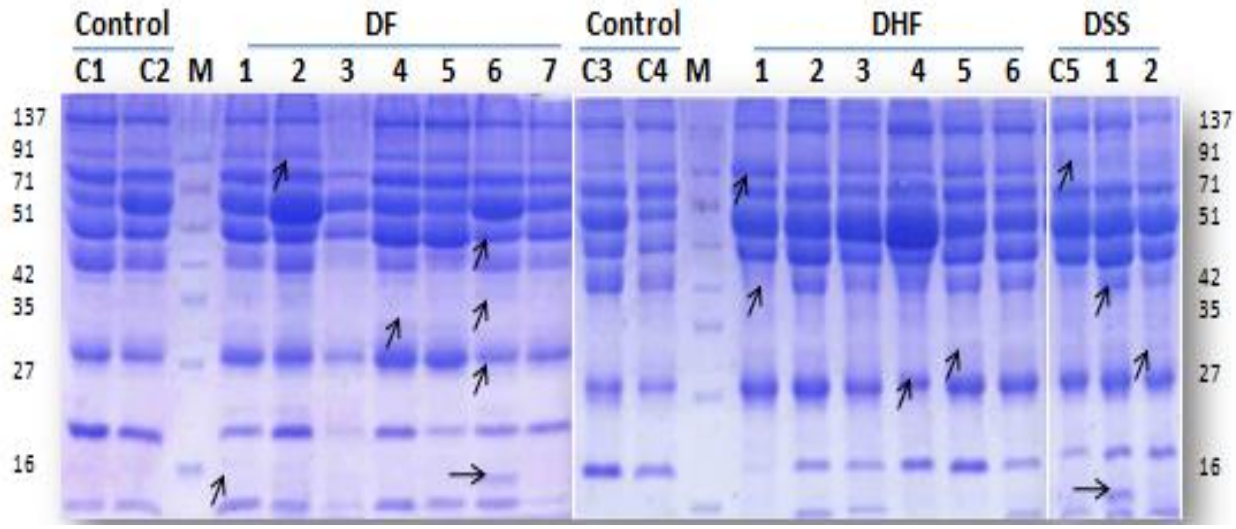


Figure 3.2: SDS-PAGE gels of dengue virus infected and control plasma samples. M: molecular weight marker; Controls: Lanes C1-C5, DF samples: Lanes DF 1-7, DHF samples: Lanes DHF 1-6 and DSS samples: Lanes DSS 1-2. DF: dengue fever; DHF: dengue hemorrhagic fever; DSS: dengue shock syndrome.

3.3.1 2D gel analysis:

To identify biomarkers for dengue fever, 2-Dimensional gel electrophoresis was performed using 15 infected and 5 control samples employing two different IPG strips (pH 4-7 and pH 3-10). Age of control patients was 3.2 ± 2.48 and infected patients was 6.87 ± 3.73. Clinical details of the samples used in 2-DE (Table 3.1). Among 15 infected patients, 7 are male and 8 are female and out of 5 control patients 2 are male and 3 are female. Platelet count of control patients was 2, 98, 600 ± 1, 16, 896 and infected samples was 67, 333 ± 40, 270. Out of 15 samples, 7 had dengue fever, 6 had dengue hemorrhagic fever and 2 had dengue shock syndrome. The samples used for 2 Dimensional gel electrophoresis experiment was shown in the below flow chart.

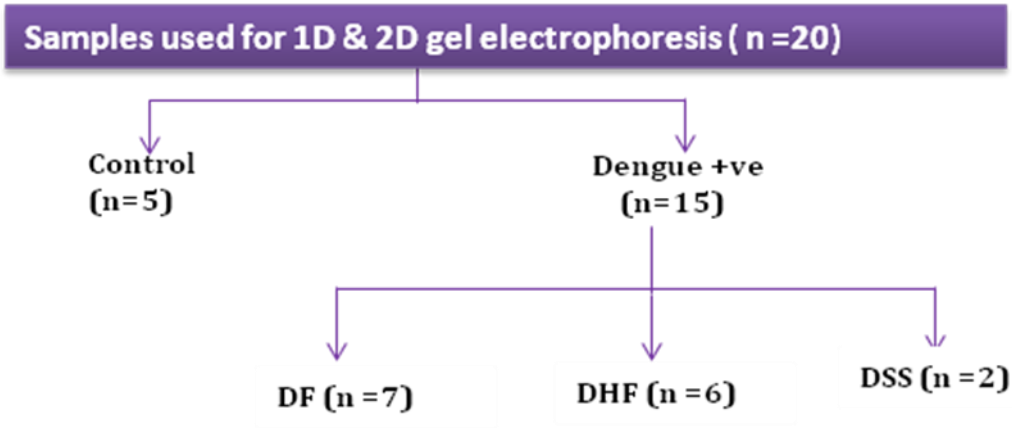


Table 3.1: Clinical and demographic features of samples used in 2-DE.

Parameter	Control (n=5)	Infected (n=15)
Age (years)	3.2 ± 2.48	6.87 ± 3.73
Sex	2	3
Male		7
Female		8
Temp	100.9 ± 1.5	101.9 ± 1.4
Diagnosis		-
Positive	5	15
Negative		-
Hematological parameters		
Platelet count/cumm	298600 ± 116896	67333 ± 40270
RBC count (mill/cumm)	4.0 ± 0.59	4.2 ± 0.59
Total WBC (cell/cumm)	9500 ± 5653	7687 ± 7393
PCV	33.64 ± 6.85	36.5 ± 4.13
Headache	1	1
Vomiting	1	4
Abdominal pain	-	4

2D gels were run as explained above. In the pH range 4-7, several protein spots were differentially expressed in dengue infected samples when compared to the controls. Among these spots, 6 protein spots which were reproduced in many gels are given for MALDI-TOF analysis and identified these protein spots as Human transthyretin, calpain-7, ZNF224, CGI-105, Keratin type1 cytoskeletal1 and Keratin type 2cytoskeletal9. All 6 spots were significantly up-regulated in infected samples and were labeled (Figure 3.3). Proteins identified by MALDI-TOF analysis in pH range 4-7 and their functions were shown (Table 3.2). Mascot score histogram of all six protein spots was significant ($p < 0.05$).

3.3.2 2-D gel electrophoresis of plasma samples (pH range 4 – 7)

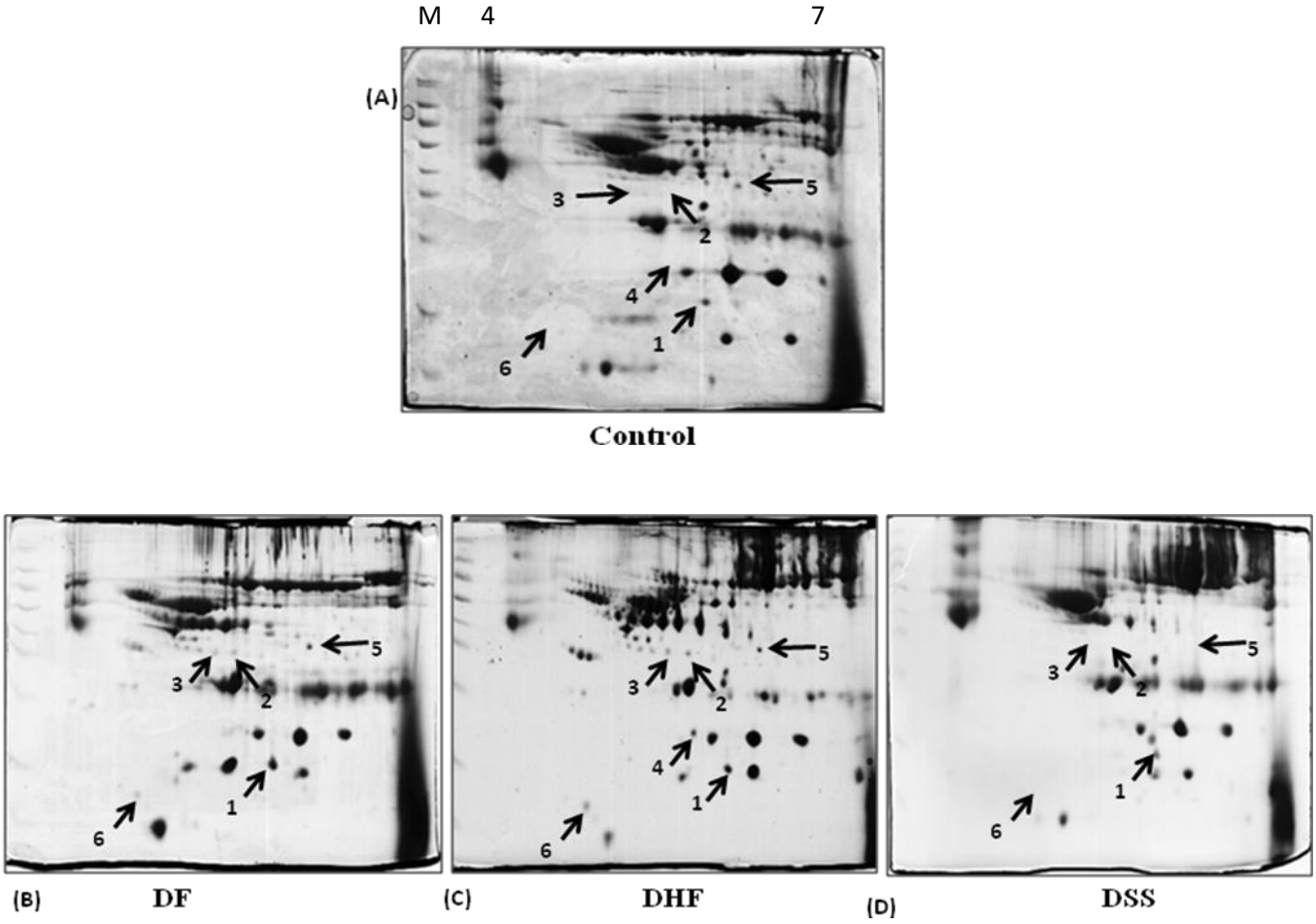


Figure 3.3: Representative 2-dimensional gel electrophoresis gels of control and infected plasma samples. (A) Control sample, (B) DF, (C) DHF and (D) DSS samples. Differentially expressed protein spots were shown by numbers with black arrows.

Table 3.2: List of differentially expressed proteins identified by 2DE (pH range 4-7) in dengue infected samples and their functions.

Spot	Proteins Identified	Peptide seq. matched	Observed Mw	Accessions numbers	Sequence coverage	Score	Functions
1	Human transthyretin	8	12869	gi377656323	84	90	Transport protein
2	Calpain-7	10	84690	gi767922834	20	72	cysteine protease
3	Zinc finger protein224	15	84906	gi313104253	28	66	Transcription repressor
4	CGI-105 protein	1	34902	gi4929679	3	22	Fumaryl acetoacetate (FAA) hydrolase family
5	Keratin, type I cytoskeletal 9	10	62255	gi239938886	27	59	Cytoskeletal protein
6	Keratin, type II cytoskeletal 1	6	66170	gi238054406	18	60	Cytoskeletal protein

Human transthyretin was found in four controls and nine infected samples. In the same way Calpain-7 was found in two control and nine infected samples, ZNF224 was found in two controls and nine infected samples, CGI-105 was found only in one infected sample, Keratin type1 cytoskeletal9 was found in two controls and eight infected samples and Keratin type2 cytoskeletal1 was found in one control and seven infected samples. Enlarged view, 3D view and the number of gels present for all six spots were shown (Figure 3.4).

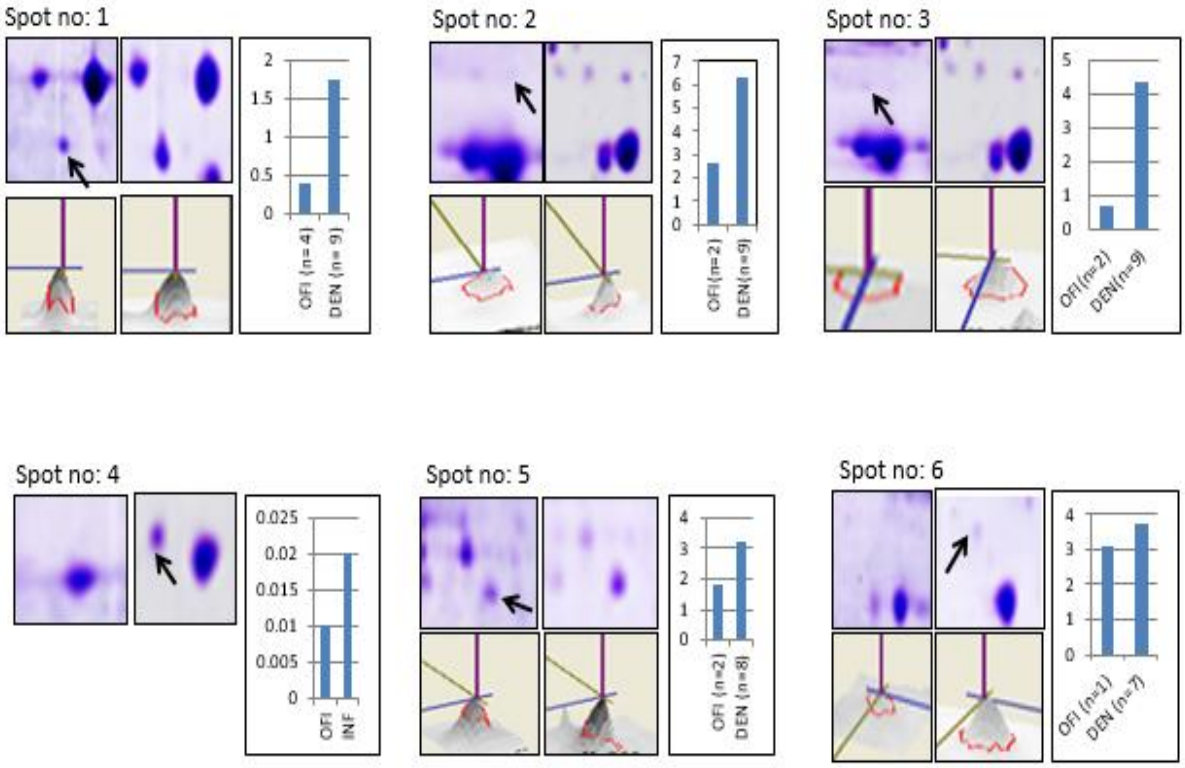
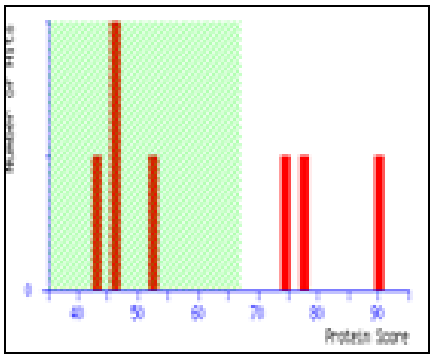
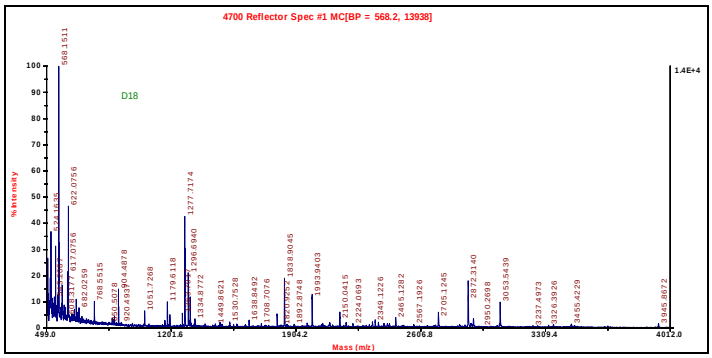


Figure 3.4: Enlarged view, 3D view and the number of gels present in each spot.
Spot no.1 (Human transthyretin); Spot no.2 (Calpain-7); Spot no.3 (ZNF224); Spot no.4 (CGI-105); Spot no.5 (Keratin type1 cytoskeletal9) and Spot no.6 (Keratin type2 cytoskeletal1).

Mascot score histogram and sequence of all the six proteins identified in the dengue-infected samples compared to control samples (pH range 4-7) were shown (Figure 3.5 A-F).



(A)

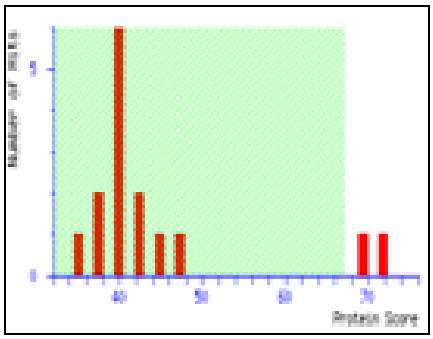


(B)

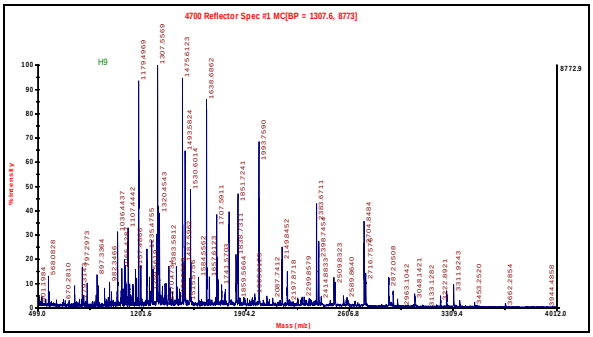
1	MLMVKVLDAV	RGSPAINVAV	HVFRKAADDT	WEPFASGKTS	ESGELHGLTT
51	EEEFVEGIYK	VEIDTKSYWK	ALGISPFHEH	AEVVFTANDS	GPRRYTIAAL
101	LSPYSYSTTA	VVTNPKE			

(C)

Figure 3.5A: Mascot score histogram, spectrum and sequence of Transthyretin. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of Transthyretin.



(A)

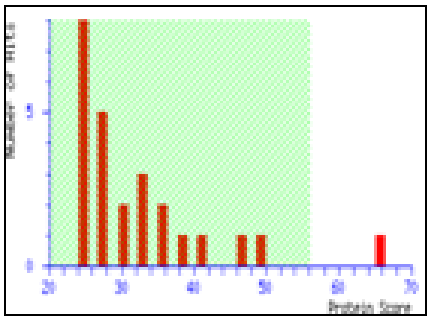


(B)

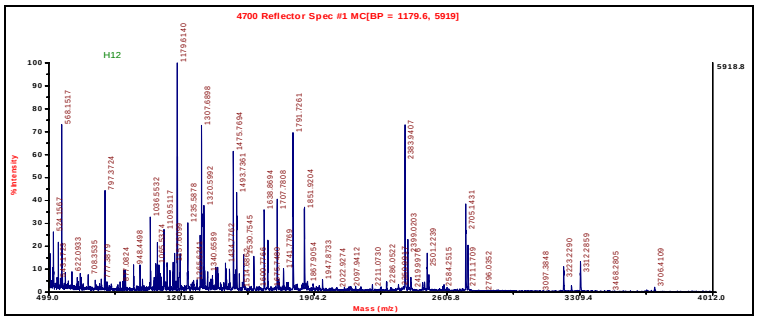
1	MTTFKEAMTF	KDVAVVFTEE	ELGLLDLAQR	KLYRDVMLEN	FRNLLSVGHQ
51	AFHRDTFHFL	REEKIWMMKT	AIQREGNSGD	KIQTEMETVS	EAGTHQEWSF
101	QQIWEKIASD	LTRSQDLMIN	SSQFSKEGDF	PCQTEAGLSV	IHTRQKSSQG
151	NGYKPSFSDV	SHFDFHQQLH	SGEKSHTCDE	CGKNFCYISA	LRIHQRVHMG
201	EKCYKCDVCG	KEFSQSSHLQ	THQRVHTGEK	PFKCVECGKG	FSRRSALNVH
251	HKLHTGEKPY	NCEECGKAFI	HDSQLQEHQR	IHTGEKPFKC	DICGKSFCGR
301	SRLNRHSMVH	TAEKPFRCDT	CDKSFRQRSA	LNSHRMIHTG	EKPYKCEECG
351	KGFICRRDLY	THHMVHTGEK	PYNCKECGKS	FRWASCLLKH	QRVHSGEKPF
401	KCEECGKGFY	TNSQCYSHQR	SHSGEKPYKC	VECGKGYKRR	LDLDFHQRVH
451	TGEKLYNCKE	CGKSFSRAPC	LLKHERLHSG	EKPFQCEECG	KRFTQNSHLH
501	SHQRVHTGEK	PYKCEKCGKG	YNSKFNLDMH	QKVHTGERPY	NCKECGKSFG
551	WASCLLKHQR	LHSGEKPFKC	EECGKRFTQN	SQLHSHQRVH	TGEKPYKCDE
601	CGKGFSWSST	RLTHQRRHSR	ETPLKCEQHG	KNIVQNSFSK	VQEKVHSVEK
651	PYKCEDCGKG	YNRRLNLDMH	QRVHMGEKTW	KCRECDMCFS	QASSLRLHQN

(C)

Figure 3.5B: Mascot score histogram, spectrum and sequence of Calpain-7. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of Calpain-7.



(A)

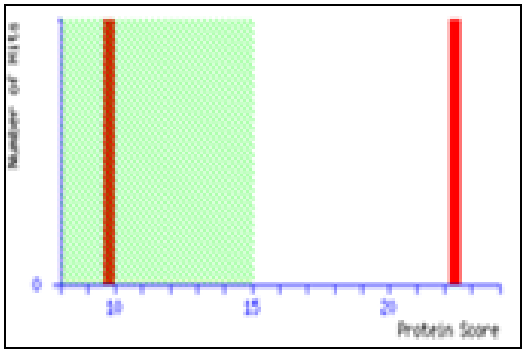


(B)

1	MDATALERDA	VQFARLAVQR	DHEGRYSEAV	FYYKEAAQAL	IYAEMAGSSL
51	ENIQEKITEY	LERVQALHSA	VQSKSADPLK	SKHQLDLERA	HFLVTQAFDE
101	DEKENVEDAI	ELYTEAVDLC	LKTSYETADK	VLQNKLKQLA	RQALDRAEAL
151	SEPLTKPVGK	ISSTSVKPKP	PPVRAHFPLG	ANPFLERPQS	FISPQSCDAQ
201	GQRYTAEEIE	VLRTTSKING	IEYVPFMNVD	LRERFAYPMP	FCDRWGKLPL
251	SPKQKTTFSK	WVRPEDLTNN	PTMIYTVSSF	SIKQTIVSDC	SFVASLAISA
301	AYERRFNKKL	ITGIIYPQNK	DGEPEYNPCG	KYMVKLHLNG	VPRKNTYKLN
351	ISNLKIQNTP	VIIDDQLPVD	HKGELLCSYS	NNKSELWVSL	IEKAYMKVMG
401	GYDFPGSNSN	IDLHALTGWI	PERIAMHSDS	QTFSKDNSFR	MLYQRFHKGD
451	VLITASTGMM	TEAEGEKWGL	VPTHAYAVLD	IREFKGLRFI	QLKNPWSHLR
501	WKGRYSENDV	KNWTPELQKY	LNFDPRTAQK	IDNGIFWISW	DDLCQYYDVI

(C)

Figure 3.5C: Mascot score histogram and sequence of Zink finger protein 224. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of Zink finger protein 224.

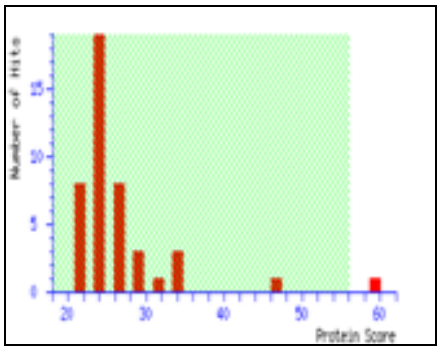


(A)

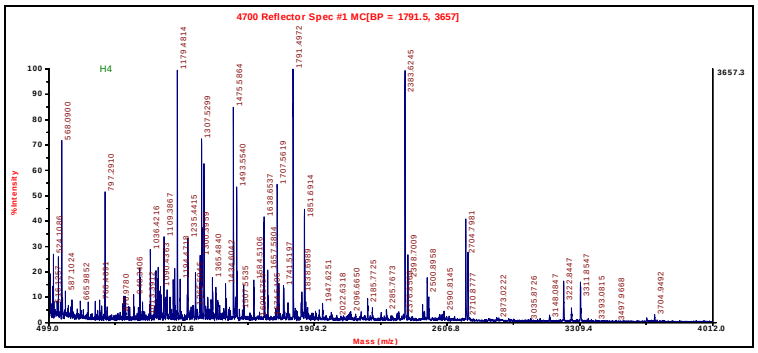
1	MLVSGRRRLL	TVLLQAQKWP	FQPSRDMRLV	QFRAPHLVGP	HLGLETGNGG
51	GVINLNAFDP	TLPKTMTQFL	EQGEATLSVA	RRALAAQLPV	LPRSEVTFLA
101	PVTRPDKVVC	VGMNYVDHCK	EQNVPVPKEP	IIPSKPASSI	VGPYDEVVLP
151	PQSQEVDWEV	ELAVVIGKKG	KHIKATDAMA	HVGGFTVAHD	VSARDWQMRR
201	NGKQWLLGKT	FDTFCPLGPA	LVTKDSVADP	HNLKICCRVN	GEVVQSGNTN
251	QMVFKTEDLI	AWVSQFVTFY	PGDVILTGTP	PGVGVFRKPP	VFLKKGDEVQ
301	CEIEELGVII	NKVV			

(B)

Figure 3.5D: Mascot score histogram and sequence of CGI-105. (A) Mascot score histogram and (B) Peptide matches of CGI-105.



(A)

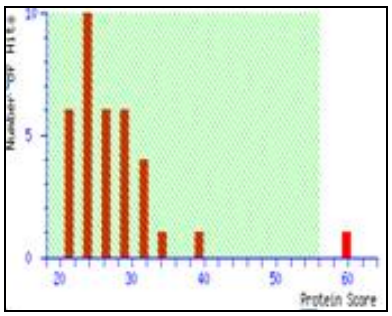


(B)

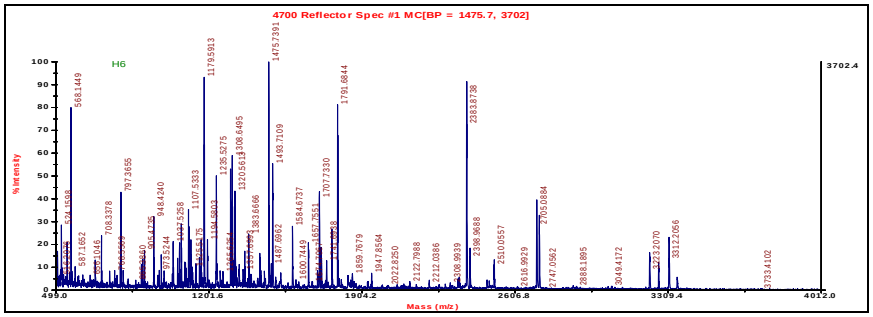
1	**MSCR**QFSSSY	LSRSGGGGGG	GLGSGGSIRS	SYSRFSSSGG	GGGGGR**FSSS**
51	**SGYGGGSSRV**	**CGRGGGGSFG**	**YSYGGGSGGG**	**FSASSLGGGF**	**GGGSR**GFGGA
101	SGGGYSSSGG	FGGGFGGGSG	GGFGGGYGSG	FGGFGGFGGG	AGGGDGGILT
151	ANEKSTMQEL	NSRLASYLDK	VQALEEANND	LENKIQDWYD	KKGPAAIQKN
201	YSPYYNTIDD	LKDQIVDLTV	GNNKTLLDID	NTRMTLDDFR	**IKFEMEQNLR**
251	QGVDADINGL	RQVLDNLTME	KSDLEMQYET	LQEELMALKK	NHKEEMSQLT
301	GQNSGDVNVE	INVAPGKDLT	K**TLNDMRQEY**	**EQLIAK**NRKD	IENQYETQIT
351	QIEHEVSSSG	QEVQSSAKEV	TQLRHGVQEL	EIELQSQLSK	KAALEKSLED
401	TKNRYCGQLQ	MIQEQISNLE	AQITDVR**QEI**	**ECQNQEYSLL**	**LSIKMR**LEKE
451	IETYHNLLEG	GQEDFESSGA	GKIGLGGR**GG**	**SGGSYGR**GSR	**GGSGGSYGGG**
501	**GSGGGYGGGS**	**GSR**GGSGGSY	GGGSGSGGGS	GGGYGGGSGG	GHSGGSGGGH
551	SGGSGGNYGG	GSGSGGGSGG	GYGGGSGSR**G**	**GSGGSHGGGS**	**GFGGESGGSY**
601	**GGGEEASGSG**	**GGYGGGSGK**S	SHS		

(C)

Figure 3.5E: Mascot score histogram, spectrum and sequence of Keratin type I cytoskeletal 9. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of Keratin type 1cytoskeletal9.



(A)



(B)

1	MSRQFSSRSG	YRSGGGFSSG	SAGIINYQRR	TTSSSTRRSG	GGGGRFSSCG
51	GGGGSFGAGG	GFGSRSLVNL	GGSKSISISV	ARGGGRGSGF	GGGYGGGGFG
101	GGGFGGGGFG	GGGIGGGGFG	GFGSGGGGFG	GGGFGGGGYG	GGYGPVCPPG
151	GIQEVTINQS	LLQPLNVEID	PEIQKVKSRE	REQIKSLNNQ	FASFIDKVRF
201	LEQQNQVLQT	KWELLQQVDT	STRTHNLEPY	FESFINNLRR	RVDQLKSDQS
251	RLDSELKNMQ	DMVEDYRNKY	EDEINKRTNA	ENEFVTIKKD	VDGAYMTKVD
301	LQAKLDNLQQ	EIDFLTALYQ	AELSQMQTQI	SETNVILSMD	NNRSLDLDSI
351	IAEVKAQYED	IAQKSKAEAE	SLYQSKYEEL	QITAGRHGDS	VRNSKIEISE
401	LNRVIQRLRS	EIDNVKKQIS	NLQQSISDAE	QRGENALKDA	KNKLNDLEDA
451	LQQAKEDLAR	LLRDYQELMN	TKLALDLEIA	TYRTLLEGEE	SRMSGECAPN
501	VSVSVSTSHT	TISGGGSRGG	GGGGYGSGGS	SYGSGGGSYG	SGGGGGGGRG
551	SYGSGGSSYG	SGGGSYGSGG	GGGGHGSYGS	GSSSGGYRGG	SGGGGGGSSG
601	GRGSGGGSSG	GSIGGRGSSS	GGVKSSGGSS	SVKFVSTTYS	GVTR

(C)

Figure 3.5F: Mascot score histogram, spectrum and sequence of Keratin type II cytoskeletal 1. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of Keratin type 2 cytoskeletal 1

3.3.3 2D gel electrophoresis of plasma samples (pH range 3 – 10)

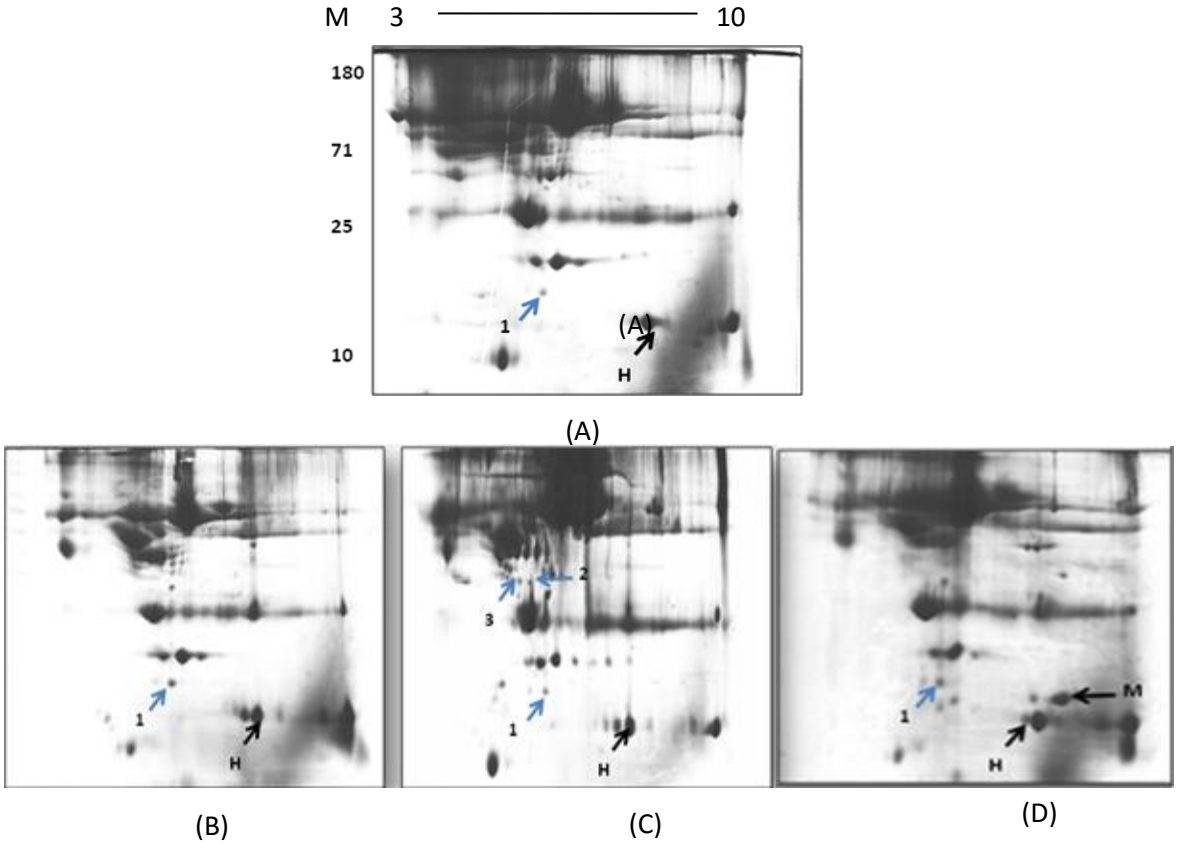


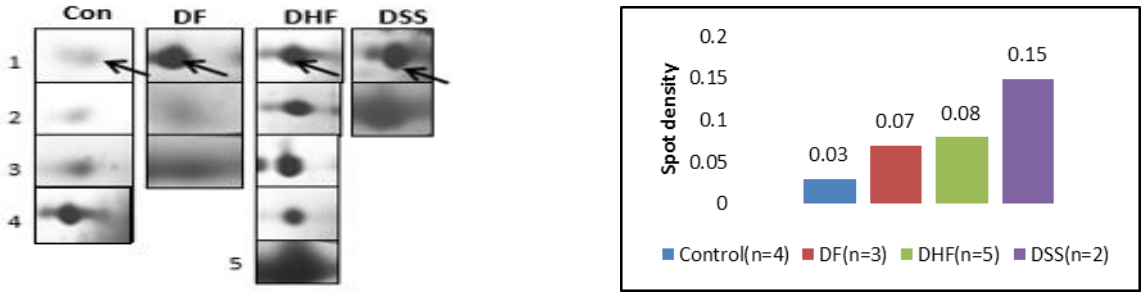
Figure 3.8: Representative 2-dimensional gel electrophoresis gels of control and infected plasma samples. (A) Control sample, (B) DF, (C) DHF and (D) DSS samples. Differentially expressed protein spots were shown by alphabets with black arrows and protein spots observed in pH range 4-7 were shown in numbers with blue arrows.

The samples used in 4 -7 pH range were repeated in 3- 10 pH range for better resolution of spots. Interestingly, along with some of the protein spots which were found in pH range 4-7, some other protein spots were also found in the pH range 3-10. Among these protein spots, only two spots were repeated in infected gels and those two spots were labeled (Figure 3.8). These spots were identified by MALDI-TOF analysis as Human hemoglobin beta and Myoglobin (Table 3.3).

Table 3.3: List of differentially expressed proteins identified by 2DE (pH range 3 – 10) in dengue infected samples and their functions.

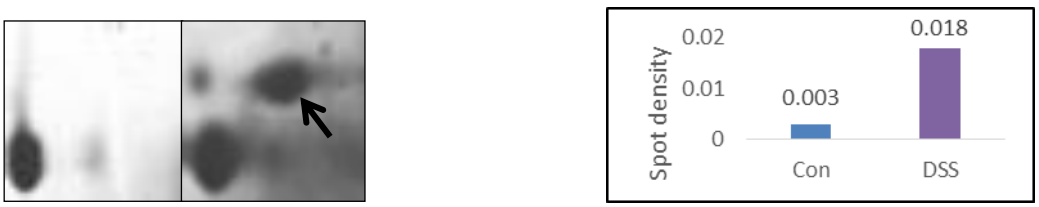
Spot	Proteins Identified	Peptide. seq. matched	Observed Mw	Accessions numbers	Sequence coverage	Score	Functions
H	Haemoglobin beta	3	15980	gi2239149	32	191	Transport oxygen
M	Myoglobin	4	17099	gi229361	28	416	Transport oxygen

Hemoglobin beta was found to be upregulated in three DF, five DHF and two DSS samples and average spot density value of that spot in three subtypes of fever was shown (Figure 3.6). Myoglobin was found only in one (DSS) sample. The average spot density values of myoglobin (Figure 3.7). These two proteins were significantly up-regulated in infected samples when compared to control samples. Hemoglobin beta and myoglobin proteins were observed with molecular weights of 15, 980 and 17, 099 and these two proteins are involved in the transport of oxygen.



(A) (B)

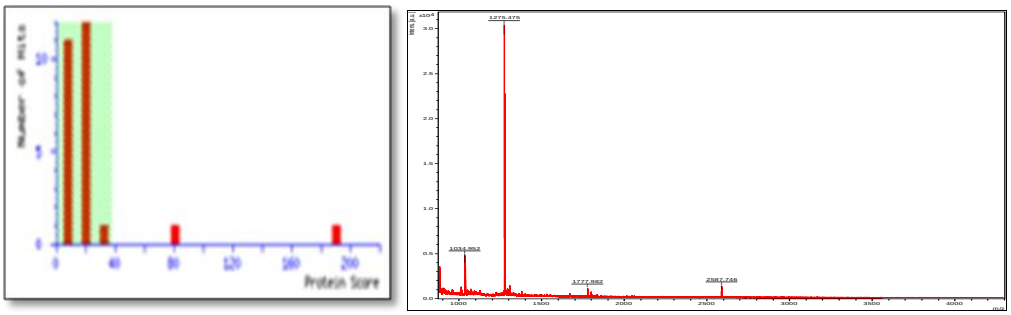
Figure 3.6: Differential expression of Hemoglobin beta spot. (A) Enlarged view and (B) Average spot density value of Hemoglobin beta.



(A) (B)

Figure 3.7: Differential expression of myoglobin spot. (A) Enlarged view and (B) average spot density value of myoglobin beta.

Mascot score histogram and sequence of Hemoglobin beta and Myoglobin (Figure 3.9).

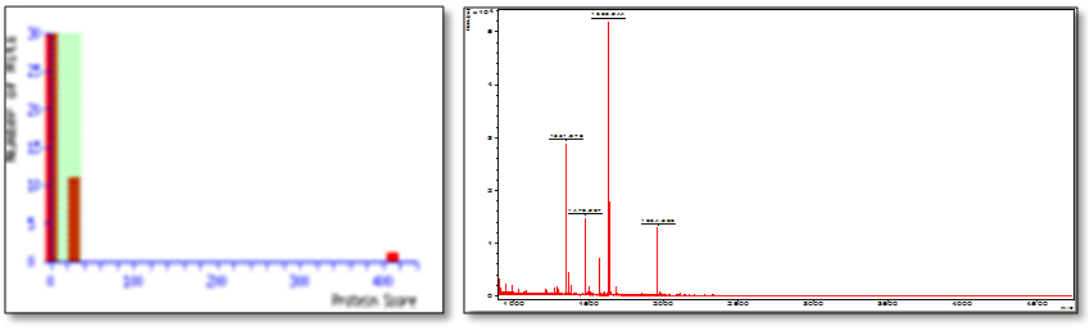


(A) (B)

```
1   VHLTPEEKSA VTALWGKVDV DEVGGEALGR LLVVYPWTER  FFESFGDLST
51  PDAVMGDPKV KAHGKKVLGA FSDGLAHLDD LKGTFATLSE  LHCDKLHVDP
101 EDFRLLGDVL  VCVLAHHFGK  EFTPPVEAAY EKVVAGVADA LAHKYH
```

(C)

Figure 3.9A: Mascot score histogram, spectrum and sequence of Hemoglobin beta. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of Hemoglobin beta.



(A) (B)

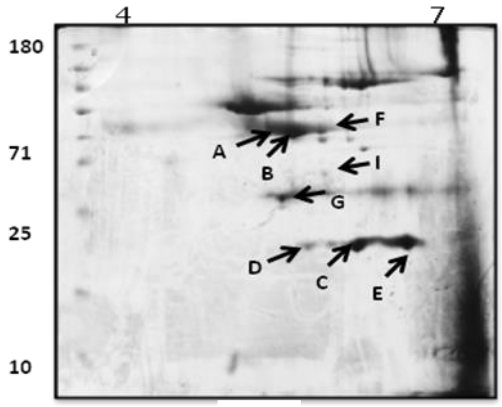
```
 1 GLSDGEWQLV LNVWGKVEAD IPGHGQEVLI RLFKGHPETL EKFDKFKHLK
51 SEDEMKASED LKKHGATVLT ALGGILKKKG HHEAEIKPLA QSHATKHKVP
101 IKYLEFISEC IIQVLQSKHP GDFGADAQGA MNKALELFRK DMASNYKELG
151 FQG
```

(C)

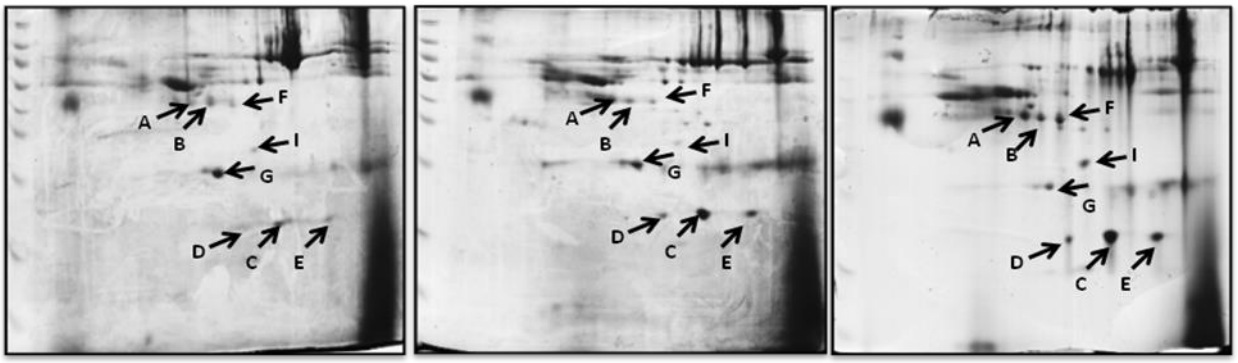
Figure 3.9B: Mascot score histogram, spectrum and sequence of Myoglobin. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of Myoglobin.

3.3.4 Proteins identified in disease progression samples

Along with those proteins identified in dengue virus infected samples, we also interested to know the protein profiles during disease progression. In this regard, we have collected disease progression samples of 15 infected (day1 to day6) based on the availability of patients in the hospital. Among these samples, one dengue hemorrhagic fever patient sample was compared with the control sample and some differentially expressed protein spots were found in day1 to day3 samples when compared to two control samples. During disease progression, some proteins were found to be differentially regulated. Among them, eight protein spots were selected and given for MALDI-TOF analysis. These eight protein spots were indicated by arrows (Figure 3.10). The enlarged view of the eight spots in control and infected samples from day1 to day3 was shown (Figure 3.11). This patient was given platelet transfusion at the day of hospital admission. The clinical details of the dengue hemorrhagic patient and control samples were shown (Table 3.4). Mascot score histogram and peptide matches of eight proteins were shown (Figure 3.12)



(A)



(B) (C) (D)

Figure 3.10: 2-dimensional gel electrophoresis gels of control and disease progression samples. Each sample containing 100 µg of protein was loaded on 2D gel (A) Control, (B) day1, (C) day-2 and (D) day-3 samples. Differentially expressed protein spots were shown by black arrows.

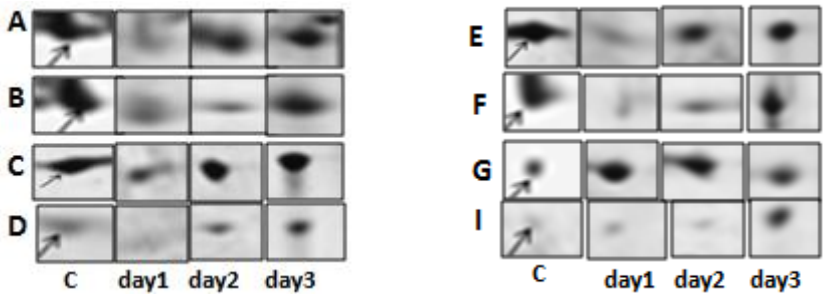


Figure 3.11: Enlarged view of expression patterns of protein spots in control and infected samples in disease progression samples (day1-day3). Spots in the first vertical lane from top to bottom in two figures (A-G and I) indicate control samples, second lane spots are of the first day, third lane spots are of the second-day and fourth lane spots are of the third day. C: Control; day1: First day; day2: Second day and day3: Third-day samples.

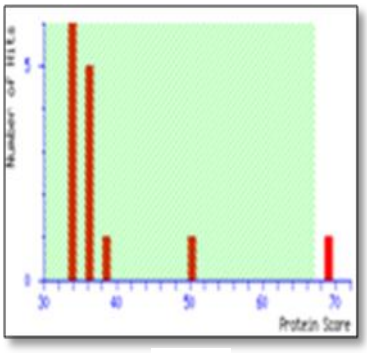
Table 3.4: Clinical characteristics of control and dengue hemorrhagic fever patients.

Parameter	Control	1st Day	2nd Day	3rd Day
Platelet count	195000/cumm	*30000/cumm	*90000/cumm	160000/cumm
RBC	3.31/cumm	*3.4/cumm	*2.9/cumm	*3.2/cumm
WBC	3100 cell/cumm	7300 cell/cumm	7300 cell/cumm	8700 cell/cumm
PCV	25.2 %	*31%	*26%	*28%
Temp	101 F	92.2 F		
SGOT (NR: 15 – 40 U/L)	42	52		
SGPT (NR: 5– 45 U/L)	26	18		
Thrombocytopenia	No	Yes	yes	No
Pleural effusion	No	yes		
Ascites	No	yes		
Fast breathing	No	yes		
vomiting	No	yes		
Distended liver	No	yes		
BP	106/69 (70mm of Hg)	80/50 (70mm of Hg)		
Tachycardia	No	yes		
APTT	-	*64.1		
PT	-	13		

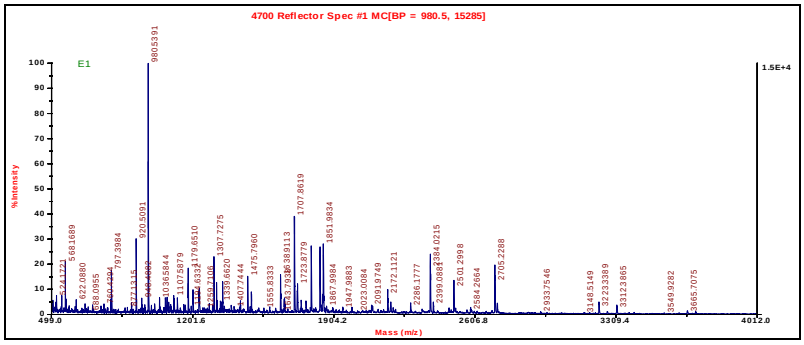
We found that some protein spots were differentially expressed in three days of infected samples. Among these spots, eight protein spots were selected for MALDI-TOF analysis and found those spots as RIP, Ankyrin repeat domain, olfactory receptor, Mitochondrial ribosomal protein L17, ATP-dependent DNA helicase, Very large A-kinase anchor protein, Potassium channel subfamily k member and Immunoglobulin heavy chain variable region (Table 3.5).

Table 3.5: List of differentially expressed proteins identified by 2DE in samples with dengue disease progression.

Spot	Proteins Identified	Peptide seq. matched	Observed Mw	Accessions numbers	Sequence coverage	Score	Functions
A	RIP	15	151527	gi554505515	13	69	Inflammation
B	Ankyrin repeat domain	9	32922	gi767909098	37	67	Anchor protein
C	Olfactory receptor 2T7	8	34948	gi206557832	58	63	Helps in virus transmission
D	Mitochondrial Ribosomal Protein L17	9	13713	gi1591638	64	69	Protein synthesis
E	ATP-dependent DNA helicase PIF1	10	70610	gi557357743	22	62	potent unwinder of G4 structures
F	Very large A kinase anchor protein	12	333107	gi727863588	5	57	Anchor protein
G	Potassium channel Subfamily K member	4	31926		45	45	Vasodilation
I	Immunoglobulin heavy chain variable region	4	9631	gi14594233	67	67	Antigen binding



(A)

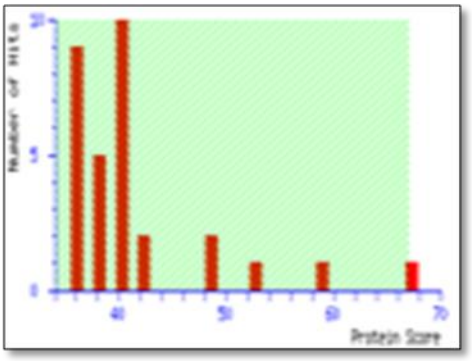


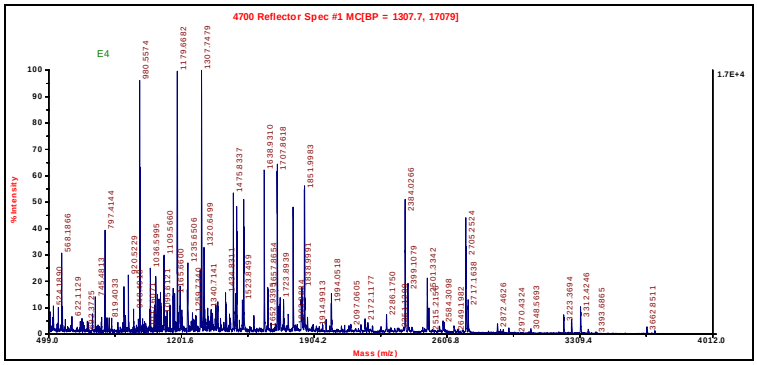
(B)

```
   1 MSAAKENPCR KFQANIFNKS KCQNCFKPRE SHLLNDEDLT QAKPIYGGWL
  51 LLAPDGTDFD NPVHRSRKWQ RRFFILYEHG LLRYALDEMP TTLPQGTINM
 101 NQCTDVVDGE GRTGQKFSLC ILTPEKEHFI RAETKEIVSG WLEMLMVYPR
 151 TNKQNQKKKR KVEPPTPQEP GPAKVAVTSS SSSSSSSSSI PSAEKVPTTK
 201 STLWQEEMRT KDQPDGSSLS PAQSPSQSQP PAASSLREPG LESKEEESAM
 251 SSDRMDCGRK VRVESGYFSL EKTKQDLKAE EQQLPPPLSP PSPSTPNHRR
 301 SQVIEKFEAL DIEKAEHMET NAVGPSPSSD TRQGRSEKRA FPRKRDFTNE
 351 APPAPLPDAS ASPLSPHRRA KSLDRRSTEP SVTPDLLNFK KGWLTKQYED
 401 GQWKKHWFVL ADQSLRYYRD SVAEEAADLD GEIDLSACYD VTEYPVQRNY
 451 GFQIHTKEGE FTLSAMTSGI RRNWIQTIMK HVHPTTAPDV TSSLPEEKNK
 501 SSCSFETCPR PTEKQEAELG EPDPEQKRSR ARERRREGRS KTFDWAEFRP
 551 IQQALAQERV GGVGPADTHE PLRPEAEPGE LERERARRRE ERRKRFGMLD
 601 ATDGPGTEDA ALRMEVDRSP GLPMSDLKTH NVHVEIEQRW HQVETTPLRE
 651 EKQVPIAPVH LSSEDGGDRL STHELTSLLE KELEQSQKEA SDLLEQNRLL
 701 QDQLRVALGR EQSAREGYVL QATCERGFAA MEETHQKKIE DLQRQHQREL
 751 EKLREEKDRL LAEETAATIS AIEAMKNAHR EEMERELEKS QRSQISSVNS
 801 DVEALRRQYL EELQSVQREL EVLSEQYSQK CLENAHLAQA LEAERQALRQ
 851 CQRENQELNA HNQELNNRLA AEITRLRTLL TGDGGGEATG SPLAQGKDAY
 901 ELEVLLRVKE SEIQYLKQEI SSLKDELQTA LRDKKYASDK YKDIYTELSI
 951 AKAKADCDIS RLKEQLKAAT EALGEKSPDS ATVSGYGPAV LAPEDGLAMS
1001 LHFMTLGGSS LSPTEGKGSG LQGHIIENPQ YFSDACVHHI KRRDIVLKWE
1051 LGEGAFGKVF LAECHNLLPE QDKMLVAVKA LKEASESARQ DFQREAELLT
1101 MLQHQHIVRF FGVCTEGRPL LMVFEYMRHG DLNRFLRSHG PDAKLLAGGE
1151 DVAPGPLGLG QLLAVASQVA AGMVYLAGLH FVHRDLATRN CLVGQGLVVK
1201 IGDFGMSRDI YSTDYYRVGG RTMLPIRWMP PESILYRKFT TESDVWSFGV
1251 VLWEIFTYGK QPWYQLSNTE AIDCITQGRE LERPRACPPE VYAIMRGCWQ
1301 REPQQRHSIK DVHARLQALA QAPPVYLDVL G
```

(C)

Figure 3.12A: Mascot score histogram, spectrum and sequence of RIP. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of RIP.



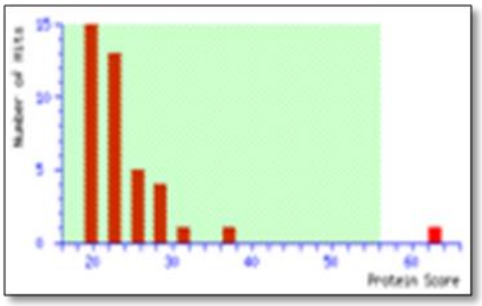


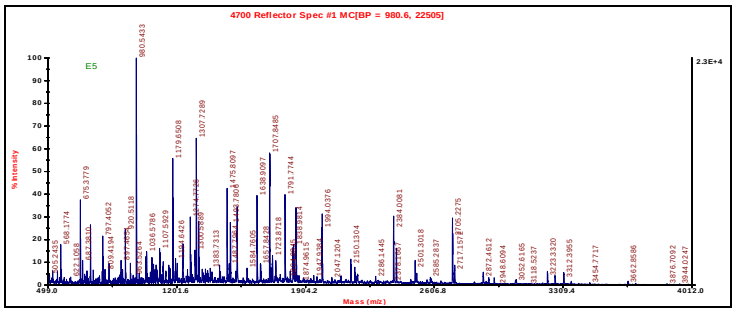
(A) (B)

```
  1 MCFCLPDFYR IWKLFVFLEL MESEGPPESE SSEFFSQQEE ENEEEEAQEP
 51 EETGPKNPLL QPALTGDVEG LQKIFEDPEN PHHEQAMQLL LEEDIVGRNL
101 LYAACMAGQS DVIRALAKYG VNLNEKTTRG YTLLHCAAAW GRLETLKALV
151 ELDVDIEALN FREERARDVA ARYSQTECVE FLDWADARLT LKKYIAKVSL
201 AVTDTEKGSG KLLKEDKNTI LSACRAKNEW LETHTEASIN ELFEQRQQLE
251 DIVTPIFTKM TTPCQVKSAK SVTSHDQKRS QDDTSN
```

(C)

Figure 3.12B: Mascot score histogram, spectrum and sequence of Ankyrin repeat domain. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of Ankyrin repeat domain.



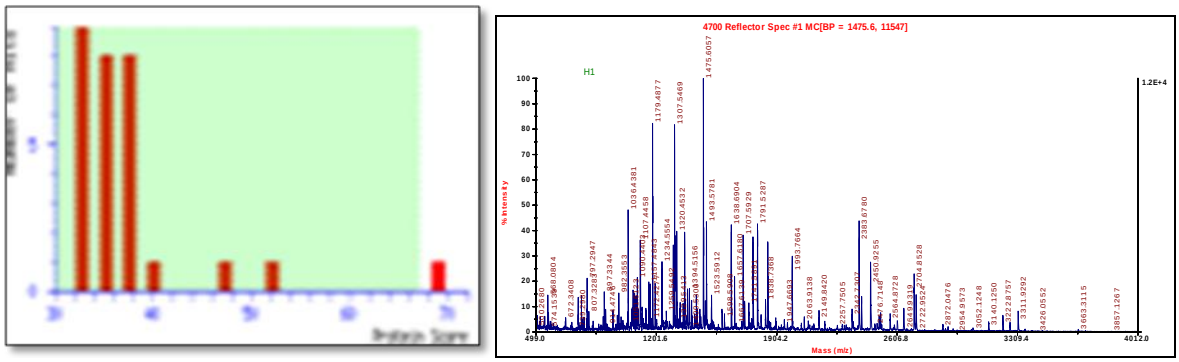


(A) (B)

```
  1 MPTLSFWVCS ATPVSPGFFA LILLVFVTSI ASNVVKIILI HIDSRLHTPM
 51 YFLLSQLSLR DILYISTIVP KMLVDQVMSQ RAISFAGCTA QHFLYLTLAG
101 AEFFLLGLMS CDRYVAICNP LHYPDLMSRK ICWLIVAAAW LGGSIDGFLL
151 TPVTMQFPFC ASREINHFFC EVPALLKLSC TDTSAYETAM YVCCIMMLLI
201 PFSVISGSYT RILITVYRMS EAEGRRKAVA TCSSHMVVVS LFYGAAMYTY
251 VLPHSYHTPE QDKAVSAFYT ILTPMLNPLI YSLRNKDVTG ALQKVVGRCV
301 SSGKVTTF
```

(C)

Figure 3.12C: Mascot score histogram, spectrum and sequence of olfactory receptor 2T7. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of olfactory receptor 2T7.

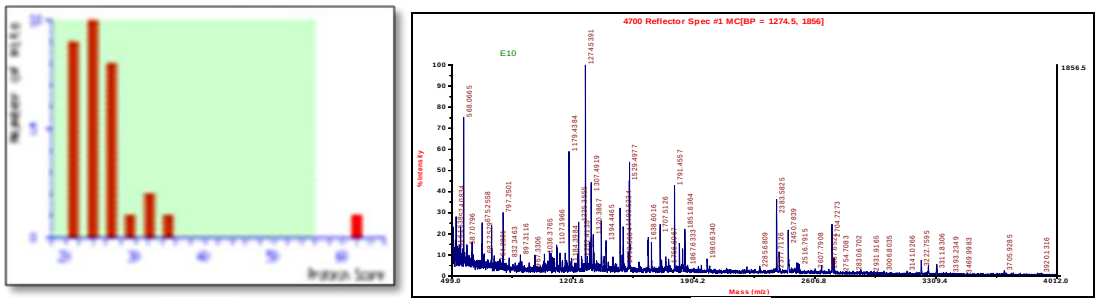


(A) (B)

```
  1 GSSGSSGLLR NLLTGLVRHE RIEAPWARVD EMRGYAEKLI DYGKLGDTNE
 51 RAMRMADFWL TEKDLIPKLF QVLAPRYKDQ TGGYTRMLQI PNRSLDRAKM
101 AVIEYKGNCL PPLPLPSGPS SG
```

(C)

Figure 3.12D: Mascot score histogram, spectrum and sequence of Mitochondrial Ribosomal Protein L17. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of Mitochondrial Ribosomal Protein L17.

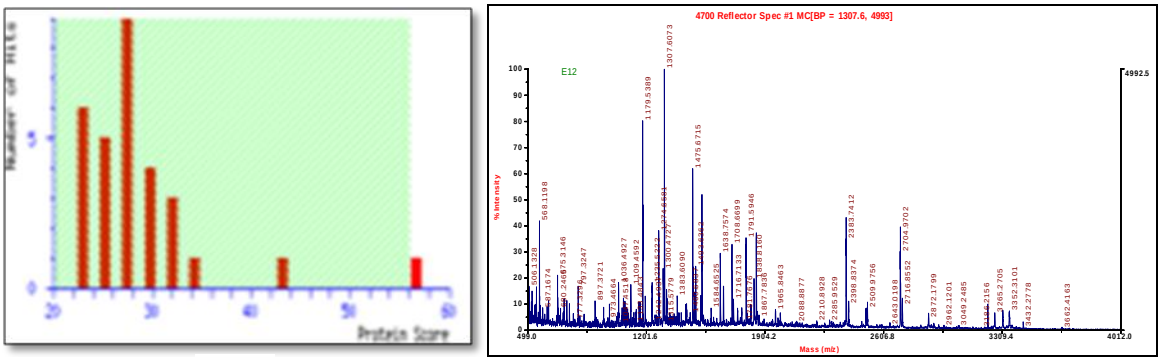


(A) (B)

```
   1 MLSGIEAAAG EYEDSELRCR VAVEELSPGG QPRRRQALRT AELSLGRNER
  51 RELMLRLQAP GPAGRPRCFP LRAARLFTRF AEAGRSTLRL PAHDTPGAGA
 101 VQLLLSDCPP DRLRRFLRTL RLKLAAAPGP GPASARAQLL GPRPRDFVTI
 151 SPVQPEERRL RAATRVPDTT LVKRPVEPQA GAEPSTEAPR WPLPVKRLSL
 201 PSTKPQLSEE QAAVLRAVLK GQSIFFTGSA GTGKSYLLKR ILGSLPPTGT
 251 VATASTGVAA CHIGGTTLHA FAGIGSGQAP LAQCVALAQR PGVRQGWLNC
 301 QRLVIDEISM VEADLFDKLE AVARAVRQQN KPFGGIQLII CGDFLQLPPV
 351 TKGSQPPRFC FQSKSWKRCV PVTLELTKVW RQADQTFISL LQAVRLGRCS
 401 DEVTRQLQAT ASHKVGRDGI VATRLCTHQD DVALTNERRL QELPGKVHRF
 451 EAMDSNPELA STLDAQCPVS QLLQLKLGAQ VMLVKNLSVS RGLVNGARGV
 501 VVGFEAEGRG LPQVRFLCGV TEVIHADRWT VQATGGQLLS RQQLPLQLAW
 551 AMSIHKSQGM TLDCVEISLG RVFASGQAYV ALSRARSLQG LRVLDFDPMA
 601 VRCDPRVLHF YATLRRGRSL SLESPDDDEA ASDQENMDPI L
```

(C)

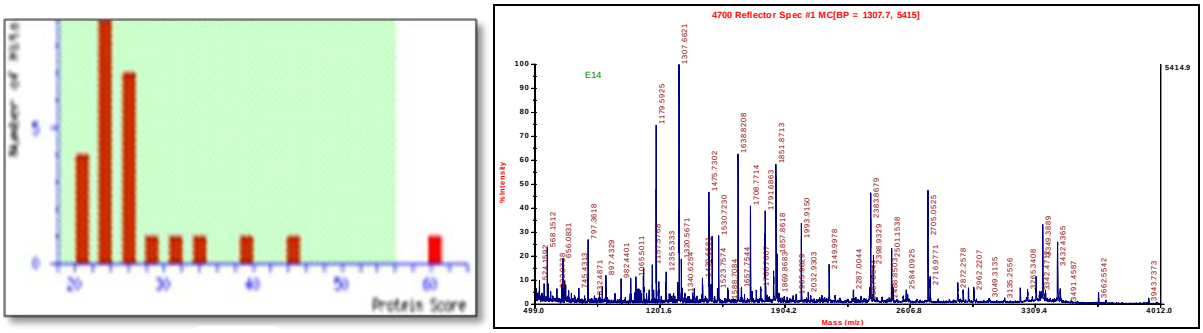
Figure 3.12E: Mascot score histogram, spectrum and sequence of ATP-dependent DNA helicase PIF1. (A) Mascot score histogram, spectrum and (B) Peptide matches of ATP-dependent DNA helicase PIF1.



(A) (B)

(C)

Figure 3.12F: Mascot score histogram, spectrum and sequence of Very large A kinase anchor protein. (A)Mascot score histogram, (B) spectrum and (C) Amino acid sequence of Very large A kinase anchor protein.

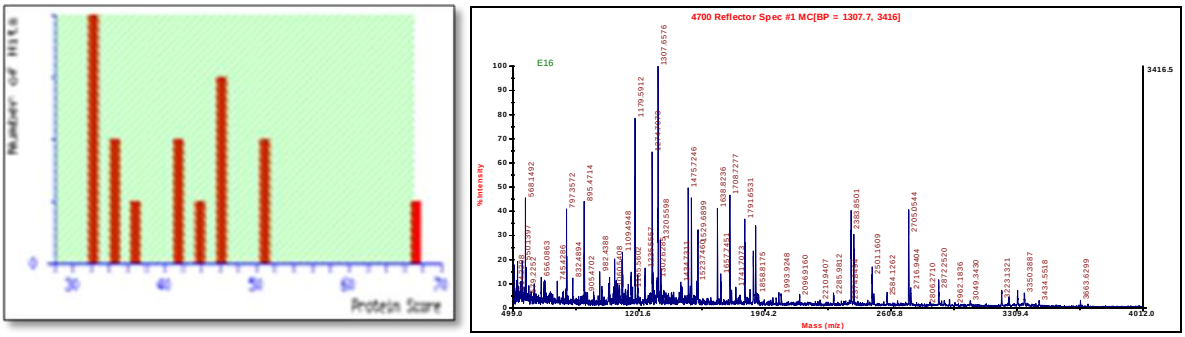


(A) (B)

```
  1 MGGLRPWSRY GLLVVAHLLA LGLGAVVFQA LEGPPACRLQ AELRAELAAF
 51 QAEHRACLPP GALEELLGTA LATQAHGVST LGNSSEGRTW DLPSALLFAA
101 SILTTTGYGH MAPLSPGGKA FCMVYAALGL PASLALVATL RHCLLPVLSR
151 PRAWVAVHWQ LSPARAALLQ AVALGLLVAS SFVLLPALVL WGLQGDCSLL
201 GAVYFCFSSL STIGLEDLLP GRGRSLHPVI YHLGQLALLG YLLLGLLAML
251 LAVETFSELP QVRAMGKFFR PSGPVTAEDQ GGILGQDELA LSTLPPAAPA
301 SGQAPAC
```

(C)

Figure3.12G: Mascot score histogram, spectrum and sequence of Potassium channel Subfamily K member. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of Potassium channel Subfamily K member.



(A) (B)

```
  1 KASGYSFTGN LLHWVRQAPG QGLEWMGWIN TNTGDTDSAQ KFEGRVTLTF
 51 DTSINTGYME MTSLRSDDTA VYYCARDRSY HYDI
```

(C)

Figure 3.12H: Mascot score histogram, spectrum and sequence of Immunoglobulin heavy chain variable region. (A) Mascot score histogram, (B) spectrum and (C) Peptide matches of Immunoglobulin heavy chain variable region.

Among eight proteins, three proteins namely RIP, mitochondrial ribosomal protein L17, and very large A kinase anchor proteins were down-regulated in first day of infected sample compared to control samples. These three proteins were showing up-regulation after first day of infection to third day. Whereas, olfactory receptor and ATP-dependent DNA helicase were down-regulated in the first day of infected sample. After first day these two proteins were increased in the second day of infection and again decreased to normal level expression. Potassium channel Subfamily K-member protein was up-regulated from first day to second day of infection and then decreased to normal expression like control sample. Ankyrin repeat domain protein is up-regulated in first day of infection. This protein was down-regulated after the first day of infection and again up-regulated. Immunoglobulin heavy chain variable region is up regulated from first day to third day of infection compared to control samples. The histogram, 3D view and spot density values of each protein spots were shown in figure 3.13.

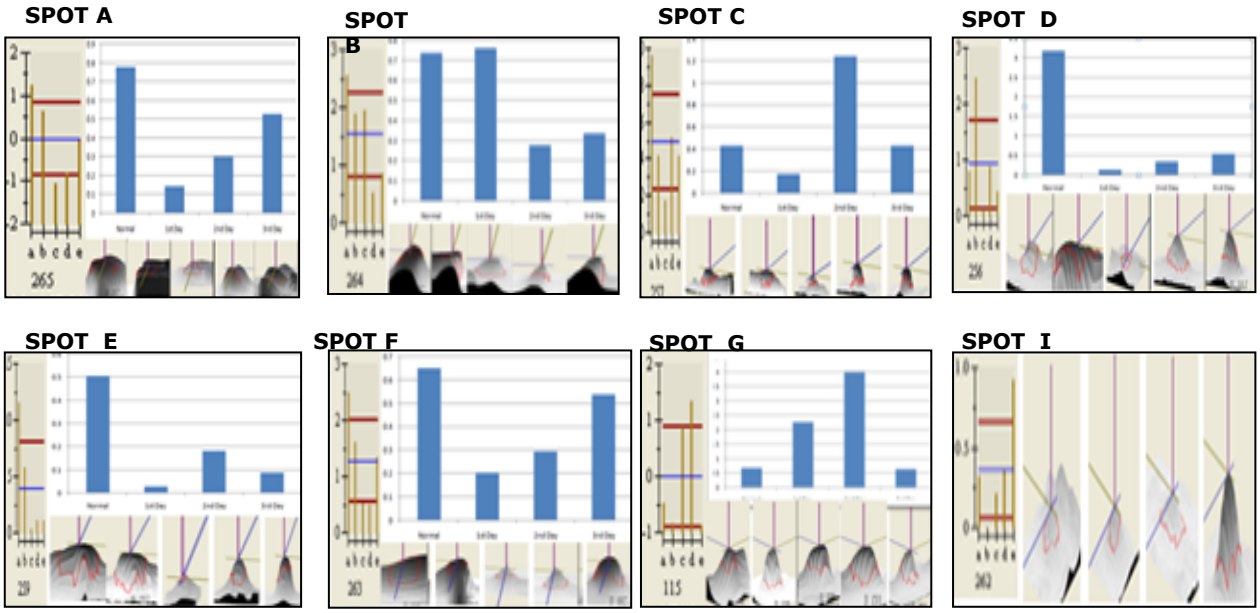


Figure 3.13: Expression pattern of protein spots during disease progression from day1 to day3.
Spot A: RIP; Spot B: Ankyrin repeat domain; Spot C: Olfactory receptor 2T7; Spot D: Olfactory receptor 2T7; Spot D: Mitochondrial Ribosomal Protein L17; Spot E: ATP-dependent DNA helicase; Spot F: Very large A kinase; Spot G: Potassium channel Subfamily K-member; Spot I: Immunoglobulin heavy chain variable region.

3.4 Discussion

Dengue virus infections occupied almost the top of the list of dreadful pathogenic infections for which there is no specific cure or vaccine at present. A vaccine to be developed should be able to target all four serotypes of this virus at a time and hence its development remains challenge. At this junction, it is time for exploring the host responses for developing specific control measures for this virus. Systematic characterization of circulating proteins in human plasma in healthy and disease may lead to the development of new biomarkers for diagnosis, prognosis and treatment follow-up in several pathologies ranging from infectious diseases to cancer. Indian soil especially the states Telangana and Andhra Pradesh witnessed several dengue and other virus outbreaks during different time periods. In the present investigation, we have analyzed the dengue virus prevalence in the above states of India and procured the dengue virus infected blood samples for the analysis of gene expression during virus infection. Then the samples collected from DF, DHF, DSS patients and controls were subjected to 2-DE. The protein expression levels were compared between all three groups. 2-DE image analysis was chosen to compare the differential expression profile of the three groups.

We have identified eight proteins using 2D GE followed by MALDI-TOF in dengue infected samples compared to controls. They are Human transthyretin, Calpain-7, ZNF224, CGI-105, Keratin type1 cytoskeletal1 and Keratin type 2cytoskeletal9, hemoglobin beta and myoglobin. Mascot scores of all eight proteins were significant ($p<0.05$) and also eight differentially expressed proteins were identified in disease progression samples. They are RIP, Ankyrin repeat domain, olfactory receptor, Mitochondrial ribosomal protein L17, ATP-dependent DNA helicase, Very large A-kinase anchor protein, Potassium channel subfamily k member and Immunoglobulin heavy chain variable region. We expect that these proteins can be utilized as markers for dengue viral infections. The importance and functions of above identified proteins are discussed below.

Transthyretin is a 55kDa homotetramer. It is a transport protein which is present in the serum and cerebrospinal fluid that carries the thyroxine and retinol binding protein. Transthyretin was secreted into the blood by the liver and into the cerebrospinal fluid by the choroid plexus. TTR was originally called pre-albumin (115) because it ran faster than albumin on electrophoresis gels. Human Transthyretin is considered as an acute-phase protein whose expression is reduced in several circumstances such as inflammation and trauma (116). When HepG2 cells were treated with IL-6, IL-1 or TNF-α cytokines, the mRNA levels of this protein were decreased (117).

Transthyretin can inhibit IL-1 production by monocyte and endothelial cells, thus, presenting anti-inflammatory properties (118). This protein was found to be down-regulated in plasma from severe acute respiratory syndrome (SARS patients analyzed by 2-D DIGE and MS and in sera from lung cancer patients identified by surface enhanced laser desorption ionization time-of-flight mass spectrometry (119, 120). In the present study, transthyretin levels were found to be increased in plasmas of dengue fever patients.

Calpains are intracellular Ca2+ dependent cysteine proteases which are plays a important role in signal transduction, apoptosis, and the cell cycle, as well as membrane trafficking (121). There are 15 calpain genes in the human genome and they are categorized into two groups 1) domain structures (classical calpains and non-classical calpains) and 2) tissue distribution (ubiquitous calpains and tissue-specific calpains) (122).

cDNA of ZNF224 encodes a protein which belonging to the Kruppel-like zinc-finger protease class. It is one of the largest families of transcriptional factors which are divided into many subclasses based on the number and type of zinc finger they contain (123-124). ZNF224 specifically binds to the AldA-NRE motif through its array of C2H2 zinc fingers and inhibits the transcription of a eukaryotic promoter bearing the AldA-NRE sequence. Therefore, ZNF224 functions as repressor protein of aldolase A gene expression, previously defined as p97 (125-129).

CGI-105 is a protein which is a member of the fumaryl acetoacetate (FAA) hydrolase family and this contains 2A (130, 131). Presently there was no report regarding the expression and function of the CGI-105 gene in any species such as humans, mice and, as well as bovine. Keratin type 1 cytoskeleton 9 and Keratin type 2 cytoskeletal 1 are the cytoskeletal proteins that may be resulted because of contamination while handling.

The molecular weight of hemoglobin beta was 15,980 Da. It is involved in the transport of oxygen from lungs to various peripheral tissues. Myoglobin is an iron and oxygen-binding protein particularly found in the muscle tissue of vertebrates and in all mammals. It is similar to hemoglobin, which is also the iron- and oxygen-binding protein in blood, mainly in the red blood cells. In humans, myoglobin is observed in the circulation only when muscle injury occurs. It is observed in an abnormal condition, and can be diagnostically relevant when found in blood.

In conclusion, the application of proteomic analysis in human disease is moving rapidly, and without a doubt, the findings would improve our understanding of the functions of individual

proteins in the development of disease including the identification and elucidation of structure-function interrelations that define health and diseased conditions. Using the above data we have chosen two proteins i.e. Calpain7 and Hemoglobin beta (HBB) for the development of dengue diagnostic test for dengue virus infections. More importantly, both these proteins were over expressed in dengue patients compared to control samples, leading to hypothesis that these two proteins could possibly be candidate markers of dengue viral infections.

CHAPTER 4

Standardization and evaluation of tests for dengue virus diagnosis, disease progression and infections with different serotypes

(a) DAC- ELISA

(b) Dot blot

4.1 Introduction

Eight proteins were found to be upregulated in dengue virus infected samples and identified them using 2D GE followed by MALDI-TOF. They are Human transthyretin (TTR), Calpain-7, ZNF224, CGI-105, Keratin type1 cytoskeletal9 and Keratin type 2 cytoskeletal1, hemoglobin beta and myoglobin. Among eight proteins identified in dengue virus infected samples Calpain-7 and hemoglobin beta were selected based on their functional relatedness to viral infections for evaluation of diagnostic tests.

Calpain-7 was upregulated in three DF, five DHF and two DSS samples when compared to controls. Calpains are a group of calcium-sensitive cysteine proteases that are ubiquitously expressed in mammals. Calpain-7 was found to be interacted with (ESCRT)-III related proteins suggesting its involvement of calpain-7 in the ESCRT system. The ESCRT system was originally identified as machinery contributing to multivesicular endosome (multivesicular body) formation in the endocytic pathway (132, 133). Carpp et al reported that ESCRT system utilized by several enveloped viruses to facilitate their budding from cellular membranes (134) ESCRT machinery has been proposed to have additional roles in other membrane deformation／fission events, such as retrovirus budding and membrane fission of daughter cells in cytokinesis (135). So we expect to have similar roles for Calpain-7 in dengue virus multiplication mainly in release from the cellular membranes.

Human hemoglobin beta was upregulated in three DF, five DHF and two DSS samples when compared to the control samples. Hemoglobin beta was involved in transport of oxygen from lungs to various peripheral tissues. Hemoglobin subunit Beta interacts with the capsid protein and antagonizes the growth of classical Swine Fever Virus (136). The interactions between dengue capsid-Hemoglobin beta (HBB) had been predicted based on structural similarity (137). Human transthyretin was found in four controls and nine infected samples. Transthyretin was found to be up-regulated in dengue patients (138).

Dot blot assay is a relatively older technique but provides an inexpensive way for identification of certain specific proteins. It is very rapid, easy to perform and does not require any expensive equipment. We hope that either of these two proteins can be utilized as markers for dengue viral infections.

This study aims to identify the levels of Calpain-7 and hemoglobin beta by DAC-ELISA and Dot blot in samples seropositive for Dengue virus to compare the diagnostic performance of these two markers by ROC curve analysis.

4.2 Materials and methods

4.2.1 Reagents

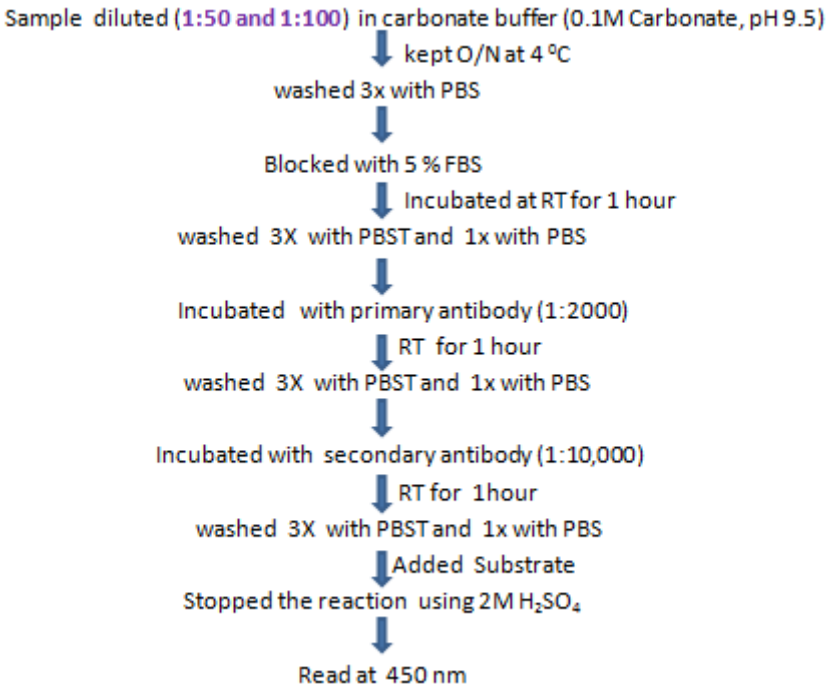
PVDF membrane, ELISA plates, TMB substrate, PBS pH 7.5, $H2SO_4$, Tween-20, Fetal bovine albumin, Sodium chloride, Sodium Bicarbonate, Sodium carbonate Anhydrous, Potassium chloride, Sodium phosphate Dibasic Anhydrous, Potassium Dihydrogen phosphate.

4.2.2 Antibodies

Goat anti-human Calpain-7 polyclonal antibody (CAPN7) (Sancta Cruz), Mouse anti-human hemoglobin beta monoclonal antibody (HBB) (Sancta Cruz), Rabbit anti-goat HRP conjugated secondary antibody and Rabbit anti-mouse HRP conjugated secondary antibody.

4.2.3 Standardization of DAC- ELISAs

ELISA plates were coated with 100 µl/well of plasma samples in 0.1M Carbonate buffer, pH 9.5 at 4°C overnight. The wells were washed three times with PBS and then each well was blocked with 100 µL of PBS, pH 7.4, containing 5 % fetal bovine serum for one hour at room temperature. Washed the wells three times with washing buffer (PBS + 0.05 % Tween-20) and once with PBS and incubated with 50 µL of 1:2000 diluted primary antibody (Sancta Cruz) for one hour at room temperature. The wells were washed as indicated above and incubated with 50 µL/well of 1:10,000 diluted secondary antibody conjugated with HRP conjugate. After further washing the antigen-antibody reaction was developed by addition of 50 µL/well of TMB substrate. The color was developed for 10 min at room temperature. The reaction was stopped by addition of 50 µL of 2M H_2SO_4 and the plate was read at a wavelength of 450 nm with an ELISA reader. This protocol was standardized with slight modification. The protocol used for experiment was shown in the below flow chart.



4.2.4 Standardization of Dot blots

10 µl of antigen was directly spotted onto PVDF membrane. The membrane with antigen spots was air dried for 30 minutes at room temperature. The antigen in the membrane was blocked with a blocking buffer (5% FBS, pH 7.4) for one hour over a shaker. Washing of the membrane was done three times with TBS pH 7.4 containing 0.02% Tween 20 and at the end once with TBS. The membrane was incubated with 1: 2000 diluted primary antibody in TBS, pH 7.4 for one hour with continuous shaking. Washing of the membrane was then repeated as above and incubated the membrane at room temperature with 1:10,000 diluted secondary antibody in TBS, pH 7.4. The membrane was again washed as above and the antigen-antibody reaction was developed by the addition of substrate. The results were read as the development of a black colored dot on PVDF membrane. Here colored dots indicate positive reaction and colorless dot indicated as non-reactive. This protocol was standardized with slight modification (). The protocol standardized for the experiment was shown in the below flow chart.

The highest dilution of antigen gave the best dot intensities was 1:8000. It was considered as optimum antigen concentration for use in validation experiments. This dilution of the plasma samples gave the least background color in both infected and control samples.

4.2.5 Samples collection

As we mentioned above, along with infected samples collected at the first day of admissions into the hospital, totally fifteen infected samples were also collected from day1 to day 6. Clinical and demographic characters of every case from day 1 to day 6 were also recorded in a case record form including symptoms, signs and laboratory investigations. The detailed clinical examination was done at the time of admission followed by serial monitoring to assess the progression of the disease.

4.2.6 Serotyping analysis

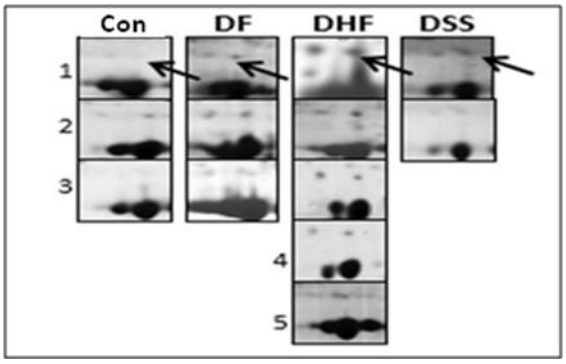
As we discussed above serotyping analysis was performed by the co-researchers in the lab for some samples and found that all four serotypes were prevalent in this outbreak. Among these samples DEN1 serotype was dominant in this outbreak. We have compared the ELISA and Dot blot analysis of Calpain-7 and Hemoglobin beta using serotyped samples with serotyping results.

4.2.7 Statistical analysis:

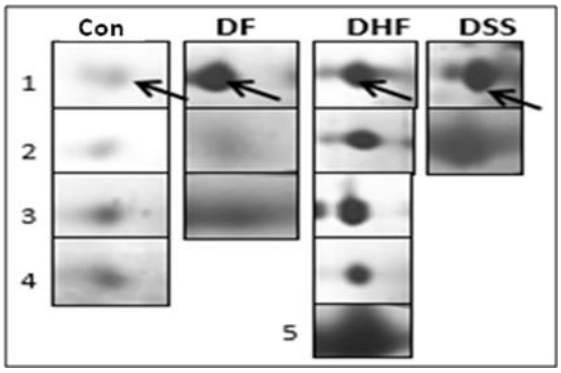
Cutt off levels of Calpain-7 and Hemoglobin beta evaluated by both ELISA and dot blot method were determined using receiver operator characteristic curve(ROC).Sensitivity, specificity, positive and negative predictive values were calculated for cut-off values. SPSS version 13, Sigmaplot version 11 and Medcalc version 15 softwares were used for obtaining ROC curves.

4.3 Results

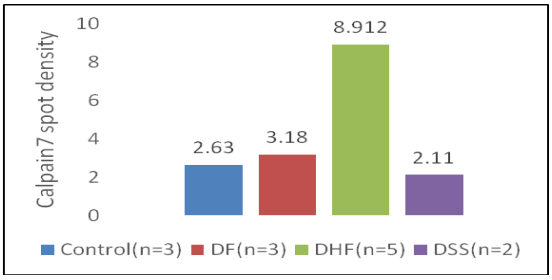
The two proteins selected for standardization and evaluation were Calpain-7 and Hemoglobin beta. The presence of these two proteins in three grades of dengue fever and controls and their average spot density volume was shown in figure 4.1.



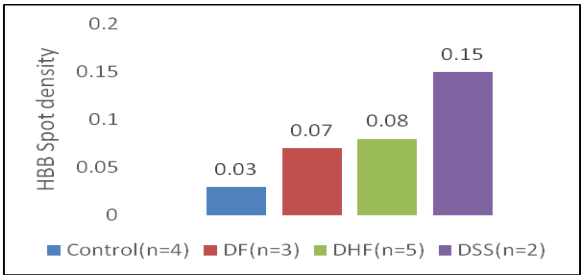
(1A)



(2A)



(1B)



(2B)

Figure 4.1: Enlarged view and average spot density values of Calpain-7 and Hemoglobin beta.
(1A) Enlarged view of Calpain-7 (1B) Average spot density values of calpain-7.
(2A) Enlarged view of Hemoglobin beta and (2B) Average spot density values Hemoglobin beta.

4.3.1 DAC- ELISA

The optimal amount of protein concentration used for Calpain-7 and hemoglobin beta in DAC-ELISA was determined to be 1: 50 and the optimal dilution of primary antibody were 1:2,000. Standardization experiment was performed with 25 infected and 5 control samples. Out of 25 infected samples, 10 were DF, 10 DHF, and 5 were DSS samples. In the standardization of DAC-ELISA using Calpain-7 and hemoglobin beta antibodies to detect these two protein levels in the infected and control samples, the absorption levels of Calpain-7 and hemoglobin beta were found to be significantly increased in three subtypes of dengue fever samples when compared to control samples (Figure 4.2A). In the same way, hemoglobin beta protein levels were also found to be significantly increased in three subtypes of dengue fever patients when compared to control patients (Figure 4.2B).

(A) (B)

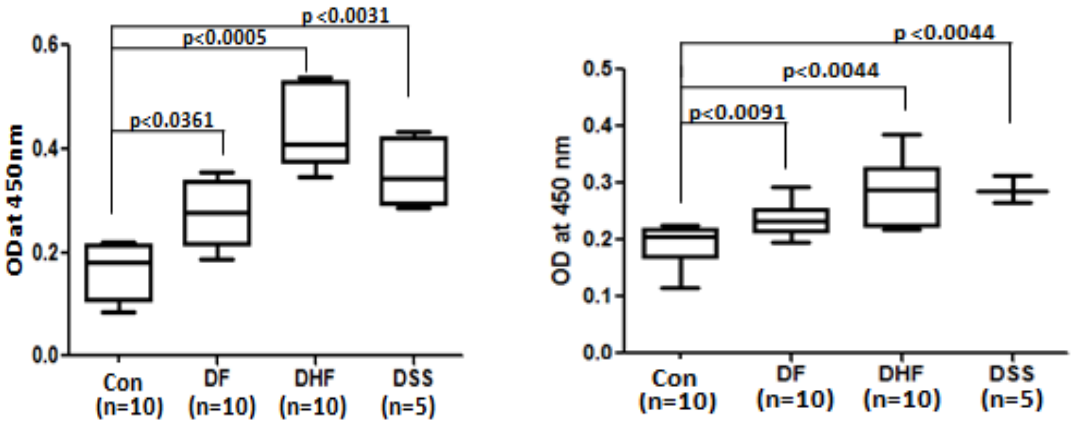


Figure 4.2: The elevated absorption values of Calpain-7 and Hemoglobin beta in three subtypes of dengue and control patients by DAC-ELISA. (A) Calpain-7 (B) Hemoglobin beta. Con: control; DF: dengue fever; DHF: dengue hemorrhagic fever; DSS: dengue shock syndrome.

4.3.2 Evaluation of DAC-ELISA in dengue infected and control samples

Evaluation of DAC-ELISA was performed by increasing the number of samples. The number of infected and control samples used for evaluation were 133 and 50 samples. Among 133 infected, 64 had dengue fever, 59 had dengue hemorrhagic fever, and 7 had dengue shock syndrome.

In the evaluation of DAC-ELISA using Calpain-7 and hemoglobin beta antibodies, the Calpain-7 and hemoglobin beta levels were also significantly increased in dengue patients compared to control patients (Figure 4.3A and 4.3B). The Calpain-7 and hemoglobin beta proteins were also significantly increased in 3 subtypes of dengue patients such as DF, DHF and DSS patients compared to control patients (Figure 4.4A and 4.4B).

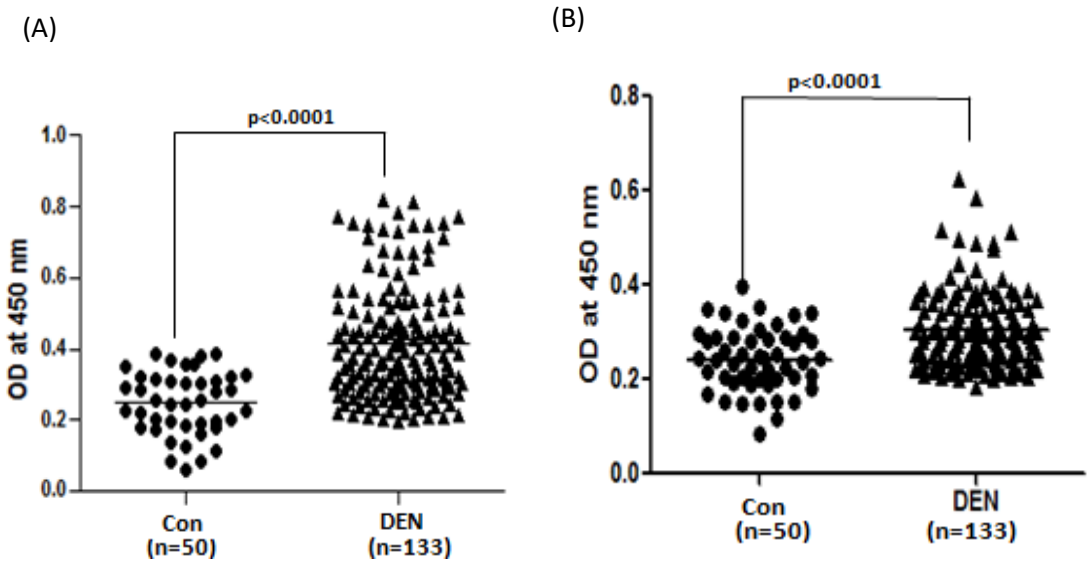


Figure 4.3: The elevated Calpain-7 and hemoglobin beta protein absorption values in the plasma of dengue and control patients by DAC-ELISA. (A) Calpain-7 and (B) Hemoglobin beta.

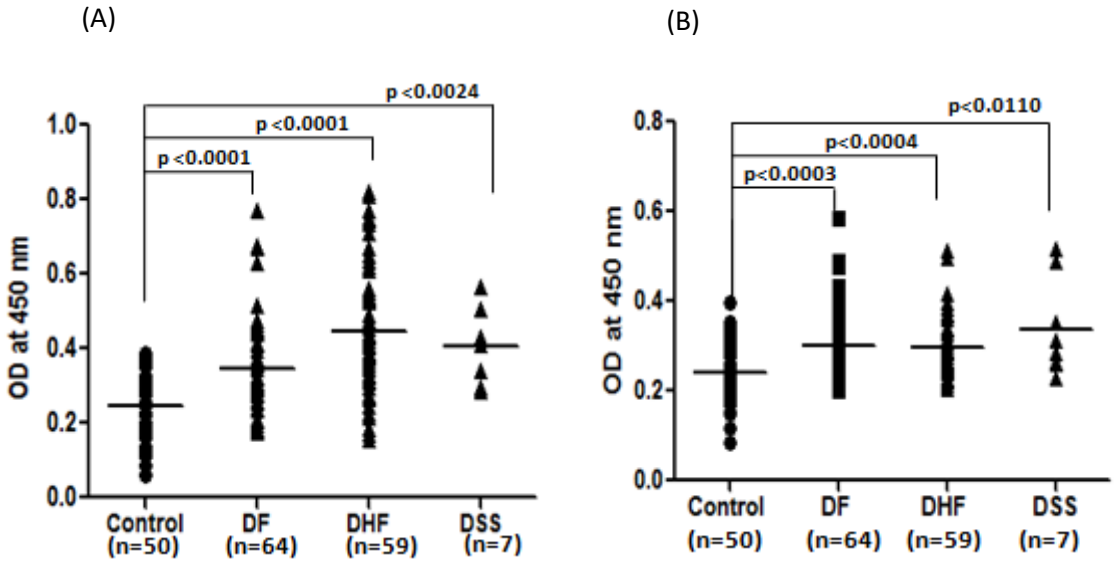


Figure 4.4: The elevated Calpain-7 and hemoglobin beta protein absorption values in three subtypes of dengue and control patients by DAC-ELISA. Con: control; DF: dengue fever; DHF: dengue hemorrhagic fever; DSS: dengue shock syndrome. The Calpain-7 and hemoglobin beta proteins in three subtypes of dengue patients were also significantly higher than that found in control patients.

4.3.3 Standardization of Dot-Blot in dengue infected and control patient samples

Preliminary standardization of antigen concentration was determined using six dilutions of plasma samples (1:1,000, 1:2,000, 1:4,000, 1:8,000, 1: 16,000, 1:32,000) and antibody dilution of 1:1000, 1:2000 and 1:3000 was done with three subtypes of dengue fever and controls. The optimal amount of antigen concentration used for Calpain-7 and hemoglobin beta in DAC- ELISA was determined to be 1:8,000 and the optimal dilution of antibody was 1:2,000. Standardization of Dot blot for Calpain-7 was performed with four controls and three infected (1 DF, 1 DHF and 1 DSS) samples and the dot intensity of Calpain-7 was found to be significant between dengue and control patient samples (Figure 4.5).

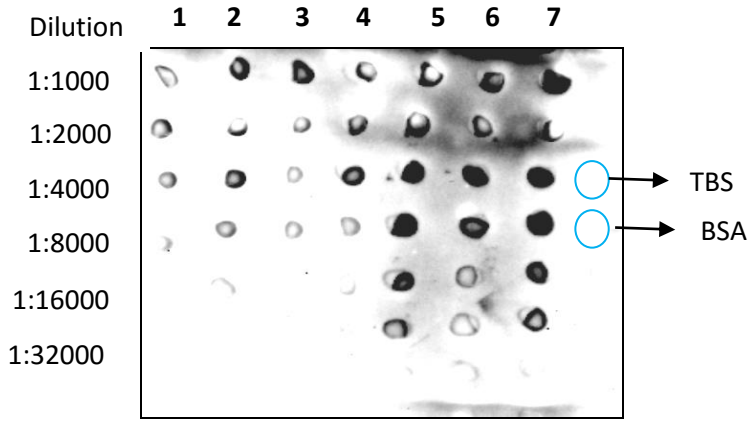


Figure 4.5: Standardization of Dot-blot test for Calpain-7 in dengue and control patient samples. Lanes (Top to bottom) 1-4: control and Lane 5-7: DF, DHF and DSS. Arrows indicate TBS buffer and BSA.

In the same way, standardization of Dot blot for hemoglobin beta was performed with four controls, two DF, three DHF and five DSS samples and the dot intensity of hemoglobin beta was found to be a significant between dengue and control patient samples (Figure 4.6A and 4.6B).

(A) (B)

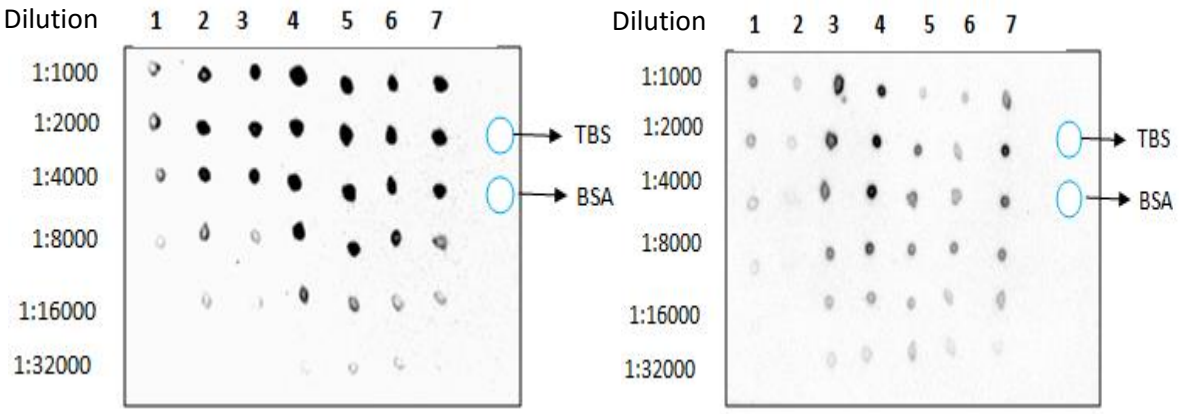
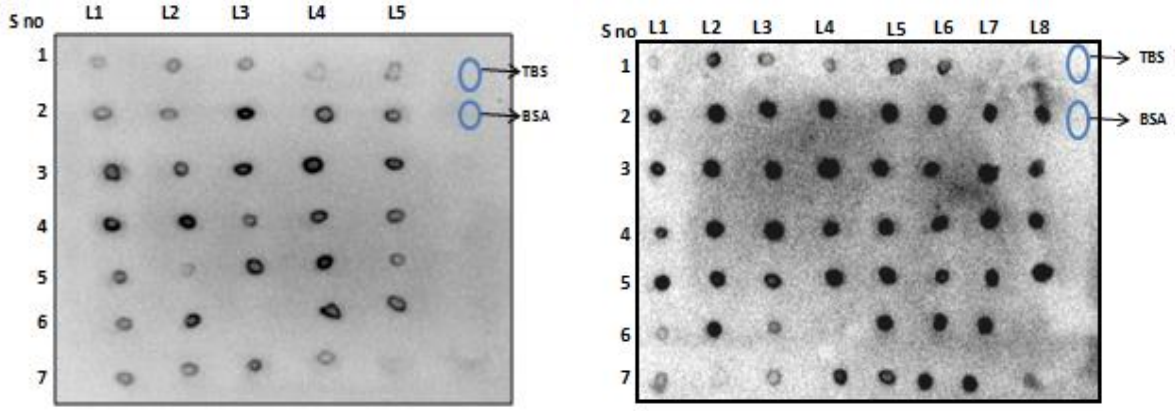


Figure 4.6: Standardization of Dot-blot test for hemoglobin beta in dengue and control patient samples. (A) Lanes (Top to bottom) 1-2: control samples; Lanes 3-4: DF; Lanes 5-7: DSS. Arrows indicate TBS buffer and BSA.

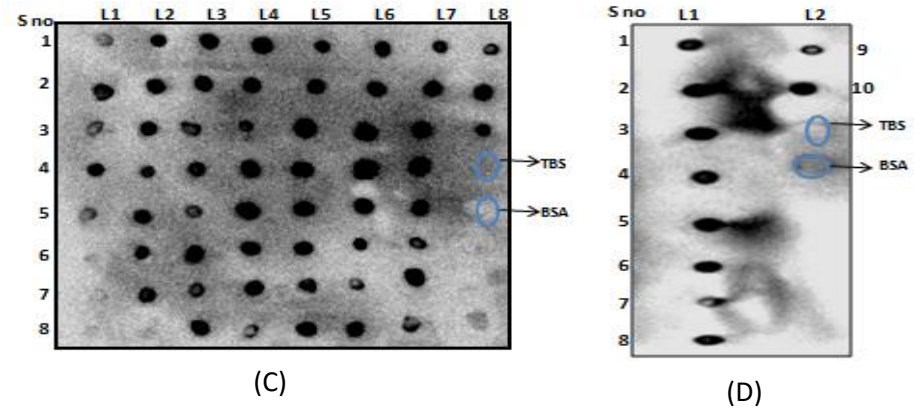
4.3.4 Evaluation of Dot blot in dengue infected and control patient samples.

Evaluation of Dot blot was performed by increasing the number of samples. The number of infected and control samples used for evaluation were 123 and 34 samples. Among 133 infected samples 54 are dengue fever, 59 are dengue hemorrhagic fever and 10 are dengue shock syndrome samples.

In the evaluation of Dot blot using a Calpain-7 antibody, the intensity values of Calpain-7 protein were increased in three subtypes of dengue patients compared to control patients (Figure 4.7).



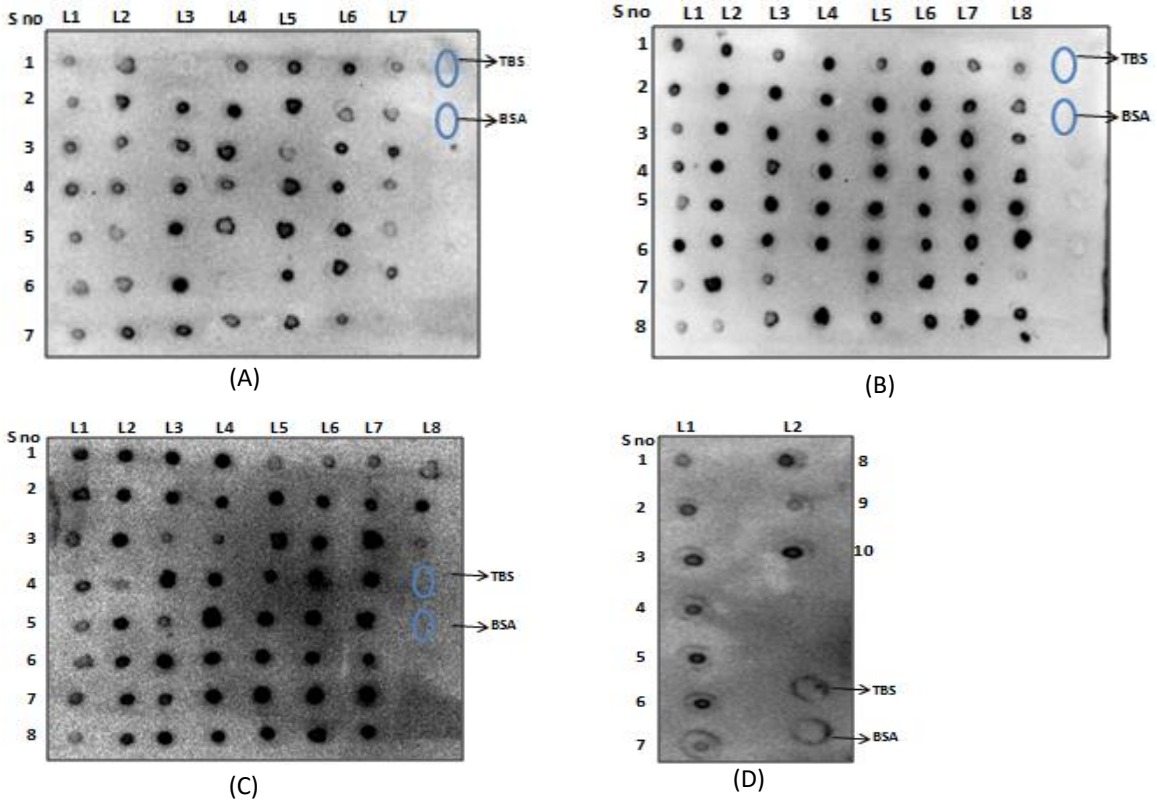
(A) (B)



(C) (D)

Figure 4.7: Dot blot test for Calpain-7 in dengue and control samples. (A) Control, (B) DF, (C) DHF and (D) DSS samples. Individual patient samples were dotted on PVDF membrane on different lanes (Top to bottom). The membrane was incubated with secondary antibody coupled to HRP (1:10,000). Exposure time: 10 sec.

In the evaluation of Dot blot using hemoglobin beta antibody, the intensity values of Calpain-7 protein was also increased in three subtypes of dengue patients compared to control patients (Figure 4.8).



(A) (B)

(C) (D)

Figure 4.8: Dot blot test for hemoglobin beta in dengue and control samples. (A) Control, (B) DF, (C) DHF and (D) DSS samples. Individual patient samples were dotted on PVDF membrane on different lanes (Top to bottom). The membrane was incubated with secondary antibody coupled to HRP (1:10,000). Exposure time: 10 sec.

In dot blot, the intensities of these two proteins were high in infected samples compared to control samples. The average dot intensity of Calpain-7 was significantly high in DF and DHF but not significant in DSS samples compared to control samples whereas for, hemoglobin beta the average dot intensity was significantly high in DHF and DSS samples but not significant in DF samples compared to control samples (Figure 4.9A and 4.9B).

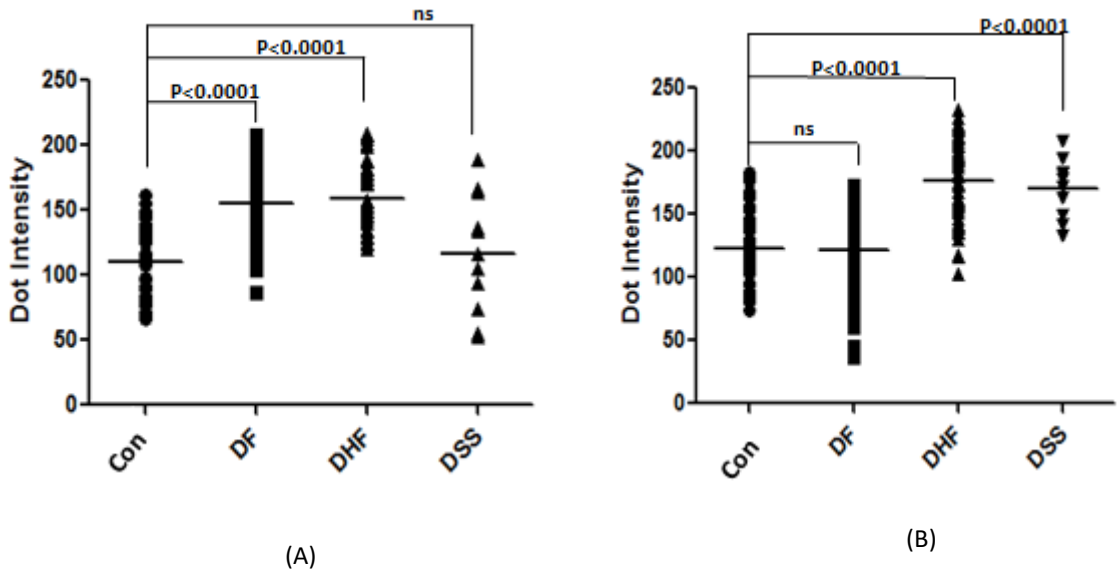
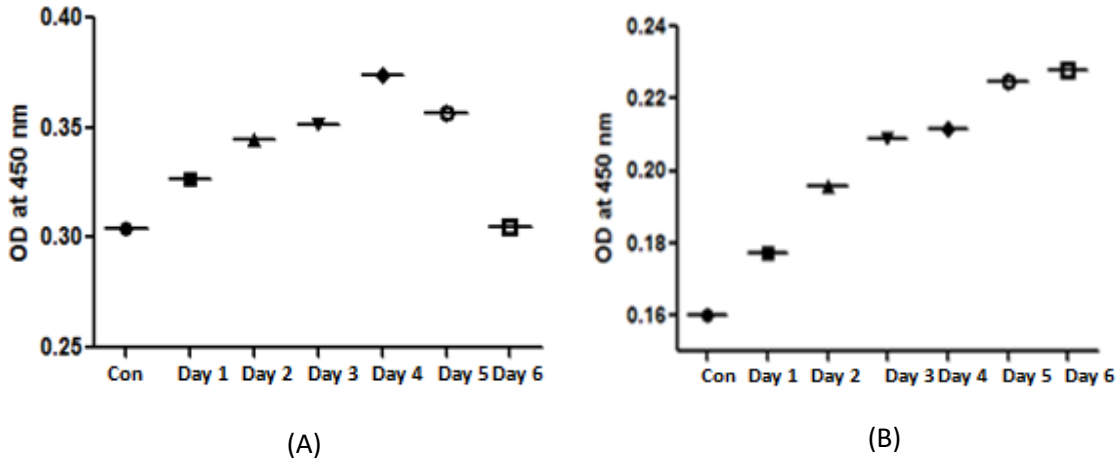


Figure 4.9: The average dot intensities of Calpain-7 and hemoglobin beta in infected and control samples. (A) Calpain-7 and (B) Hemoglobin beta.

4.3.5 Evaluation of Calpain-7 and hemoglobin beta protein in days progression samples.

As we discussed above, the optimal amount of protein concentration used for Calpain-7 and hemoglobin beta in DAC- ELISA was determined to be 1: 50 and the optimal dilution of polyclonal antibody was 1:2, 000. By using this we performed ELISAs with Calpain-7 and Hemoglobin beta antibodies to detect these two proteins in 10 controls and 10 infected samples from day 1 to day 6.

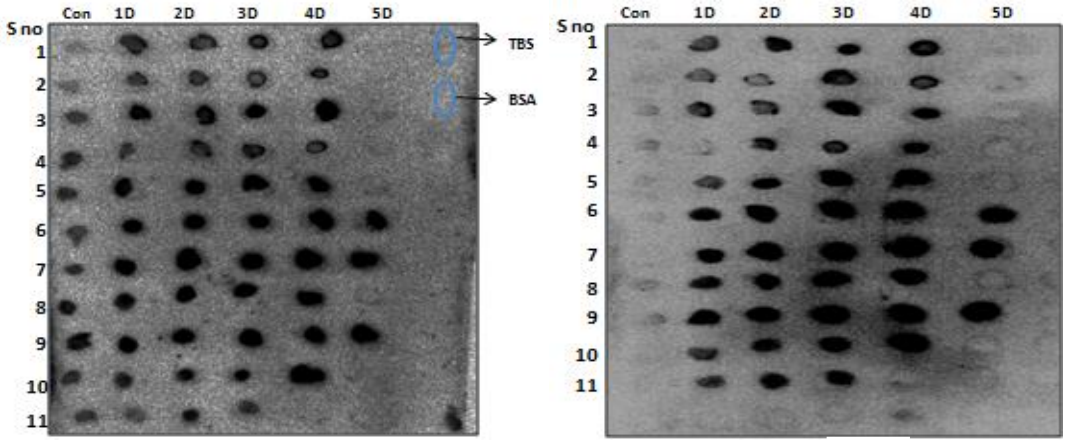
The Calpain-7 protein levels were increased from day 1 to day 4 compared to control and decreased to normal by day 6 and Hemoglobin beta levels are also increased from day 1 to day 6. This data suggested that these proteins may have some role in disease severity. (Figure 4.10A, 4.10B).



(A) (B)

Figure 4.10: The elevated absorption values of Calpain-7 and Hemoglobin beta in the plasma of dengue samples from day 1 to day 6 and control patients by DAC-ELISA. (A) Calpain-7and (B) Hemoglobin beta.

The optimal amount of antigen concentration used for Calpain-7 and hemoglobin beta in Dot blot was determined to be 1: 8,000 and the optimal dilution of antibody was 1:2, 000. By using this concentration of antigen, dot blot was performed with 11 infected and 11 control samples from day 1 to day 5 to detect these two proteins in disease progression samples. The dot intensities of Calpain-7 were found to be increased from day 1 to day 5 in some infected samples when compared to control samples (Figure 4.11A). Hemoglobin beta also increased from day 1 to day 5 (Figure 4.11B).



(A) (B)

Figure 4.11: Dot blot test for Calpain-7 and hemoglobin beta in control and disease progression samples dengue samples. (A) Calpain-7 and (B) Hemoglobin beta. (Top to bottom) con: control; 1D: day 1; 2D: day2; 3D: day3; 4D: day4 and 5D: day5. Individual patient samples were dotted on PVDF

membrane on different lanes (Top to bottom). Membrane was incubated with secondary antibody coupled to HRP (1:10,000). Exposure time: 10 sec.

DAC- ELISA results of Calpain-7 and Hemoglobin beta were compared among different serotype samples. Calpain-7 levels were significantly high in DEN1, DEN2, and DEN4 and in co-infections compared to control samples but not significant in DEN3 samples which may be due to low sample size (Figure 4.12).

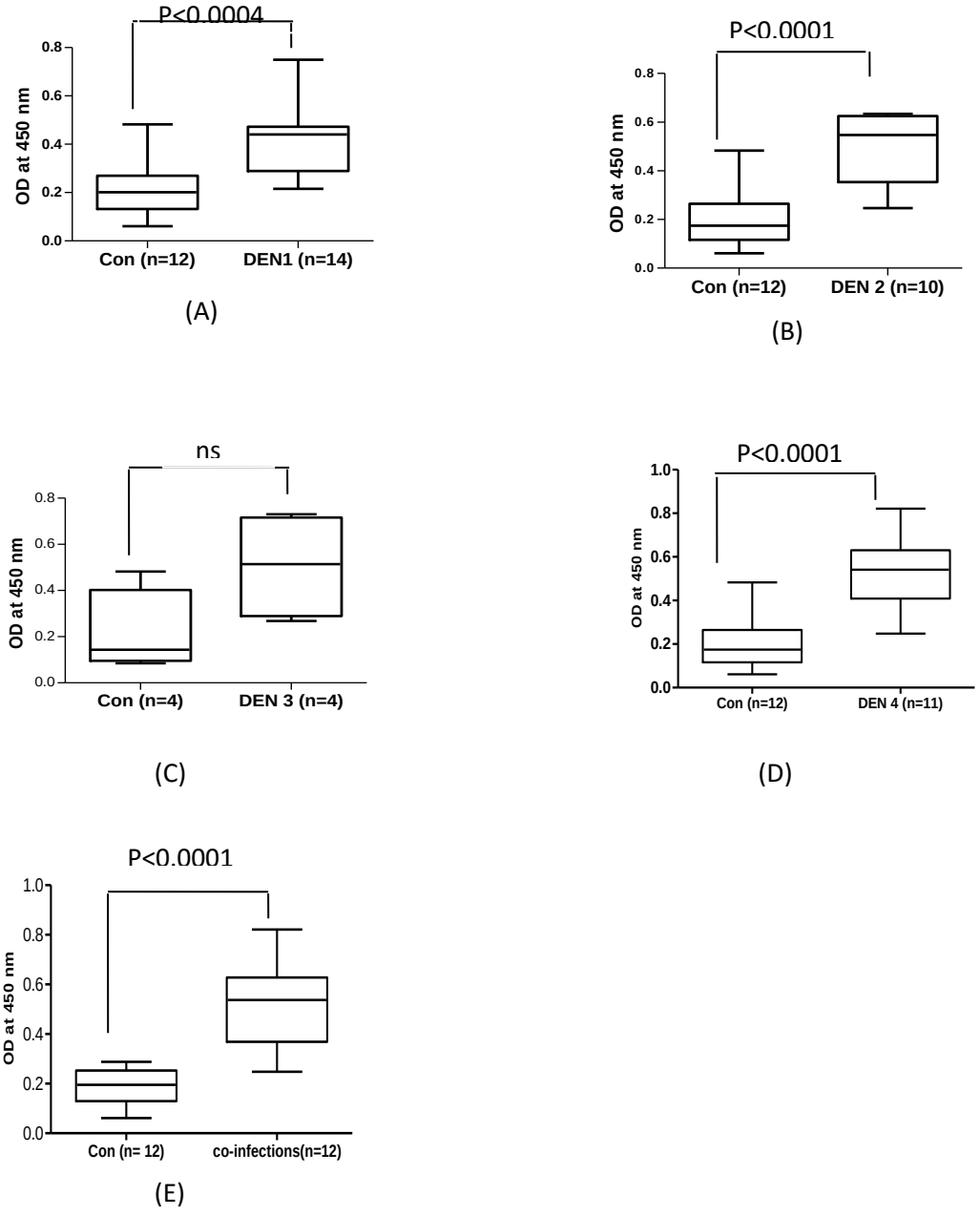


Figure 4.12: Comparison of Calpain-7 DAC- ELISA results among different serotype samples. (A): DEN 1; (B): DEN 2; (C): DEN3; (D):DEN4 and (E): co-infections.

In the same way, hemoglobin beta levels were also compared among different serotype samples and found that hemoglobin beta levels were not significantly increased in all four serotypes and in co- infections (Figure 4.13).

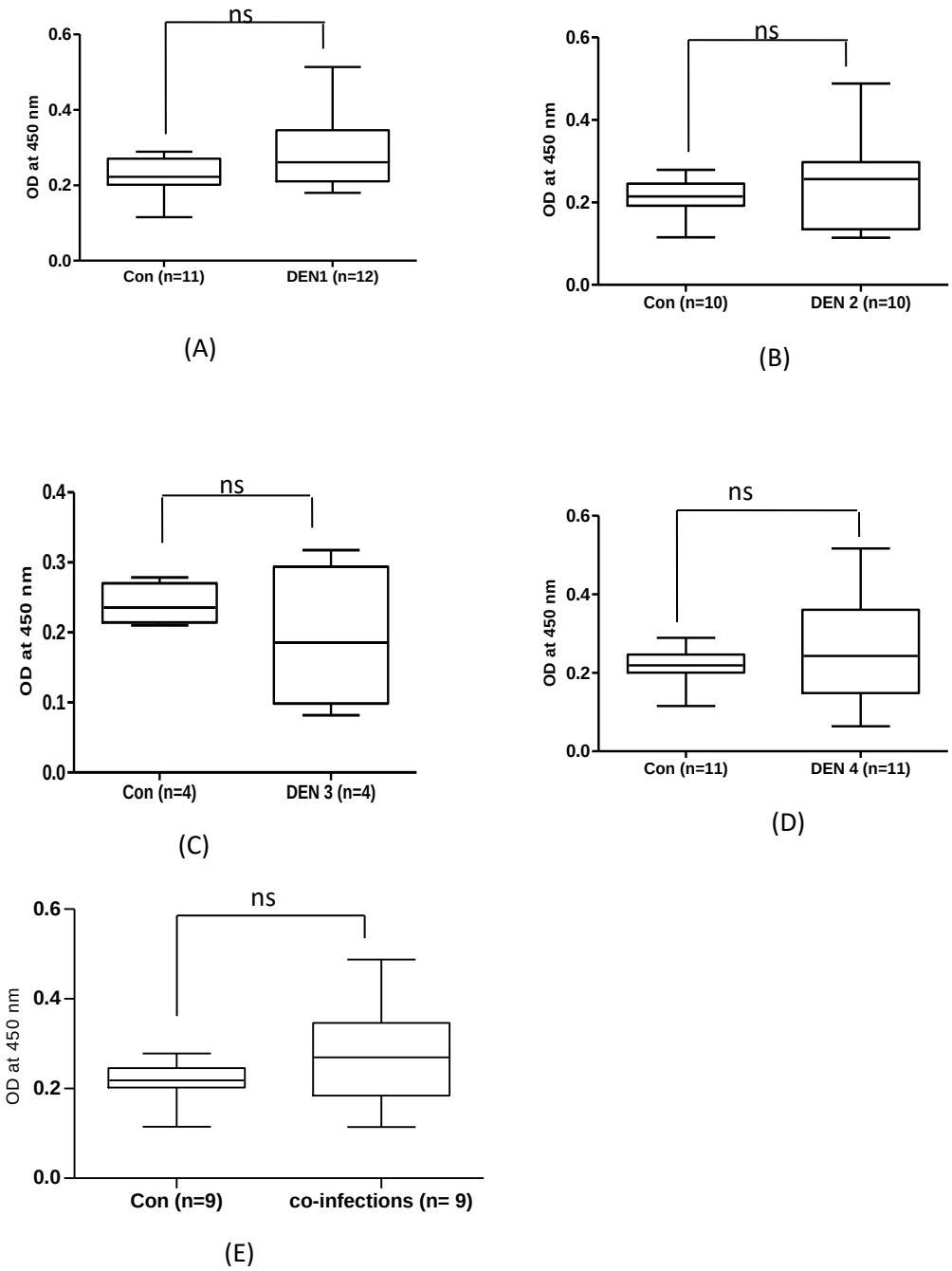


Figure 4.13: Comparison of Hemoglobin beta levels by DAC- ELISA among different serotype samples (A): DEN 1; (B): DEN 2; (C): DEN3; (D): DEN4 and (E): co-infections.

Dot blot intensity values of Calpain-7 were compared among different serotype samples and found that Calpain-7 levels were significantly increased in only DEN 1 and DEN2 serotypes but not significant in DEN 3 and DEN 4 and in co-infections (Figure 4.14).

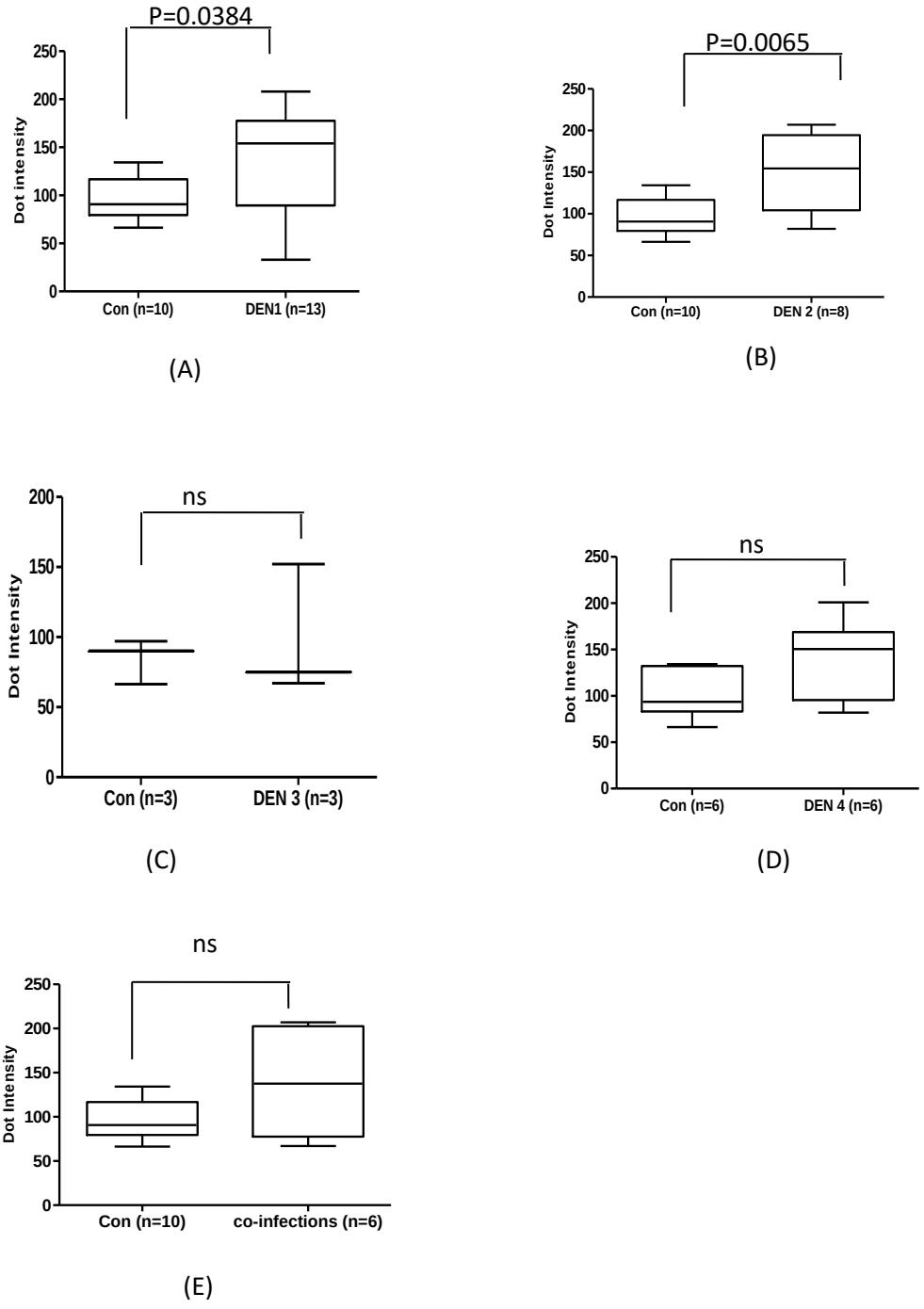


Figure 4.14: Comparison of Calpain-7 levels by Dot blot among different serotype samples. (A): DEN 1; (B): DEN 2; (C): DEN3; (D):DEN4 and (E): co-infections.

Whereas for hemoglobin beta, dot intensity levels were not significantly increased in all four serotypes and in co-infections (Figure 4.15).

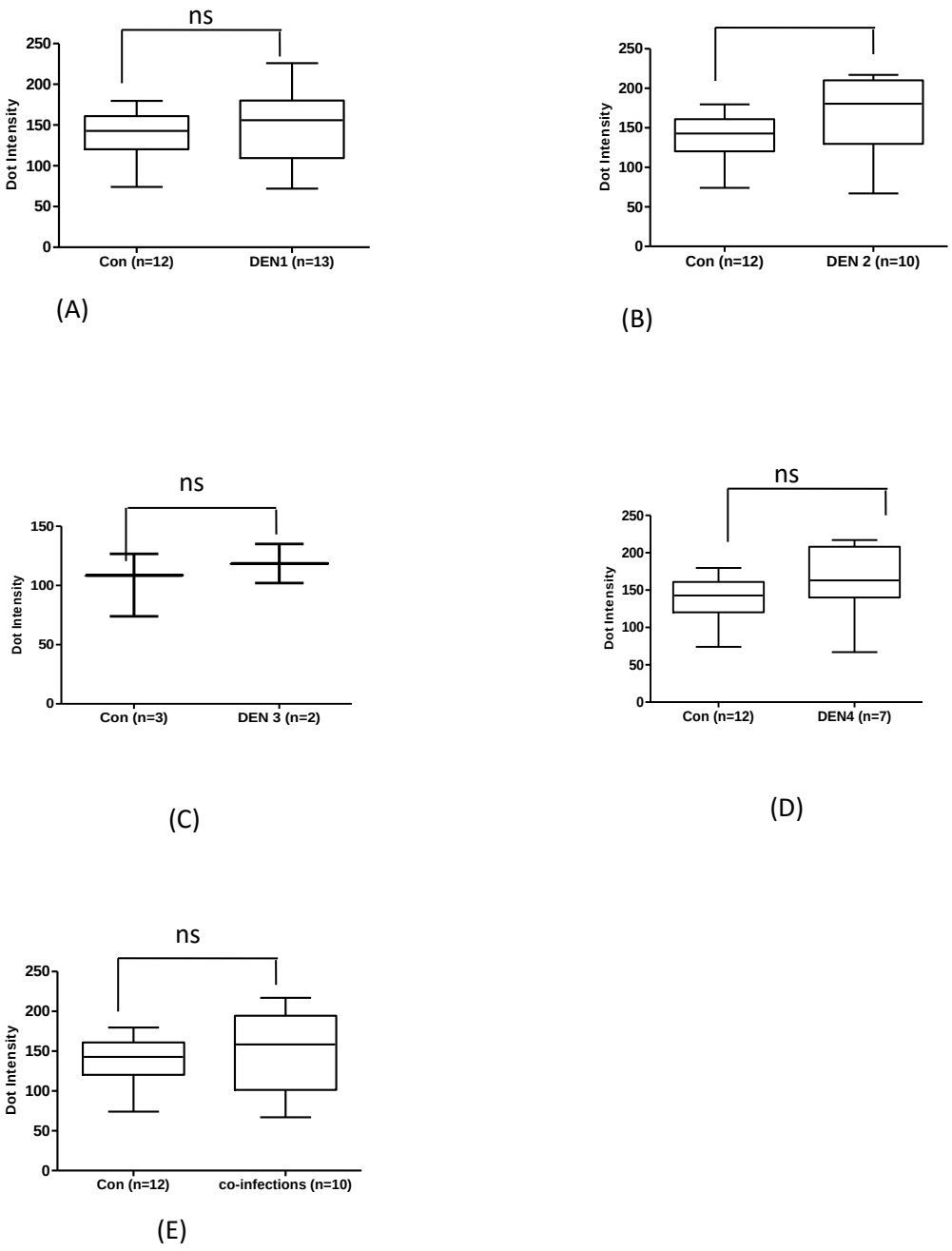


Figure 4.15: Comparison of hemoglobin beta levels by Dot blot among different serotype samples. (A): DEN 1; (B): DEN 2; (C): DEN3; (D):DEN4 and (E): co-infections. Hemoglobin beta result were not significantly increased in all four serotypes and in co-infections compared to control samples.

4.3.6 ROC (Receiver operator characteristic) curve

Sensitivity and specificity are important measures of the diagnostic accuracy of a test. The diagnostic accuracy of a test is used to discriminate disease cases from healthy cases and is evaluated using Receiver Operating Characteristic (ROC) curve analysis (Metz et al., 1978). It is used to compare the diagnostic performance of two or more laboratory diagnostic tests (Grinner et al., 1981). It is a graph of sensitivity and 1- specificity for a range of cut-off values. Sensitivity is expressed in percentage and defines the proportion of true positive subjects with the disease in a total group of subjects with the disease

True positive (TP): subjects with the disease with the value of a parameter of interest above the cut-off.

Specificity: a proportion of subjects without the disease with the negative test result in a total of Subjects without the disease.

False positive (FP): subjects without the disease with the value of a parameter of interest above the cut-off.

AUC is a combined measure of sensitivity and specificity. The area under the curve can have any value between 0 and 1 and it is a good indicator of the goodness of the test. A perfect diagnostic test has an AUC 1.0. Whereas a non-discriminating test has an area 0.5. Generally, we can say that the relation between AUC and diagnostic accuracy.

4.3.7 ROC curve analysis for DAC-ELISA

The AUC, sensitivity, specificity, positive predictive value and negative predictive values of Calpain-7 protein by DAC-ELISA were represented in Table 4.1. The cut-off values for this protein in three subtypes of dengue fever were 0.22, 0.23 and 0.22. The AUC value for DF, DHF and DSS was 0.834, 0.913 and 0.959. The sensitivity values of DF, DHF and DSS was 93, 96 and 100. The specificity values of DF, DHF and DSS were 63, 66.67 and 86. The ROC curves of three subtypes of dengue fever.

Variable	Cut off	AUC 95% CI	Sensitivity	Specificity	PPV	NPV	P Value
Total Calpain7	>0.32	0.849(0.747-0.922)	59/(42.1-75.2)	91.89(78.1-98.3)	75.6(49.4-92.6)	84.3(72.2-92.6)	0.01
DF	>0.22	0.834(0.71-0.9)	93(77.9-99.2)	63(43.9-80.1)	52(33.6-69.7)	96(80.6-99.8)	0.01
DHF	>0.24	0.913(0.81-0.97)	96.67(82.8-99.9)	66.67(47.2-82.7)	55.1(36.3-72.8)	97.9(84.3-100)	0.01
DSS	>0.22	0.959(0.70-100)	100(59-100)	86(42.1-99.6)	75(26.7-98)	100(64.6-100)	0.01

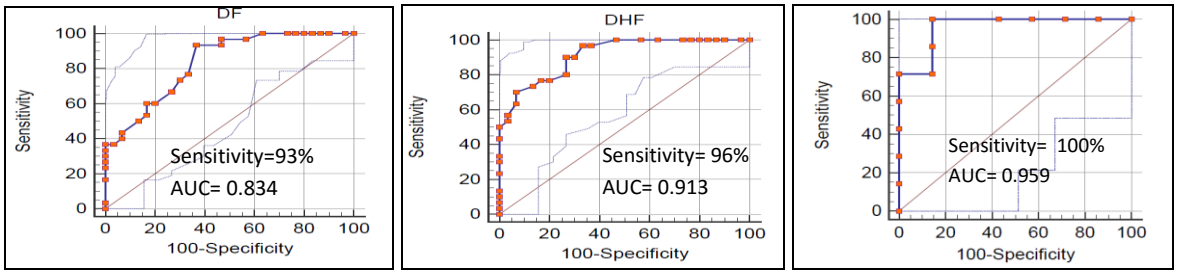
Table 4.1: ROC curve analysis of Calpain-7 by DAC-ELISA.

In the same way, the AUC, sensitivity, specificity, positive predictive value and negative predictive values of hemoglobin beta protein by DAC-ELISA were represented in Table 4.2. The cut-off values for this protein in three subtypes of dengue fever was 0.21, 0.25 and 0.25. The AUC value for DF, DHF and DSS was 0.663, 0.682 and 0.703. The sensitivity values of DF, DHF and DSS were 91.93, 54.29 and 85. The specificity values of DF, DHF and DSS were 31.43, 83.33 and 50.

Variable	Cut off	AUC 95% CI	Sensitivity	Specificity	PPV	NPV	P Value
Total HBB	>0.3	0.687(0.575-0.785)	34.15/(20.1-50.6)	92.68(80.1-98.5)	66.3(35.3-89.5)	76.9(65.2-86.2)	0.001
DF	>0.21	0.663(0.54-0.77)	91.93(76.9-98.2)	31.43(16.9-49.3)	36(23.3- 50.4)	89.7(65.6-98.9)	0.01
DHF	>0.25	0.682(0.53-0.81)	54.29(36.6-71.2	83.33(51.6-97.9)	57.9(28.6-83.6)	81.2(64.1-92.5)	0.01
DSS	>0.25	0.703(0.42-0.9)	85(47.3-99.7)	50(15.7-84.3)	42(13.5-76.1)	90(43.9-100)	0.14

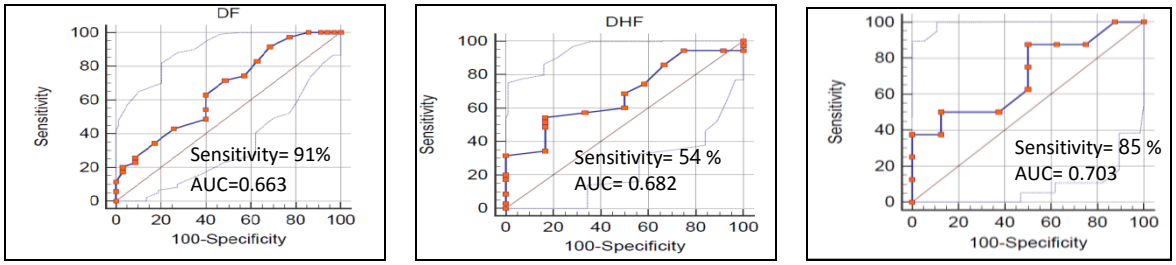
Table 4.2: ROC curve analysis of Hemoglobin beta by DAC-ELISA.

When we compare DAC-ELISA values of these two proteins by ROC curve analysis, the AUC values for Calpain-7 were ranged from 0.834 to 0.959 (Figure 4.16). It indicates excellent performance of the test and AUC values for Hemoglobin beta were ranged from 0.663 to 0.703. It indicates sufficient performance of the test but compared to Hemoglobin beta, Calpain-7 has excellent diagnostic performance (Figure 4.17).



(A) (B) (C)

Figure 4.16: ROC curve analysis of DAC-ELISA of Calpain-7. (A): DF; (B): DHF; and (C): DSS.



(A) (B) (C)

Figure 4.17: ROC curve analysis of DAC-ELISA of Hemoglobin beta. (A): DF; (B): DHF; and (C): DSS.

4.3.8 ROC Curve analysis of Dot blot

The AUC, sensitivity, specificity, positive predictive value and negative predictive values of Calpain-7 protein by Dot blot (Table 4.3). The cut-off values for this protein in three subtypes of dengue fever was >136.43, >116.6 and >97.17. The AUC value for DF, DHF and DSS was 0.691, 0.892and 0.755. The sensitivity values of DF, DHF and DSS was 46, 96.15 and 35.71. The specificity values of DF, DHF and DSS were 96, 65.38and 71.43.

Variable	Cut off	AUC 95% CI	Sensitivity	Specificity	PPV	NPV	P Value
Total Calpain7	>136.43	0.722 (0.58-0.83)	53.85(33.4-73.4)	96(80.4-99.9)	85(49.7-99.1)	83(68.5-92.9)	0.01
DF	>136.43	0.691(0.547-0.812)	46(26.6-66.6)	96(80.4-99.9)	83(44.6-98.9)	80(66.1-91.2)	0.01
DHF	>116.6	0.892(0.775-0.961)	96.15(80.4-99.9)	65.38(44.3-82.8)	54(34.1-73)	97.6(81.8-100)	0.01
DSS	>97.17	0.755(0.46-0.93)	35.71(42.1-99.6)	71.43(29-96.3)	55.9(16.1-90.5)	92(51.5-100)	0.01

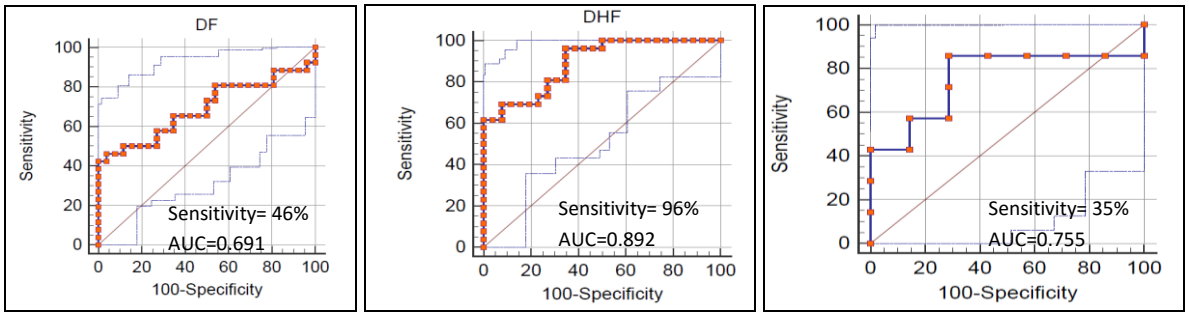
Table 4.3: ROC curve analysis of Calpain-7 by Dot blot.

In the same way, AUC, sensitivity, specificity, positive predictive value and negative predictive values of Hemoglobin beta protein by Dot were represented in Table 4.4. The cut-off values for this protein in three subtypes of dengue fever was ≤102.13, >153.97 and >124.18. The AUC value for DF, DHF and DSS was 0.526, 0.864and 1.0. The sensitivity values of DF, DHF and DSS was 30, 74.19and 100. The specificity values of DF, DHF and DSS were 86.67, 90.32 and 100.

Variable	Cut off	AUC 95% CI	Sensitivity	Specificity	PPV	NPV	P Value
Total HBB	≤155.92	0.512(0.382-0.641)	77.42(58.9-90.4)	3.23(0.08-16.7)	25.3(14.6-38.6)	25.3(1.6-73.3)	0.87
DF	≤102.13	0.526(0.392-0.656)	30(14.7-49.4)	86.67(69.3-96.2)	48.7(19-79.1)	74.6(60.1-85.9)	0.74
DHF	>153.97	0.864(0.753-0.938)	74.19(55.4-88.1)	90.32(74.2-98)	76.4(50.8-92.9)	89.2(76.2-96.5)	0.01
DSS	>124.18	1(0.794-1.0)	100(63.1-100)	100(63.1-100)	100(46-100)	100(72-100)	0.01

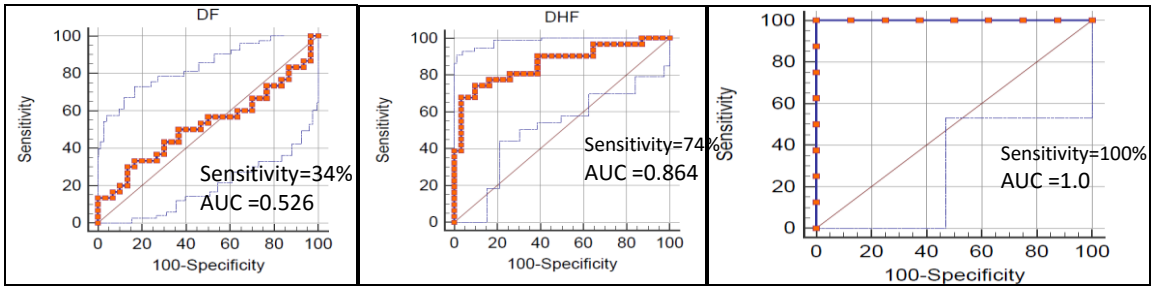
Table 4.4: ROC curve analysis of Hemoglobin beta by Dot blot.

When we compared Dot blot values of these two proteins by ROC analysis, the AUC value for Calpain-7 was between 0.691 to 0.755 (Figure 4.18) and the AUC value of Hemoglobin beta was between 0.526 to 1.0 but may be due to low sample size AUC value of DSS was 1.0. Both tests have good diagnostic performance (Figure 4.19).



(A) (B) (C)

Figure 4.18: ROC curve analysis of Dot blot of Calpain-7. (A): DF; (B): DHF; and (C): DSS.



(A) (B) (C)

Figure 4.19: ROC curve analysis of Dot blot of hemoglobin beta. (A): DF; (B): DHF; and (C): DSS.

Finally, when we compared DAC-ELISA values of Calpain-7 with hemoglobin beta, Calpain-7 has AUC value between 0.834 to 0.959 and sensitivity of DHF was 96.67 %. It has excellent diagnostic performance. Whereas in Dot blot, Calpain-7 has highest sensitivity of 96.13 in DHF and hemoglobin beta has highest sensitivity of 91% in DF.

4.4 Discussion

Virus or virus components are being used for the diagnosis of dengue virus infections. Platelet count, virus isolation, RT-PCR, DD-RT PCR, NS1 detection are the techniques under practice for this virus diagnosis. In addition to the above methods, detection of host responses as a result of viral infections becoming an alternative in dengue viral infections as well as many other cases. In such instances, these host related components can be considered as targets for therapeutics. Studies for identifying differentially regulated genes in dengue virus infected mosquitoes, cell

lines showed interesting observations. Up-regulation of genes involved in innate immunity was noticed in the HepG2 cell lines infected with dengue virus 2 and controlling the virus infection. Although several investigations were carried out to understand the immuno-pathogenesis of dengue virus infections, several aspects remains unclear.

The two proteins Calpain-7 and hemoglobin beta were considered for standardized and evaluation of DAC-ELISA and Dot blot using a large set of infected and control samples. These two proteins were significantly increased in three subtypes of dengue fever compared to control samples by DAC-ELISA and were shown (Figure 4.3). In the same way, these two proteins were also significantly increased in infected samples by Dot blot was shown (Figure 4.6 and 4.7).

During disease progression, the Calpain-7 protein levels were increased from day 1 to day 4 compared to control and decreased to normal by day 6 (Figure 5.1A) which suggested that this protein may have some role in dengue viral infections. Whereas for Hemoglobin beta, it is increased from day 1 to day 6 (Figure 5.1B).

DAC- ELISA results of Calpain-7 and Hemoglobin beta were compared among different serotypes, where Calpain-7 levels were significantly high in DEN1, DEN2, and DEN4 and in co-infections compared to control samples but not significant in DEN3 samples may be due to low sample size (Figure 5.3). Whereas, hemoglobin beta levels were not significantly increased in all four serotypes and in co- infections.

In the same way, Dot blot intensity values of Calpain-7 were compared among different serotypes and found that Calpain-7 levels were significantly increased in only DEN 1 and DEN2 serotypes but not significant in DEN 3 and DEN4 and in co-infections (Figure 5.5). Whereas, dot intensity levels of hemoglobin beta were not significantly increased in all four serotypes and in co-infections. From this, we can say that DAC-ELISA and Dot blot results of Calpain-7 and hemoglobin beta are not much correlated to serotypes.

When we compare DAC-ELISA values of Calpain-7 with hemoglobin beta, Calpain-7 has AUC values ranged from 0.834 to 0.959 and sensitivity for DHF was 96.67 %. It has excellent diagnostic performance. Whereas in Dot blot, Calpain-7 has highest sensitivity of 96.13 for DHF and hemoglobin beta has highest sensitivity of 91% in DF.

Based on the accuracy of DAC-ELISA and Dot blot, ELISA with Calpain-7 antibody is recommended as a better diagnostic test for dengue virus infections.

CHAPTER 5

Summary and Conclusions

World health Organization (WHO) reported that Southeast Asia and Western Pacific regions reports more cases with dengue infections and contribute for nearly 75% of the dengue disease burden. Among Southeast Asian countries, India is refereed as hyper-endemic which reports frequent outbreaks with case fatality rates as high as 3-5%. For the proper treatment, prevention and control of any infectious disease, thorough characterization of the causal agent and an ideal diagnostic tool are required. Although dengue was earlier considered as mild self limiting disease, current reports are indicating high mortality. Lack of precise diagnostic tool for the dengue virus is one of the drawbacks in treating infected people at right time. The symptoms mimic with many of the other diseases like chikungunya, malaria and leptosporosis. Hence there is a demand for the development of specific diagnostic tool for this disease during early stages of infection. Identification of differentially regulated proteins in dengue virus target cells (platelets, monocytes, macrophages, neutrophils) which can be used as biological markers is a promising approach in this direction. These markers can be used to diagnose the disease or can be considered as targets for designing specific antivirals. Viremia occurs within 4 days of dengue virus infection where as antibody response will be there only after two weeks. Hence virus related antibody based tests drags time. Detection of non structural protein -1 (NS1) of dengue virus is also considered as one of the diagnostic tool which is also having limitations. Since the currently using virus specific antibody based diagnostic tests take time to confirm the disease and hence host antigen based tests are gaining importance for the detection of the disease during early stages of the infection. Hence, the aim of the present study is in identification of differentially regulated proteins in virus infected blood/serum samples which could be used as biological markers. These markers can be used to diagnose the disease or considered as targets for designing antivirals. The study also proceeded for the standardization and evaluation of diagnostic tests using the above identified proteins.

The major outlines of the findings of the present study are as follows.

Available information about dengue from Southeast Asia was collected from Pro-MED mail (Program for Monitoring Emerging Diseases) as case reports. Pro-MED mail is the informal online source reporting dengue information and the information is available from ten countries from Southeast Asia. The number of cases in each report from Southeast Asia was grouped for every country from the year 2012-2015. Confirmed dengue fever and death case information was also collected from NVBDCP (National vector-borne disease control program) from 2010-2015. Information about chikungunya and Japanese information also obtained from the NVBDCP along

with dengue information. The data indicated that in the year 2012 and 2013 India occupied second place in disease burden and in 2014 third but in 2015 India held first place in disease burden.

NVBDCP is the official information source for dengue cases which reports only from India. 28,292 confirmed dengue positive cases and 110 deaths were reported by NVBDCP in 2010. Similarly, in 2011, 18, 860 cases and 169 deaths, in 2012, 50,222 cases and 242 deaths, in 2013, 75,808 cases and 193 deaths, in 2014, 40,571 cases and 137 deaths and in 2015, 99,913 cases and 220 deaths were reported by NVBDCP . Reports of both sources showed increased number of infections and deaths which suggest the increasing burden of dengue infections during recent years.

The study also noticed the increased burden of dengue from South India especially from Telangana and Andhra Pradesh from 2009-2015 and particularly in 2015 nearly five thousand cases were reported from Telanagana and Andhra Pradesh. In Telangana, disease burden-wise Khammam district occupied first place followed by Hyderabad in 2015. So Hyderabad district was considered as one of the hot spot regions for dengue viral infections and was chosen for the collection of dengue virus infected samples used in the present study. Along with dengue, other viral infections such as Japanese encephalitis and chikungunya infections also prevalent and dengue disease burden is more when we compared to other diseases.

A total of 199 pediatric cases admitted with clinical symptoms of dengue infection during the period 2014-2015. 40 infected samples in 2014 and 109 in 2015 were collected along with 50 other febrile illness control samples. In total, 149 dengue positive samples were collected in two years for which the serotype analysis was also carried out in our laboratory. All four dengue serotypes and co-infections with more than one serotype were detected.

We have identified eight proteins using 2D GE followed by MALDI-TOF in dengue infected samples compared to controls. They are Human transthyretin, calpain-7, ZNF224, CGI-105, Keratin type1 cytoskelital9 and Keratin type 2cytoskelital1, hemoglobin beta and myoglobin. Mascot scores of all eight proteins are significant ($p<0.05$). Further we have also identified eight differentially expressed proteins in disease progression samples. They are RIP, Ankyrin repeat domain, olfactory receptor, Mitochondrial ribosomal protein L17, ATP-dependent DNA helicase, Very large A-kinase anchor protein, Potassium channel subfamily k member, and Immunoglobulin heavy chain variable region.

Among the above identified proteins, Calpine 7 and Hemoglobin beta were selected based on their functional relatedness to dengue for the standardization and evaluation of diagnostic tests.

The chosen tests for the study were DAC-ELISA and Dot-ELISA.

During the standardization step, only limited samples were used to examine the levels of candidates for its ability to discriminate between dengue infected and controls using DAC- ELISA and Dot Blot assays. For the evaluation of the above tests the sample size was increased.

Calpain-7 demonstrated the greatest potential to distinguish between control and dengue infected samples compared to the hemoglobin beta.

The data using the disease progression samples indicated that the levels of calpine 7 were found to be increased from day 1 to day 4 and resume to the normal level by day 6.

Calpain-7 and Hemoglobin beta were compared using samples with infections of different serotypes and found that only Calpain-7 levels were significantly high in DEN1, DEN2, and DEN4 and co- infections compared to control samples but not significant in DEN3 samples may be due to low sample size.

The statistical analysis using ROC curve analysis suggested that calpine 7 has significant AUC and sensitivity values compared to the hemoglobin beta of both DAC-ELISA and Dot ELISA.

Based on the overall data of DAC-ELISA and Dot blot, ELISA with Calpain-7 antibody is recommended as diagnostic test for dengue virus infections.

References:

1. Halstead SB. (2008) Dengue (Tropical Medicine: Science and Practice). River Edge, *N.J: Imperial College Press*, pp. 1–10.
2. Duane J. Gubler Epidemic Dengue/Dengue Hemorrhagic Fever: A Global Public Health Problem in the 21st Century.
3. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL. (2013) The global distribution and burden of dengue. *Nature*, 496, 504-7.
4. World Health Organization. Handbook for clinical management of dengue. Geneva: WHO, 2012. Available at www.who.int/denguecontrol/9789241504713/en/ (accessed 14 December 2013).
5. World Health Organization Dengue and Severe Dengue. Media Centre Fact Sheet. WHO, 2013.Available from: http://www.who.int/mediacentre/factsheets/fs117/en/. [Last accessed on 2014 Feb 08].
6. Thavara U, Tawatsin A, Chansang C, Kong-ngamsuk W, Paosriwong S, Boon-Long J. (2001) Larval occurrence, oviposition behavior and biting activity of potential mosquito vectors of dengue on Samui Island, Thailand. *J Vector Ecol*, 26, 172-80.
7. Perich MJ, Davila G, Turner A, Garcia A, Nelson M. (2000) Behavior of resting *Aedes aegypti* (culicidae: diptera) and its relation to ultra-low volume adulticide efficacy in recent years. Panama City, Panama. *J Med Entomol*, 37, 541-6.
8. Gibbons RV, Vaughn DW. (2002) Dengue: An escalating problem. *BMJ*, 324, 1563-6.
9. McBride WJ, Bielefeldt-Ohmann H. Dengue viral infections: Pathogenesis and epidemiology. *Microbes Infect*, 2, 1041-50.
10. Gubler DJ. (2004) The changing epidemiology of yellow fever and dengue, 1900 to 2003: full circle? *Comp Immunol Microbiol Infect Dis*, 27, 319–330.
11. World Health Organization. (2014) Dengue and severe dengue. Fact sheet no. 117, March Geneva: WHO, 2014. http://www.who.int/ mediacentre/factsheets/fs117/en.
12. Nimmannitya S. (2009) Dengue and dengue haemorrhagic fever; in Cook GC, Zumla AI (Eds): *Manson's Tropical Diseases*, 22nd ed. London, Saunders. pp 753–762.

13. Dash PK, Parida MM, Saxena P, Abhyankar A, Singh CP, Tewari KN. (2006) Reemergence of dengue virus type-3 (subtype-III) in India: Implications for increased incidence of DHF & DSS. *Virol J*, July. 3: 55.
14. Broor S, dar L, Sengupta S, Chakraborty M, Wali J P, Biswas A, Kabra S K, Jain Y, Seth P. (1997) Recent dengue epidemic in Delhi, India. In Factors in the emergence of arbovirus diseases, edited by:saluzzo JE, Dode B, Paris : *Elsevier*, 123-27
15. Dar l, Broor S, Sengupta S, Xess I, Seth P. (1999) The first major outbreak of dengue hemorrhagic fever in Delhi, India. *Emerg Infect Dis*, 5, 589-90.
16. Gupta N, Srivastava S, Jain A, Chaturvedi UC. (2012) Dengue in India. *Indian J of Med Res*, 136, 373 –390.
17. Chakravarti A, Matlani M, Kashyap B, Kumar A. (2012) Awareness of changing trends in epidemiology of dengue fever is essential for epidemiological surveillance. *Indian J Med Res*, 30, 222–226.
18. Karamchandani PV. (1946) Dengue group of fevers in India. *Lancet*, 1(6386):92.
19. Ramakrishanan SP, Gelfand HM, Bose PN, Sehgal PN, Mukharjee RN. (1964) The epidemic of acute haemorrhagic fever, Calcutta: epidemiological inquiry. *Indian J Med Res*, 52, 633–650.
20. Sarkar JK, Pavri KM, Chatterjee SN, Chakravarty SK, Anderson CR. (1964) Virological and serological studies of cases of haemorrhagic fever in Calcutta. *Indian J Med Res*, 52, 684–691.
21. Balaya S, Paul SD, D Lima LV, Pavri KM. (1969) Investigations on an outbreak of dengue in Delhi in 1967. *Indian J Med Res*, 57, 767–774.
22. Ghosh BN. (1968) A study on the epidemic of dengue-like fever in Pondicherry (1964–65 and 1965–66) *J Indian Med Assoc*, 51, 261–264.
23. Gupta N, Srivastava S, Jain A, Chaturvedi UC. (2012) Dengue in India. *Indian J of Med Res*, 136, 373–390.
24. Chakravarti A, Matlani M, Kashyap B, Kumar A. (2012) Awareness of changing trends in epidemiology of dengue fever is essential for epidemiological surveillance. Indian J Med Res, 30, 222–226.
25. Gurugama P, Garg P, Perera J, Wijewickrama A, Senevirathe SL. (2010) Dengue viral infections. *Indian J Dermatol*, 55(1), 68-78

26. Christian Julián Villabona-Arenas, Paolo Marinho de Andrade Zanotto (2011) *Infection, Genetics and Evolution*, 11 (5), 878-885.
27. Chang GJ. (1997) Molecular biology of dengue viruses. In: Dengue and dengue hemorrhagic fever. Gubler DJ, Kuno G, eds. Cambridge: *CAB International*, p. 175-98
28. Wahala WM, Silva AM. (2011) The human antibody response to dengue virus infection. *Viruses*, 3, 2374–2395
29. Simmons CP, Farrar JJ, Nguyen vV, Wills B. (2012) Dengue. *N Engl J Med*, 366, 1423.
30. World Health Organization. Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2nd ed. Geneva: WHO; 1997
31. Guzman MG, Kouri G. (2002) Dengue: an update. *Lancet Infect Dis*, 2, 33–42
32. Seet RCS, Ooi E, Wong HB, Paton NI. (2005) An outbreak of primary dengue infection among migrant Chinese workers in Singapore characterized by prominent gastrointestinal symptoms and a high proportion of symptomatic cases. *J Clin Virol*, 33, 336–40.
33. Henchal EA, Putnak JR. (1990) The dengue viruses. *Clin Microbiol Rev*, 3, 376-96
34. Rush B. (1789) An account of the bilious remitting fever, as it appeared in Philadelphia in the summer and autumn of the year 1780. In: Garrison-Morton, ed. *Medical inquiries and observations*. Philadelphia: Pritchard & Hall, p. 104.
35. Guzman MG, Kouri G. (2002) Dengue: an update. *Lancet Infect Dis*, 2, 33–42.
36. Gubler DJ. (1998) Dengue and dengue hemorrhagic fever. *Clin Microbiol Rev*, 11, 480–96
37. N. Gupta, S. Srivastava, A. Jain, and U. C. Chaturvedi (2012) "Dengue in India," The *Indian Journal of Medical Research*, vol.136, no. 3, pp. 373–390.
38. Bharaj P, Chahar HS, Pandey A, Diddi K, Dar L, Guleria R. (2008) Concurrent infections by all four dengue virus serotypes during an outbreak of dengue in 2006 in Delhi, India. *Virol J*, 5:1.
39. Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ. (1997) Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med*, 3, 86671.
40. Tassaneetrithep B, Burgess TH, Granelli-Piperno A, Trumpfheller C, Finke J, Sun W. (2003) DC-SIGN (CD209)mediates dengue virus infection of human dendritic cells. *J Exp Med,* 197, 8239.

41. Miller JL, de Wet BJ, Martinez-Pomares L, Radcliffe CM, Dwek RA, Rudd PM. (2008) The mannose receptor mediates dengue virus infection of macrophages. *PLoS Pathog,* 4: e17
42. Heinz FX, Allison SL. (2003) Flavivirus structure and membrane fusion. *Adv Virus Res*, 59, 6397.
43. Clyde K, Kyle JL, Harris E. (2006) Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J Virol, 80, 1141831.
44. Green S, Rothman A. (2006) Immunopathological mechanisms in dengue and dengue hemorrhagic fever. *Curr Opin Infect Dis*, 19, 429–36.
45. Halstead SB, O'Rourke EJ. (1977) Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. *J Exp Med*, 146, 201–17.
46. King CA, Marshall JS, Alshurafa H, Anderson R. (2000) Release of vasoactive cytokines by antibody-enhanced dengue virus infection of a human mast cell/basophil line. *J Virol*, 74, 7146-50
47. Mitrakul C, Poshyachinda M, Futrakul P, Sangkawibha N, Ahandrik S. (1977) Hemostatic and platelet kinetic studies in dengue hemorrhagic fever. *Am J Trop Med Hyg,* 26, 975–984.8 Choudhury N:Bl
48. Hayes EB, Gubler DJ. (1992)Dengue and dengue hemorrhagic fever. *Pediatr Infect Dis J*, 11, 311-7
49. Dengue: guidelines for diagnosis, treatment, prevention and control. Geneva (Switzerland): World Health Organization; 2009. Figure 2.1: The course of dengue illness.p.25.Availablefrom:http://whqlibdoc.who.int/publications/2009/9789241547871_eng.pdf?ua=1(accessed 2014 Jul 27).
50. Chen LH, Wilson ME. (2010) Dengue and chikungunya infections in travelers. *Curr Opin Infect Dis*, 23 (5), 438–444.
51. Simmons CP, Farrar JJ, Nguyen vV, Wills B. (2012) Dengue. *N Engl J Med*, 366 (15), 1423–1432.
52. Section 27: Viral infections of skin and mucosa. In: Wolff K, Johnson RA. Fitzpatrick's color atlas and synopsis of clinical dermatology.6th ed. New York (NY): McGraw-Hill Professional; 2009. Subsection "Infectious exanthems: dengue fever"; p. 810–812.20. Gould EA, Solomon T. Pathogenic flaviviruses.

53. Wright S, Jack M. Tropical medicine (chapter 21. In: Knoop KJ, Stack LB, Storrow AB, Thurman RJ (2009) Atlas of emergency medicine. 3rd ed. New York (NY): *McGraw-Hill Professional* p. 649-687.
54. Gould EA, Solomon T. (2008) Pathogenic flaviviruses. *Lancet*, 371(9611), 500–509.
55. Ranjit S, Kissoon N. (2011) Dengue hemorrhagic fever and shock syndromes. *Pediatr Crit Care Med*, 12(1), 90–100
56. Rodenhuis-Zybert IA, Wilschut J, Smit JM. (2010) Dengue virus life cycle: viral and host factors modulating infectivity. *Cell Mol Life Sci*, 67(16), 2773–2786.
57. King A., Innis B.L., Caudle L. (1991) B-cells are the principal circulating mononuclear cells infected by dengue virus. *Faseb J*, 5a, 9998.
58. Guzman M.G. and Kouri G. (1996) Advances in dengue diagnosis. *Clin Diagn Lab Immnunol*, 3, 621-7.
59. W.F. Wright, B.S. Pritt (2012) Update: the diagnosis and management of dengue virus infection in North America, Diagn. *Microbiol Infect Dis*, 73, 215–220.
60. K.-I. Yamada, T. Takasaki, M. Nawa, I. Kurane (2002) Virus isolation as one of the diagnostic methods for dengue virus infection, *J. Clin. Virol*, 24, 203–209.
61. Jarman RG, Nisalak A, Anderson KB. (2011) Factors influencing dengue virus isolation by C6/36 cell culture and mosquito inoculation of nested PCR-positive clinical samples. *Am J Trop Med Hyg*, 84, 218-223.
62. Gibbons R, Vaughn D (2002) Dengue: an escalating problem. BMJ, 324, 1563-1566.
63. Lam SK. (1986) Isolation of dengue viruses by intracerebral inoculation of mosquito larvae*. J Virol Methods*, 14, 133 - 40.
64. Rosen L, Gubler D. (1974) The use of mosquitoes to detect and propagate dengue viruses. *Am J Trop Med Hyg*, 23, 1153 - 60.
65. Yamada K, Takasaki T, Nawa M, Kurane I. (2002) Virus isolation as one of the diagnostic methods for dengue virus infection. *J Clin Virol*, 24, 203-209.
66. Suwandono A, Kosasih H, Nurhayati N, Kusriastuti R, Harun S. (2004) Four dengue virus serotypes found circulating during an outbreak of dengue fever and dengue haemorrhagic fever in Jakarta, Indonesia*. Trans R Soc Trop Med Hyg*, 100, 855-862.

67. Kumaria R, Chakravarti A. (2005) Molecular detection and serotypic characterization of dengue viruses by single-tube multiplex reverse transcriptase- polymerase chain reaction. *Diagn Microbiol Infect Dis*, 52, 311-316.
68. Lanciotti, R. S., Calisher, C. H., Gubler, D. J., Chang, G. J. & Vorndam, V. (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J. Clin. Microbiol*, 30, 545 - 551).
69. Johnson, B. W., Russell, B. J. & Lanciotti, R. S. (2005) Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. *J. Clin. Microbiol,* 43, 4977–4983.
70. Dussart (2006) Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. *Clin. Vaccine Immunol*, 13, 1185–1189
71. Innis, B. L. (1989) An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am. J. Trop. Med Hyg*, 40, 418–427.
72. Kuno, G., Gomez, I. & Gubler, D. J. (1991) An ELISA procedure for the diagnosis of dengue infections. *J. Virol. Meth*, 33, 101–113.
73. M.D.L. Oliveira, M.T.S. Correia, F.B. Diniz (2009b) A novel approach to classify serum glycoproteins from patients infected by dengue using electrochemical impedance spectroscopy analysis, *Synth Met*, 159, 2162–2164.
74. Simmons CP, Farrar JJ, Chau NVV, Wills B. (2012) Dengue. *N Engl J Med*, 366, 1423–32.
75. San Martin JL, Brathwaite O, Zambrano B, Solorzano JO, Bouckenooghe A, Dayan GH. (2012) The epidemiology of dengue in the Americas over the last three decades: A worrisome reality. *Am J Trop Med Hyg*, 82(1), 128–35.
76. WHO (2009) Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control. World Health Organization.
77. Bhatt S, Gething PW, Brady OJ, Messina JP, FarlowAW, Moyes CL. (2013). The global distribution and burden of dengue. *Nature*, 496, 504–507.
78. Simmons CP, Farrar JJ, Nguyen v V, Wills B. (2012) Dengue. *NEngl J Med*, 366, 1423–1432.
79. Dash AP, Bhatia R, Sunyoto T, Mourya DT. (2013) Emerging and re-emerging arboviral diseases in Southeast Asia. *J Vector Borne Dis*, 50, 77–84.

80. Kumar A, Rao CR, Pandit V, Shetty S, Bammigatti C, Samarasinghe CM. (2010) Clinical manifestations and trend of dengue cases admitted in a tertiary care hospital, Udupi district, Karnataka. *Indian J Community Med*, 35, 386–90.
81. Garg A, Garg J, Rao YK, Upadhyay GC, Sakhuja S. (2011) Prevalence of dengue among clinically suspected febrile episodes at a teaching hospital in North India. *J Infect Dis and Immu*, 3(5), 85–9.
82. Barde PV, Kori BK, Shukla MK, Bharti PK, Chand G, Kumar G. (2015) Maiden outbreaks of dengue virus 1 genotype III in rural central India. *Epidemiol Infect*, 143, 412–8.
83. Barde PV, Godbole S, Bharti PK, Chand G, Agarwal M, Singh N. (2012) Detection of dengue virus 4 from Central India. *Indian J Med Res*, 136, 491–4.
84. Mahadev PV, Prasad SR, Ilkal MA, Mavale MS, Bedekar SS, Banerjee K. (1997) Activity of dengue-2 virus and prevalence of *Aedes aegypti* the Chirimiri colliery area, Madhya Pradesh, India. *Southeast Asian JTrop Med Public Health*, 28, 126–37.
85. Baruah K, Singh PK, Mohalia MM, Dhariwal AC. (2010) A study on dengue outbreak during 2009 in Bhopal and Indore districts of Madhya Pradesh, India. *J Commun Dis,* 42, 273–9.
86. Ukey P, Bondade S, Paunipagar P, Powar R, Akulwar S. (2010) Study of seroprevalence of dengue fever in Central India*. Indian J Community Med*, 35, 517–9.
87. Gubler DJ. Dengue, Urbanization and Globalization (2011) The Unholy Trinity of the 21(st) Century. *Trop Med Health*, 39(Suppl 4), 3–11.
88. Gubler DJ. (2012) The economic burden of dengue. *Am J Trop Med Hyg*, 86(5), 743–744.
89. Beatty ME, Beutels P, Meltzer MI. (2011) Health economics of dengue: a systematic literature review and expert panel's assessment. *Am J Trop Med Hyg*, 84(3), 473–488.
90. Gubler DJ. The changing epidemiology of yellow fever and dengue, 1900 to 2003: full circle? *Comp Immunol Microbiol Infect Dis*, 2004, 27, 319-30.
91. Anderson K, Chunsuttiwat S, Nisalak A, Mammen MP, Libraty DH, Rothman AL, Green S, Vaughn DW, Ennis FA, Endy TP. (2007) Burden of symptomatic dengue infection in children at primary school in Thailand: a prospective study. Lancet, 369, 1452-1459.
92. Guzman MG. (2002) Effect of age on outcome of secondary dengue 2 infections. Int J Infect Dis 6, 118-124.

93. Díaz-Quijano FA, Waldman EA. (2012) Factors associated with dengue mortality in Latin America and the Caribbean, 1995-2009: an ecological study. *Am J Trop Med Hyg*, 86, 328-334.
94. Hammond SN, Balmaseda A, Perez L, Tellez Y, Saborio SI, Mercado JC, Videa E, Rodriguez Y, Pérez MA, Cuadra R, Solano S, Rocha J, Idiaquez W, González A, Harris E. (2005) Differences in dengue severity in infants, children and adults in a 3-year hospital-based study in Nicaragua. *Am J Trop Med Hyg*, 73, 1063-1070
95. Alvarez ME, Ramirez-Ronda CH. (1985) Dengue and hepatic failure. *Am J Med*, 79, 670–674.
96. Kuo CH, Tai DI, Chang-Chien CS, Lan CK, Chiou SS, Liaw YF. (1992) Liver biochemical tests and dengue fever. *Am J Trop Med Hyg*, 47, 265–270.
97. Lei, H. Y., T. M. Yeh, H. S. Liu, Y. S. Lin, S. H. Chen, and C. C. Liu. (2001) Immunopathogenesis of dengue virus infection. J Biomed Sci, 8, 377-88.
98. Rigau-Perez, J. G., G. G. Clark, D. J. Gubler, P. Reiter, E. J. Sanders, and A.V. Vorndam (1998) Dengue and dengue haemorrhagic fever. Lancet, 352, 971-7.
99. Senanayake, S. (2006) Dengue fever and dengue haemorrhagic fever--a diagnostic challenge. *Aust Fam Physician*, 35,609-12.
100. Teles, F. R., D. M. Prazeres, and J. L. Lima-Filho (2005) Trends in dengue diagnosis. Rev Med Virol, 15, 287-302.
101. Halstead, S. B. (1988) Pathogenesis of dengue: challenges to molecular biology. Science, 239, 476–481.
102. Monath, T. P. (1994) Dengue: the risk to developed and developing countries.Proc. Natl. Acad. Sci. U.S.A, 91, 2395–2400.
103. Dotzlaw H, Schulz M, Eggert M, Neeck G. (2004) A pattern of protein expression in peripheral blood mononuclear cells distinguishes rheumatoid arthritis from healthy individuals. *BiochemBiophys Acta*, 1696, 121—9
104. O Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem, 250, 4007—21.
105. Thayan, R.; Huat, T. L.; See, L. L.; Tan, C. P.; Khairullah, N. S.; Yusof, R.; Devi, S. (2009) The use of two - dimension electrophoresis to identify serum biomarkers from

patients with dengue haemorrhagic fever. *Trans. R. Soc. Trop. Med. Hyg*, 103, 413–419.

106. Albuquerque L.M. Trugilho M.R. Chapeaurouge A. (2009) Two - dimensional difference gel electrophoresis (DiGE) analysis of plasmas from dengue fever patients. *J Proteome Res,* 8, 5431–5441.

107. Sandipan Ray, Rajneesh Srivastava, Karnika Tripathi (2012) Serum Proteome Changes in Dengue Virus-Infected Patients from a Dengue-Endemic Area of India: Towards New Molecular Targets? *OMICS*, 16(10), 527–536.

108. Srivastava R. Ray S. Vaibhav V. (2012) Serum profiling of leptospirosis patients to investigate proteomic alterations. *J Proteomics*, doi: 10.1016/j. jprot.

109. Ray S. Kamath K.S. Srivastava R. (2012a) Serum proteome analysis of vivax malaria: An insight into the disease pathogenesis and host immune response. *J Proteomics*, 75, 3063–3080.

110. Chen J.H. Chang Y.W. Yao C.W. (2004) Plasma proteome of severe acute respiratory syndrome analyzed by two-dimensional gel electrophoresis and mass spectrometry. *Proc Natl Acad Sci USA*, 101, 17039–17044.

111. Sun J.F. Shi Z.X. Guo H.C. Li S. Tu C.C. (2011) Proteomic analysis of swine serum following highly virulent classical swine fever virus infection, *Virol J*, 8, 107.

112. Louis S. Ramagli Quantifying Protein in 2-D PAGE Solubilization Buffers Methods in Molecular Biology, Vol. 112, 2-D Proteome Analysis Protocols.

113. Kakisaka T, Kondo T, Okano T, Fujii K, Honda K, Endo M, Tsuchida A, Aoki T, Itoi T, Moriyasu F, Yamada T, Kato H, Nishimura T, Todo S, Hirohashi S. (2007) Plasma proteomics of pancreatic cancer patients by multi-dimensional liquid chromatography and two-dimensional difference gel electrophoresis (2D-DIGE): up-regulation of leucine-rich alpha-2-glycoprotein in pancreatic cancer, 852(1-2), 257-67.

114. Andrej Shevchenko, Ole N. Jensen, Alexandre V. Podtelejnikov,Francis Sagliocco, MatthiasWilm, Ole Vorm, Peter Mortensen, Anna Shevchenko, Helian Boucherie, and Matthias Mann(1996) Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. *Proc Natl Acad Sci U S* A, 93(25), 14440–14445.

115. Prealbumin at the US National Library of Medicine Medical Subject Headings (MeSH)

116. Myron Johnson, A.; Merlini, G.; Sheldon, J.; Ichihara, K. (2007) Clinical indications for plasma protein assays: transthyretin (prealbumin) in inflammation and malnutrition. *Clin. Chem. Lab. Med*, 45, 419–426.

117. Wang, Z.; Burke, P. A. (2007) Effects of hepatocyte nuclear factor-4alpha on the regulation of the hepatic acute phase response. *J. Mol. Biol*, 371, 323–335.

118. Borish, L.; King, M. S.; Mascali, J. J.; Johnson, S.; Coll, B.; Rosenwasser, L. J. (1992) Transthyretin is an inhibitor of monocyte and endothelial cell interleukin-1 production. *Inflammation*, 16, 471–484

119. Wan, J.; Sun, W.; Li, X.; Ying, W.; Dai, J.; Kuai, X.; Wei, H.; Gao, X.; Zhu, Y.; Jiang, Y.; Qian, X.; He, F. (2006) Inflammation inhibitors were remarkably up-regulated in plasma of severe acute respiratory syndrome patients at progressive phase. *Proteomics,* 6, 2886 – 2894.

120. Liu, L.; Liu, J.; Dai, S.; Wang, X.; Wu, S.; Wang, J.; Huang, L.; Xiao, X.; He, D. (2007) Reduced transthyretin expression in sera of lung cancer. *Cancer Sci*, 98, 1617–1624.

121. Goll DE, Thompson VF, Li H, Wei W & Cong J (2003) The calpain system. *Physiol Rev,* 83, 731–801.

122. Ono Y & Sorimachi H (2012) Calpains: an elaborate proteolytic system. Biochim Biophys Acta, 1824, 224–236.

123. Turner, J. and Crossley, M. (1999) Mammalian Krüppel-like transcription factors: more than just a pretty finger *Trends Biochem. Sci*. 24, 236 - 240.

124. Philipsen, S. and Suske, G. (1999) A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res*. 27, 2991- 3000.

125. Witzgall, R., O'Leary, E., Leaf, A., Onaldi, D. and Bonventre, J.V. (1994) The Krüppel-associated box-A (KRAB-A) domain of zinc finger proteins mediates transcriptional repression. *Proc. Natl. Acad. Sci. USA* 91, 4514-4518.

126. Margolin, J.F., Friedman, J.R., Meyer, K.H.K., Vissing, H., Thiesen, H. and Rauscher III, F. (1994) Proc. *Natl. Acad. Sci.* USA 91, 4509-4513.

127. Agata, Y., Matsuda, E. and Shimizu, A. (1999) Two novel Krüppel-associated box-containing zinc-finger proteins, KRAZ1 and KRAZ2, repress transcription through functional interaction with the corepressor KAP-1 (TIF1beta/KRIP-1). *J. Biol. Chem*, 274, 16412-16422.

128. Schultz, D., Friedman, J. and Rauscher III, F. (2001) Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. *Genes Dev*, 15, 428-443.

129. Lupo, A., Costanzo, P., Medugno, L., Romeo, I., Salvatore, F. and Izzo, P. (1997) Negative regulation of the mouse aldolase A gene. A cell cycle-dependent DNA binding activity functions as a silencer of gene transcription. *J. Biol. Chem*, 272, 31641-31647.

130. R.L. Strausberg, E.A. Feingold, L.H. Grouse, J.G. Derge, R.D. Klausner, F.S. Collins, L. Wagner, C.M. Shenmen, G.D. Schuler. (2002) Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc. Natl. Acad. Sci. U. S*. A, 99, pp. 16899–16903.

131. B.A. Manjasetty, F.H. Niesen, H. Delbruck, F. Gotz, V. Sievert, K. Bussow, J. Behlke, U. (2004) Heinemann X-ray structure of fumarylacetoacetate hydrolase family member Homo sapiens FLJ36880 *J. Biol. Chem,* 385, pp. 935–942.

132. Saksena S, Sun J, Chu T & Emr SD. (2007) ESCRTing proteins in the endocytic pathway. Trends Biochem Sci, 32, 561–573.

133. Williams RL & Urbe S. (2007) the emerging shapes of the ESCRT machinery. *Nat Rev Mol Cell Biol*, 8, 355–368.

134. Lindsay N. Carpp, Ricardo Galler, Myrna C. Bonaldo (2011) Interaction between the yellow fever virus nonstructural protein NS3 and the host protein Alix contributes to the release of infectious particles. *Microbes and Infection*, 13, 85 - 95.

135. McDonald B & Martin-Serrano J. (2009) No strings attached: the ESCRT machinery in viral budding and cytokinesis. *J Cell Sci*, 122, 2167–2177.

136. Dan Li, Hong Dong, Su Li, Muhammad Munir. (2013) Hemoglobin Subunit Beta Interacts with the Capsid Protein and Antagonizes the Growth of Classical Swine Fever Virus. *Journal of Virology*, p. 5707–5717.

137. Doolittle JM, Gomez SM. (2011) Mapping protein interactions between Dengue virus and its human and insect hosts, *PLoS,* 5: e954

138. Kumar Y, Liang C, Bo Z, Rajapakse JC, Ooi EE. (2012) Serum Proteome and Cytokine Analysis in a Longitudinal Cohort of Adults with Primary Dengue Infection

Reveals Predictive Markers of DHF. *PLoS Negl Trop Dis,* 6(11), e1887. doi:10.1371/journal.pntd.0001887.