Molecular Typing of Dengue Virus Isolated from Field Caught Mosquito Specimens by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

A dissertation submitted to the University of Dhaka in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Zoology

Genetics and Molecular Biology Branch
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Bangladesh
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Certificate

This is to certify that the research work embodying the results reported in this PhD thesis entitled "**Molecular Typing of Dengue Virus Isolated from Field Caught Mosquito Specimens by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**" submitted by **Shaila Nazneen** (Registration number 29/2005-06; Re admission 51/2010-11), has been carried out under our supervision in the **Genetics and Molecular Biology Branch**, Department of Zoology, University of Dhaka. It is further certified that the research work presented here is original and suitable for submission for the partial fulfillment of the degree of Doctor of Philosophy in Zoology, University of Dhaka, Dhaka-1000.

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Dedicated to
My Beloved Daughter

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Abstract

The Dengue viruses are borne by the *Aedes* mosquito. They cause dengue fever in most tropical areas of the world. The detection and successful typing of dengue virus (DENV) from field caught *Aedes* mosquito is important for virological surveillance and implementation of epidemiologic control measure. We have applied a rapid and sensitive, nested reverse transcription-polymerase chain reaction (RT-PCR) assay for the type-specific detection of dengue viruses in artificially infected and in field caught mosquitoes for first time in Bangladesh.

Small scale bionomics study was done in few selected areas of Dhaka city to help find the recent dengue prevalent season, dengue vector breeding sites and the peak season of vector population. Findings of bionomics study have increased the chance of getting dengue infected mosquitoes from field. In bionomics study of *Ae. aegypti* higher number of mosquito population (85.15% eggs, larvae or pupae) were found in artificial container than in natural container (14.85%), indicating recent creation and availability of huge breeding ground for *Aedes aegypti*. Increased use of non biodegradable plastics, pot gardening, water logging and water storage habit of Dhaka city dwellers may have influenced higher mosquito densities by providing many more breeding sites. Larvae survey showed the Breteau Index of 20-40, well above the risk level for dengue virus transmission and the post monsoon period (August to October) was found to be the most affected period for larval growth. The Indoor resting adult populations start rising from August and the peak mosquito population and higher biting rate were found from September to November, which was consistent with the larvae survey.

Field studies were conducted during November-December 2010, 2011 and from July to December 2012. Adult *Aedes aegypti* mosquitoes were caught from selected dengue prevalent areas of Dhaka city and tested for the presence of dengue virus by ELISA and RT-PCR method. The RT-PCR assay was sensitive enough to detect dengue virus in lab infected mosquito- thorax pool. Almost all ELISA positive mosquito specimens were found to be positive for dengue virus by RT-PCR (90% sensitivity compared to ELISA). Approximately 4.8%, (9 out of 188) mosquito pools (9-13 mosquitoes /pool) were found to be positive for dengue viruses. Of the nine RT-PCR positive *Ae. aegypti* pools six pools were (66.66%) positive for dengue virus 3

(DEN3), two (22.2%) were positive for DEN 2 virus and only one (11.11%) were positive for dengue 4 (DEN 4) viruses. No DEN1 virus positive pool could be recorded during this study. The predominant virus type responsible for the current dengue fever is DEN 3 and multiple virus serotypes also co-circulating in Dhaka city. Majority of the dengue virus-positive mosquitoes harbored DEN-3 virus, which had been the predominant dengue virus type responsible for the local dengue epidemic since 1964. Multiple serotypes were found in Dhanmondi R/A (serotype 3 and 4) and Mirpur (serotype 2 and 3). DEN-3 alone was detected in Rampura and DEN-2 in Dhaka University Campus and no dengue virus was detected in Shegunbagicha. *Ae aegypti* caught at the five indoor stations had MIR (minimum infection rate) ranging from 0.00-1.63 per 100 mosquitoes with an MIR of 0.72 for the combined stations. Dhanmondi residential area showed the highest (1.63) and Shegunbagicha showed the lowest (0.00). Dengue positive mosquito pools were found between end of the September to mid October in three dengue prevalent seasons 2010, 2011 and 2012.

These findings give us impression that the predominant perhaps the most virulent serotype virus type responsible for the current dengue fever is DEN-3 and multiple virus serotypes are also co-circulating in Dhaka city. We found that the overall rate of dengue virus infection per 100 *Ae. aegypti* females during the study period was as high as 0.72, giving us an impression that a low grade infection and transmission is currently present in Dhaka city. Evidence of more than one viral serotypes should be taken as a warning that outbreaks of DHF might occur in the future, although a low grade infection and transmission is currently present in Dhaka city. RT-PCR based surveillance of dengue viral infection in *Ae. aegypti* mosquitoes could serve as an early warning monitoring system of dengue outbreak.

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ABBREVIATIONS

Ae.	*Aedes*
ADE	Antibody –dependent enhancement
AFRIMS	Armed forces research institutes of Medical Science
BI	Breteau Index
BP	Base pare
BSMMU	Bangabandhu Sheikh Mujib Medical University
C	Capsid
°C	Degree centrigrade
CF	Complement fixation
CI	Container Index
cDNA	Complementary Deoxyribonucleic Acid
DALY	Disability Adjusted Life Years
DEN-1	Dengue-1
DEN-2	Dengue-2
DEN-3	Dengue-3
DEN-4	Dengue-4
DENV	Dengue virus
DF	Dengue fever
DFA	Direct fluorescent antibody
DNA	Deoxyribonucleic acid
DHF	Dengue Hemorrhagic fever
DSS	Dengue shock syndrome
dNTPs	Deoxynucleoside triphosphates
DUC	Dhaka University campus
E	Envelop

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EDTA	Ethylene di-amine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FITC	Fluorescin iso-thiocianate
g	Gram
h	Hour
HI	House Index
HI	Haemagglutination inhibition
HRP	Horse reddish peroxidase
ICTV	International Committee on Taxonomy of viruses
IFA	Indirect fluorescent antibody
Ig G	Immunoglobulin G
Ig M	Immunoglobulin M
Kb	Kilo base
M	Membrane
MAb	Monoclonal antibody
MAC ELISA	IgM capture enzyme linked immunosorbent assay
MEM	Minimum essential media
µl	Microlitre
µg	Microgram
mL	Mililitre
mM	Milimeter
mM	Milimol
MIR	Minimum Infection Rate
min	Minute
4G2	Mouse anti flavivirus monoclonal detector

nm	Nanometer
NS	Non-structural
NT	Neutralization test
OD	Optical density
pH	Negative logarithm of the hydrogen ion concentration
PAHO	Pan American Health Organization
PBS	Phosphate Buffer Saline
PBS-TW	PBS-Tween
PCR	Polymerase Chain Reaction
Pr M	Pre membrane
RC	Replication complex
RNA	Ribonucleic acid
rpm	Revolution per minute
RT	Reverse Transcription
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RT	Room temperature
RH	Room Humidity
SARIMA	Seasonal Autoregressive Integrated Moving Average
SD	Standard Deviation
SEARO	South East Asian Regional Organization
TBE	Tris -borate EDTA
TBS	Tris Buffer Saline
UV	Ultra Violate
USA	United States of America
V	Volt
WHO	World Health Organization
%	Percentage

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CHAPTER 1 *INTRODUCTION*

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1.1 Dengue as a Global Public Health Problem

Dengue, a mosquito-borne viral infection is regarded as a major public health problem globally. The two main clinical manifestations of dengue, namely Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS), are responsible for exacting heavy morbidity and mortality every year and continue to be serious health problem (Chan *et al.,* 1994; Gubler, 1998). The severity of this disease can be judged from simple statistics that over 40 percent (2.5 billion) of the world's population in 100 tropical and subtropical countries, continue to live under the threat of contracting dengue infection, while close to 50 million new infections and 24, 000 deaths are reported annually worldwide (Fonseca *et al*., 2002). Besides, every year the disease forces nearly 500,000 people to hospitalization, of which 90 percent are children. It is therefore, not surprising that Disability Adjusted Life Years (DALY) for dengue infection exacts a disease burden more or less equal to that of malaria (465,000) (Renganathan *et al*., 2003).

Dengue fever, a very old disease, has reemerged in the past 30 years with an expanded geographic distribution of both the viruses and the mosquito vectors, increased epidemic activity, the development of hyperendemicity (the co circulation of multiple serotypes) and the emergence of dengue hemorrhagic fever in new geographic regions. The geographical spread of dengue virus occurs largely by the movements of infected humans. Now a days, increased air travel facilitates the quick dispersion of dengue between different geographical areas. Viruses are usually introduced into a household by an index case and in the presence of vector mosquitoes may give rise to several secondary cases. As such, crowded urban areas provide ideal condition for increased dengue transmission in tropical urban cities (Gubler & Rosen, 1976). The reasons behind the resurgence of epidemic DHF are complex and are not fully understood. Unpredicted human population growth, unplanned and uncontrolled urbanization, changing life styles which influence increase in mosquito densities by providing many more breeding sites with increased use of non-biodegradable plastics are probable factors responsible for the resurgence of dengue epidemics. It is also due to increased movements of dengue viruses with the movement of viremic hosts in different parts of the world via increased air travel resulting in constant movement and introduction of new virus strains and serotypes between different urban centers. Lack of effective mosquito control, inadequate waste management and water supply,

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development of hyperendemicity and deterioration in public health infrastructure may also contribute to this resurgence (WHO, 1999; Gubler and Trent 1994).

Dengue viruses are largely found in tropical and subtropical areas and outbreak of DHF occurs in definite seasons, is more common during the rainy season and tends to be recurrent. The disease maintains a definite seasonal and cyclical epidemic pattern in different areas (Aung *et al*., 1996). In dengue epidemic areas like Thailand, Myanmar and Vietnam the incidence of large out breaks occur at 2-3 years interval (WHO, 1997). The interruption of transmission usually occurs during winter but transmission may occur throughout the year in some areas, peaking in the monsoon seasons (Ha and Huan, 1997; Daengran *et al*., 1996; Lan *et al.*, 1998).

1.2 Incidence of Dengue and Mosquito Vector in Bangladesh

Dengue was first reported as “Dacca fever” in Bangladesh in 1964 by Aziz and his colleagues. Subsequent reports suggested that dengue fever may have been occurring sporadically in Bangladesh from 1964 to 1999 (Aziz *et al.*, 1967; Islam *et al.*, 1982; Khan *et al.*, 1986; Alam *et al.*, 2000; Hossain *et al.,* 2003). An outbreak of an acute febrile illness clinically suspected as dengue and DHF occurred in and around Dhaka city during the summer of 1999 when serological evidence of dengue virus infection was found in the majority of the cases. The first epidemic of dengue was reported in the capital city, Dhaka in the year 2000 (Rahman *et al.*, 2002; Aziz *et al.*, 2002). Since then the disease has shown an annual occurrence in all major cities of the country. During January 2000 to December 2007, Bangladesh recorded a total of 22,245 dengue cases and 233 deaths (1.04%). Of these, Dhaka accounted for 20115 cases and 181 deaths (0.9%) (Choudhury *et al*., 2008). During 1982 to 1983, an entomological survey conducted in Dhaka City revealed the presence of vector mosquito but the population density of the vector was below the optimal level for propagation of epidemic outbreaks. The *Ae. aegypti* index was 16.2 and that of *Ae. albopictus* was 5.35 (Khan and Ahmed, 1986). Result of a mosquito survey carried out in Dhaka and Chittagong in 1997 indicated the Breteau Index in Dhaka 30.8 and in Chittagong 18.2 which was higher than previous index (Knudsen, 1997). In the year 2000 Chowdhury and his colleagues revealed the vector position in Dhaka city with overall Breteau Index (BI) of 22.6 (range from 0.0 to 94 in different city ward). Out of 90 ward

of Dhaka City, 46 wards were above 20 BI. The health department of Bangladesh government also reported a high BI of 50 for Dhaka City. The increased number of vector mosquitoes resulted in increased number of cases and finally caused the outbreak in the year 2000.

Dhaka is a densely populated city with a population of about 15 million. Housing, sanitation and water supply system cannot keep pace with the rapid growth of the city. People have to store water in containers, which facilitate the breeding grounds of container breeding mosquitoes especially, *Aedes* species. As a result in recent years both the population of vector and that of human have increased alarmingly in Dhaka city, thereby increasing the likelihood of epidemic DHF if and when dengue viruses with epidemic potentials are introduced.

1.3 Transmission of Dengue Virus

Dengue virus is transmitted only by certain species of day biting *Aedes* mosquitoes, particularly *Aedes aegypti* (Siler *et al*., 1926; Gould *et al*., 1968; Tien *et al.,* 1999). The principal and efficient vector of dengue is *Aedes aegypti* but other species e.g., *Aedes albopictus*, *Aedes polynesiensis*, *Aedes scutellaris* and *Aedes hansilli* also play role in transmission (Halstead, 1984; Rico Hesse, 1990; Rosen *et al*., 1954). *Aedes albopictus* is considered as the second important vector for dengue transmission but its role in epidemic transmission is yet uncertain (Knudsen, 1996). *Ae. aegypti* predominantly breeds indoors, in clean stored water and outdoors in natural and artificial containers, which trap rainwater. The female mosquito feeds during the day time with peak activity in the morning and late afternoon. After taking a blood meal on a viraemic individual, the mosquito may transmit virus directly by changing host or after 8-10 days extrinsic incubation period in the vector during which virus multiplies in their salivary glands. Once infected the vector is capable of transmitting viruses for rest of its life (Gould *et al*., 1968).

1.4 Dengue Virus and Serotypes

Dengue virus is a single-stranded positive sense RNA of about 10,700 bases that contains a single open reading frame (Chambers *et al.,* 1990). The viral genome encodes for three structural (C: capsid; PrM: pre membrane; M: membrane and E: envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5**)**. The structure of virus E

protein confers the infectivity and host-immune responses of the virus (Despres *et al*., 1993). The Dengue virus belongs to Flavivirus family having four serotypes, DEN-1, DEN-2, DEN-3 and DEN-4 that are closely related yet antigenically distinct (Westaway *et al*., 1985; Henchal & Putnak, 1990).

1.5 Signs and Symptoms of Dengue Infection

The clinical manifestations of symptoms due to an infection with this mosquito-borne virus vary from mild flu-like symptoms to dengue fever (DF), fulminating dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). Classical dengue or "break bone fever" is a self limiting disease characterized by mild to high fever lasting for 3-7 days, intense headache with pain behind the eyes, severe muscle and joint pain and a rash. DHF is characterized by high fever, disordered homeostasis including vascular changes, thrombocytopenia, coagulation disorder and occasionally circulatory failure i.e., dengue shock syndrome (DSS) (Hayes & Gubler, 1992; Nimannanita *et al.,* 1969 ; WHO, 1997). Dengue is public health problem because DHF can be fatal unless its associated plasma leakage is treated early. The untreated case fatality rate may be as high as 30- 43% (Srivastava *et al*., 1990).

1.6 Detection and Diagnosis of Dengue

In the absence of a vaccine or any specific drug for dengue treatment, an early diagnosis is considered indispensable to prevent any casualty. Serological and cell culture methods are conventionally used for detection and diagnosis of dengue in patient serum and mosquito vector. Recently use of molecular methods is increasing with time.

1.6.1 Conventional Methods for Detection of Dengue

The laboratory diagnosis of dengue infection is an essential part of any evaluation of the disease (Diezt *et al.,* 1990). Five basic serological tests are used for the diagnosis of dengue infection in routine practice. Among the serological tests, presently ELISA is the most common and widely used since it is simple and rapid. IgM capture ELISA (MAC ELISA) can measure dengue specific IgM and even in cases where single specimen is available. Detection of anti dengue IgM indicates the diagnosis of recent dengue infection in both primary and secondary

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dengue infections. Detection of an early and excess of IgG characterizes secondary infection (Monath & Heinz, 1996). Anti- dengue IgM develops earlier than IgG in primary infection and is usually detectable by day 5 of illness. IgM antibodies appear within a short period of infection and wane after 1-2 months (Innis *et al.,* 1989). In primary infection IgM/IgG ratio which is in OD units generally exceeds 1.5 in acute convalescent sera. Direct detection of dengue viral antigen in serum and fixed tissue can be done by counter current immuno-electrophoresis, immuno-chemical staining of peripheral blood mononuclear cell and detection of NS1 antibody by Western blotting (Kuno *et al*., 1991).

Isolation of virus can be accomplished provided the sample is taken in the first few days of febrile illness and processed without delay by mosquito inoculation or cell culture. Ideal samples for virus isolation are acute phase serum, plasma or washed buffy coat. Intra-thoracic or intra-cerebral inoculation of serum or plasma in mosquito is the most sensitive method of virus isolation. The system is also simple, economical and rapid and result can be obtained within 7 days (Rosen & Gubler, 1974; Thet-win, 1982). Dengue virus can grow in a variety of primary and continuous cell cultures of mosquito origin. Widely used cell lines include C6/36, AP-61, TRA -284SF, and TRA-284 SF provides the most sensitive assay (Kuno *et al*., 1985).

1.6.2 Molecular Method for Dengue Detection and Serotyping

The dengue virion RNA genome has been sequenced and primers were developed for detecting RNA of the four dengue prototype viruses (Lanciotti *et al.,* 1992; Hull *et al.,* 1984). These primers have successfully been evaluated in dengue virus RNA extracted from blood of infected patients using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) technique (Igrashi *et al.,* 1978). The most sensitive and rapid method for identification of dengue virus infection is reverse transcriptase polymerase chain reaction (RT-PCR). The technique allows for the multifold amplification of viral nucleic acid and has been used to rapidly diagnose viral disease (Maneekarn *et al*., 1993; Morita *et al*., 1991). Serotyping of dengue virus by RT-PCR can also be done using serotype specific primers and analysis of amplified sequence by agarose gel electrophoresis or hybridization with serotype specific degoxigenin labeled probes (Lanciotti *et al*., 1992; Deubel *et al*., 1990). The primary advantage of this molecular tool lies in the speed

at which specimens can be screened for the presence of dengue viruses and also by its highly sensitive and specific detection.

1.7 Vectors Surveillance and Dengue Infection

The prevention and control of dengue outbreaks depend upon the surveillance of cases and mosquito vectors. In the absence of a vaccine or any specific drug for dengue treatment, an early diagnosis is considered indispensable to prevent any casualty. Detection of viruses in human sera particularly in endemic areas is cumbersome, difficult and also not desirable. Therefore, as an alternative approach, detection of the dengue virus antigen in mosquitoes has provided a reliable tool to comprehend the types of virus circulating in nature; and help in designing vector specific control strategies (Samuel & Tyagi, 2006). Thus, it is important to evaluate the competence of vectors in each epidemic of endemic area. Vector surveillance provides estimates of population density and viral infection rates which are necessary to predict epidemics of dengue and implementing remedial measure, such as, fogging of adults and destruction of breeding places to limit an impending outbreak from spreading.

1.7.1 Methods Used for Vector Surveillance

Vector surveillance through dengue virus detection in the mosquito, regardless of the species, is generally performed by the direct fluorescent–antibody DFA test on mosquito tissues, usually brain or salivary glands (Gubler and Sather, 1988; Kuberski and Rosen, 1977). However, intrathoracic inoculation of adult mosquitoes and the use of mosquito cell cultures methods are also being used for detecting dengue virus in the vector (Guzman *et al.,* 1996, Vorndam *et al.*, 1997). Mosquito inoculation is the most sensitive method for dengue virus isolation and this is the only method sensitive enough for routine successful virologic confirmation of fatal DHF and DSS cases (Gubler *et al.,* 1979; Sumarmo *et al.,* 1983; Vaughn *et al.,* 1997). The mosquito inoculation technique has the disadvantages of being labor-intensive and requiring an insectaria to produce large numbers of mosquitoes for inoculation. Mosquito cell cultures are the most recent addition to dengue virus isolation methodology. The use of cell lines has provided a rapid,

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sensitive and economical method for dengue virus isolation. Moreover, many serum specimens can be processed easily, making the method ideal for routine virologic surveillance. But cell-culture laboratory facilities are not available everywhere and it is less sensitive than mosquito inoculation (Igarashi, 1978; Gubler *et al*., 1984; Kuno *et al*., 1985).

Serological method ELISA can be an alternative tool for epidemiological surveillance for dengue in mosquitoes (Srisuphanunt *et al.,* 2007). But these methods are laborious and time consuming, hence not suitable for screening large number of vector mosquitoes.

1.7.2 Use of RT-PCR method for Vector Surveillance

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) can detect small quantities of virus and has been employed for detecting and typing of dengue virus RNA in the field caught *Aedes* mosquitoes. It is able to monitor the infection rate in mosquitoes, both adults and larvae with a high degree of precision (Chan *et al.*, 1994; Ahmed *et al*., 1997; Fonseca, 2002). RT-PCR offers the potential for the rapid, highly sensitive and specific detection of dengue viruses up to the serotype level. RT-PCR has been successfully applied for diagnosis of dengue in sera, tissue from fatal cases, mosquito pool, infected cell cultures and mosquito larvae. RT-PCR method has also been reported for mosquito surveillance and genetic strain characterization. Use of RT-PCR in epidemiological investigations has been intensified recently as geographic locations of the virus-infected *Aedes* mosquitoes, detected as early as six weeks before the start of dengue outbreaks, were traced to have a correlation with the residence or workplaces of patients. Virologic surveillance using RT-PCR for detecting dengue virus infected *Aedes* mosquitoes in the field may serve as an additional early warning monitoring system for predicting dengue outbreaks (Chow *et al.*, 1998). Unlike most other techniques which require screening of pools of mosquitoes to detect viruses, RT-PCR carries out the job with solitary specimen.

RT-PCR has offered several advantages over the ELISA method. One major feature is its speed, a further advantage of RT-PCR is that some Den-1 isolates cannot be detected or identified by certain clone type-specific MAb but are clearly identifiable by RT-PCR (Maneekaran *et al.,* 1993). Virological surveillance by RT-PCR had detected infected *Ae. aegypti*

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as early as six weeks before the starts of dengue outbreak of 1995 and 1996 in Singapore and may serve as an early warning monitoring system for dengue outbreaks (Vincent *et al.,*1998). Hence, the potential of RT-PCR techniques for detecting the virus in the field caught mosquito vectors can be investigated in Bangladesh.

1.8 Molecular Studies on Dengue Virus in Bangladesh

As a dengue prevalent geographical region with high risk of dengue outbreak, an extensive research on virological, immunological and epidemiological aspects is essential to identify risk factors of DHF in Bangladesh. Research is also necessary to recommend easy and affordable laboratory methods for the diagnosis of dengue infection with high sensitivity and specificity. In Bangladesh so far few molecular studies were done on dengue patients' clinical blood samples to determine viral genotypes. As clinical virulence varies among the genotypes of dengue virus, a study was conducted to investigate the molecular genotypes of dengue in Bangladesh (Aziz *et al.,* 2002). The study suggested the predominance of DEN-3 infection with occasional co-infection with other types, during the outbreak of dengue. Another study was done about the molecular characterization and clinical evaluation of dengue outbreak in 2002 in Bangladesh and phylogenetic analysis revealed that the currently circulating DEN-3 viruses entered Bangladesh from neighboring countries (Islam *et al.,* 2006). All eight isolates were all DEN-3 suggesting that DEN-3 might be a recent predominant serotype in this country. But in Bangladesh no significant molecular study of dengue viral RNA on field caught mosquitoes has done so far.

1.8.1 Aims and Objectives of Present Study

With this above mentioned background we have undertaken the present study to establish RT-PCR based molecular method for detection and typing of Dengue virus in field collected mosquito specimens in Bangladesh. Our general objective is to develop a rapid, sensitive and specific molecular method for dengue vector surveillance. Specific objectives of the research are:

a) To develop RT-PCR based molecular method for rapid detection and identification of dengue virus in field caught vector specimens.

b) To identify the predominant dengue virus genotype and other serotypes of dengue viruses that are present in vector population in Bangladesh, and

c) To study the bionomics of field caught mosquito specimen *Ae. aegypti*.

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CHAPTER 2 *REVIEW OF LITERATURE*

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2.1 Historical background

Although first reports of major epidemics of an illness thought to possibly be dengue occurred on three continents (Asia, Africa, and North America) in 1779 and 1780 (Hirsch, 1883; Howe, 1977; Pepper, 1941; Rush, 1789), report of illness clinically compatible with dengue fever occurred even earlier. The earliest record found to date is in a Chinese encyclopedia of disease symptoms and remedies, first published during the Chin Dynasty (265 to 420 A.D.) and formally edited in 610 A.D. (Tang Dynasty) and again in 992 A.D. (Northern Sung Dynasty) (Nobuchi, H. 1979). The disease was called water poison by the Chinese and was thought to be somehow connected with flying insects associated with water. Outbreaks of illness in the French West Indies in 1635 and in Panama in 1699 could also have been dengue (Howe, 1977; Monath, 1996). Thus, dengue or a very similar illness had a wide geographic distribution before the 18th century, when the first known pandemic of dengue-like illness began. It is quite likely that the Philadelphia epidemic of 1780 was dengue (Carey, 1971). Since then dengue outbreaks reported sporadically from Zanzibar in 1823 and 1870, from Calcutta in 1824, 1853, 1871, 1905, from West Indies in 1827 and from Hong Kong in 1901 (Carey,1971).

In this century some largest outbreak have occurred before the World War II in the USA in 1922, in Australia in 1925-26 and in 1942, in Greece in 1927-28 and in Japan in 1942-45 at irregular intervals (Sabin, 1959). In the two decades during and after the war epidemic, dengue spreads to the Islands of the sea of Japan (Sabin, 1952), Pacific Islands, Vietnam, Malaysia, Singapore, Indonesia, Southern China, Australia, Thailand, Philippine, Myanmar and Indian subcontinent (WHO, 1993). The most dramatic change in the epidemiology of dengue in South East Asia also known as “home” of dengue viruses is the emergence of epidemic of DHF (Halstead 1988; 1992; Rudnick, 1974). During the World War II, a large number of susceptible persons were introduced into the dengue endemic war zone resulted into a number of largest outbreaks in the history (Halsted, 1980).

Since 1963 large outbreaks have also occurred in the Caribbean Islands and most of Central and South American countries (Gubler, 1987; Pinheiro, 1989). In Manila after the first recognition of DHF as a distinct disease entity in 1950s, the disease has spread too many

countries in South East Asia. Notably among them was Thailand, which recorded a serious outbreak in 1958, followed by Indonesia in 1968 and Myanmar in 1970.

2.2 The Current Situation of Dengue in the South-East Asia Region

The disease has now become endemic in South East Asia and the occurrence of outbreak is a regular feature here (Chusak & Andjaparidze, 1998). Now Dengue hemorrhagic fever is one of the leading causes of hospitalization and death among children in this region (Maung *et al*., 1992; Rojanapithayakorn, 1998; WHO, 1997). According to WHO/SEARO (2010) dengue is described as 'endemic' in many countries in the SEA Region –which means that cases occur every year, although there is significant variation between countries and within each country. In 2003, eight SEA Region countries (Bangladesh, India, Indonesia, Maldives, Myanmar, Sri Lanka, Thailand and Timor-Leste) reported dengue cases. The first serious DHF outbreak was reported from Calcutta in 1963 (Sarker *et al*., 1964; Ramakrishan *et al*., 1964). Since then the infection has spread to other major towns along the eastern coast of India. Delhi witnessed epidemics of dengue fever in 1969, 1982 and 1986 (Balya *et al*., 1969; Lall & Dhandra, 1996). DHF/DSS was reported for the first time in Delhi in 1988 and occurred both in children and adult (Acharya *et al*., 1988; Srivastava *et al*., 1990). A major epidemic occurred recently in Delhi during September to November 1996 (Aggarwal *et al*., 1998). Current epidemics of dengue have occurred in Maynmar since 1963 (Ming *et al.,* 1974; Thung *et al*., 1975, Halstead, 1992). Between 1956 and 1990 nearly 60,000 cases of DHF have been reported in Myanmer. In 2004, Bhutan reported the country's first dengue outbreak followed by the first indigenous dengue case reported in Nepal in November 2006. The number of reported cases of dengue in SEA Region countries is typically vary throughout the year and assume a regular pattern, normally in association with changes of temperature and rainfall. This pattern of disease is described as seasonal. In different countries of SEAR, the seasonal pattern of dengue differs, high number of cases is seen in India between August and November, in Indonesia, the peak season is seen in January to February and in Myanmar and Sri Lanka increased number of cases is reported between May and August.

Overall, for the majority of countries in the SEA Region, the total cases reported so far in 2010 (January –September) are still lower than the total for 2009, whereas in Thailand the

number of cases reported so far has already exceeded the number of cases reported in 2009. The exact cause for this condition in different countries is not completely clear, but weather patterns, especially relative increases in rainfall are very likely to be an important feature. Patterns of dengue cases difficult to explain and predict, because they are influenced by a large number of different factors, including, climate, movements of mosquitoes, the type of dengue viruses that are circulating, environmental factors such as temperature and humidity and human behavior (WHO/SEARO, 2010).

2.3 Dengue Status in Bangladesh

The first reported outbreak of dengue in Bangladesh occurred in 1964 known as "Dhaka fever"(Aziz *et al*, 1967); subsequent entomological and serological studies have indicated the continued presence of the mosquito vector and dengue virus in the country (Gaidamorich *et al*., 1980; Khan,1980, Islam *et al*, 1982, Khan and Ahmed, 1986). An outbreak of dengue and dengue hemorrhagic fever occurred in Dhaka, Bangladesh in 2000 (Rahman *et al*., 2002). Dengue heamorrhagic fever had not been previously reported.During late June 2000, a 28 year old patient was admitted to a hospital in Dhaka, Bangladesh, with Hemorrhagic fever, ascites, pleural effusion, and thrombocytopenia. An enzyme linked immune absorbent assay (ELISA) for anti-dengue antibodies confirmed the case as DHF (WHO; 1997). That summer, between July 2000 and December 2000 an outbreak of DHF ($> 5{,}000$ hospitalized cases and $>$ 80 deaths reported) occurred in Dhaka and other major cities of Bangladesh (Yunus, 2000; Ali *et al*., 2003). During the dengue outbreak in Bangladesh in 2002, 6132 clinical cases were reported and out of them 58 people died (Islam *et al*., 2006). In 2004, a total of 3,934 cases with 13 deaths (CFR=0.33%) were reported. The epidemic started in June (143), peaked in July (1,209) and continued in August (818). During the outbreak period, 98% of the cases were from Dhaka with a case fatality rate of 2.3%. The rest of the cases were from districts of Khulna, Jessore, Barishal, Comilla, Chittagong, Jhinaidah, Sirajgong and Madaripur. Bangladesh has been experiencing many outbreak of dengue science 1999. In 2005 there are 1048 reported cases and 4 deaths (CFR 0.38%). The number of cases and deaths reduction is about 73% and 69% as compared to 2000. The maximum transmission period was July to September each year since 2000. In 2007 till May no cases were reported (WHO, 2009). According to the Directorate of

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Health service (DHS), Bangladesh 93 persons died of dengue and 5,551 persons were identified with the diseas in year 2011. But unofficial sources say dengue claimed more than 150 lives in year 2011 (Rhaman, 2012). Experts think that the people and the government are much more conscious about the dengue menace, and environment was also not favourable for *Aedes* mosquitoes, the carriers. In rainy season the almost daily rainfall in Dhaka city was not allowing the mosquito larvae enough time to mature. Larvae need five to six days to develop into mosquitoes; rain without adequet interruption was destroying the larvae (Rhaman, 2012).

2.4 Epidemiology

Currently, dengue viruses and the vector *Aedes* mosquitoes have a worldwide distribution where dengue is endemic and over 2.5 billion people now live in these areas (Gubler, 1996). As *Ae. aegypti*, the principal vector of dengue expands its habitat across Asia, Africa, Central America, South America and Pacific, the number of cases and geographical range of dengue is increasing and DHF/DSS is occurring in new areas with increasing incidence (Halstead, 1992; Gubler & Trent, 1994). Expansion of geographical distribution of both the vector mosquitoes and the viruses resulted in increased frequencies of epidemic, development of hyperendemicity that is associated with co-circulation of more than one of the 4 dengue virus serotypes and the emergence of dengue hemorrhagic fever in new areas (Gubler, 1997). Over 2.7 million cases of DHF and 48,000 deaths were officially reported between 1956 and 1990, globally (Halsted, 1992) and in Southeast Asia nearly 1.3 million cases and 16,188 deaths were reported since 1985-1996 (WHO, 1999).

2.4.1 Mode of Transmission

It is supposed that the virus survives in nature mainly by two mechanisms: by transmission between infected vertebrate and mosquitoes and by vertical transmission in the mosquito. A year round horizontal human-mosquito-human cycle may be considered. Zoonotic cycles of dengue virus transmission also have been documented between monkey and *Aedes niveus* in Malaysia (Rudnick *et al*., 1965; Rudnick, 1974). The maintenance of dengue viruses between epidemics has not been clearly defined.

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Dengue transmission follows two general patterns (Kuno, 1995):

1) Epidemic dengue

2) Hyper endemic dengue in presence of competent vector mosquitoes.

Epidemic transmission occurs when a single newer virus strain introduced into a region where large populations of susceptible hosts and vector mosquitoes are present. Epidemic activity is currently the predominant pattern of dengue virus transmission in smaller Island nations.

Hyper endemic dengue transmission occurs due to continuous circulation of multiple dengue virus serotypes in an area. This requires a large population base or a steady movement of population into the area to maintain a pool of susceptible individuals and continuous transmission is common and transmission also varies from year to years but the variation is not as dramatic as the epidemic pattern of transmission.

2.4.2 Factors Responsible for the Increased Incidence

The factors responsible for the dramatic resurgence and emergence of epidemic dengue and DHF, appears to be closely associated with demographic and societal changes over the past 50 years (Gubler. 1987, 1997, Gubler and Sather, 1988). Two major factors have been the unprecedented global population growth and the associated unplanned and uncontrolled urbanization, especially in tropical and developing countries. The substandard housing, crowding, and deterioration in water, sewer, and waste management systems associated with unplanned urbanization have created ideal conditions for increased transmission of mosquito–borne diseases in tropical urban centers.

A third major factor has been the lack of effective mosquito control in areas where dengue is endemic (Gubler and Trent, 1994). Additionally geographic distribution and population density of *Ae. aegypti* has increased, especially in urban areas of the tropics, because of increased numbers of mosquito larval habitats in the domestic environment. The latter include non biodegradable plastics and used automobile tires, both of which have increased dramatically in prevalence during this period.

A fourth factor responsible for the global emergence of dengue and DHF is increased air travel, which provides the ideal mechanism for the transport of dengue and other urban pathogens between population centers of the world. For instance, in 1994, an estimated 40 million persons departed the United States by air, over 50% of who traveled for business or holiday to tropical countries where dengue is endemic. Many travelers become infected while visiting tropical areas but become ill only after returning home, resulting in a constant movement of dengue viruses in infected humans to all areas of the world and ensuring repeated introductions of new dengue virus strains and serotypes into areas where the mosquito vectors occur (Gubler 1996, Rigau-Perez *et al.,*1994).

A fifth factor that has contributed to the resurgence of epidemic dengue has been the decay in public health infrastructures in most countries in the past 30 years. Lack of resources has led to a critical shortage of trained specialists who understand and can develop effective prevention and control programs for vector born diseases. Coincident with this has been a change in public health policy that placed emphasis on emergency response to epidemics by using high – technology mosquito control methods rather than on preventing those epidemics by using larval source reduction through environmental hygiene, the only method that has been shown to be effective (Gubler, 1989).

In summary, demographic and societal changes, decreasing resources for vector-borne infectious disease prevention and control, and changes in public health policy have all contributed to increased epidemic dengue activity, the development of hyperendemicity and the emergence of epidemic DHF (Gubler, 1998).

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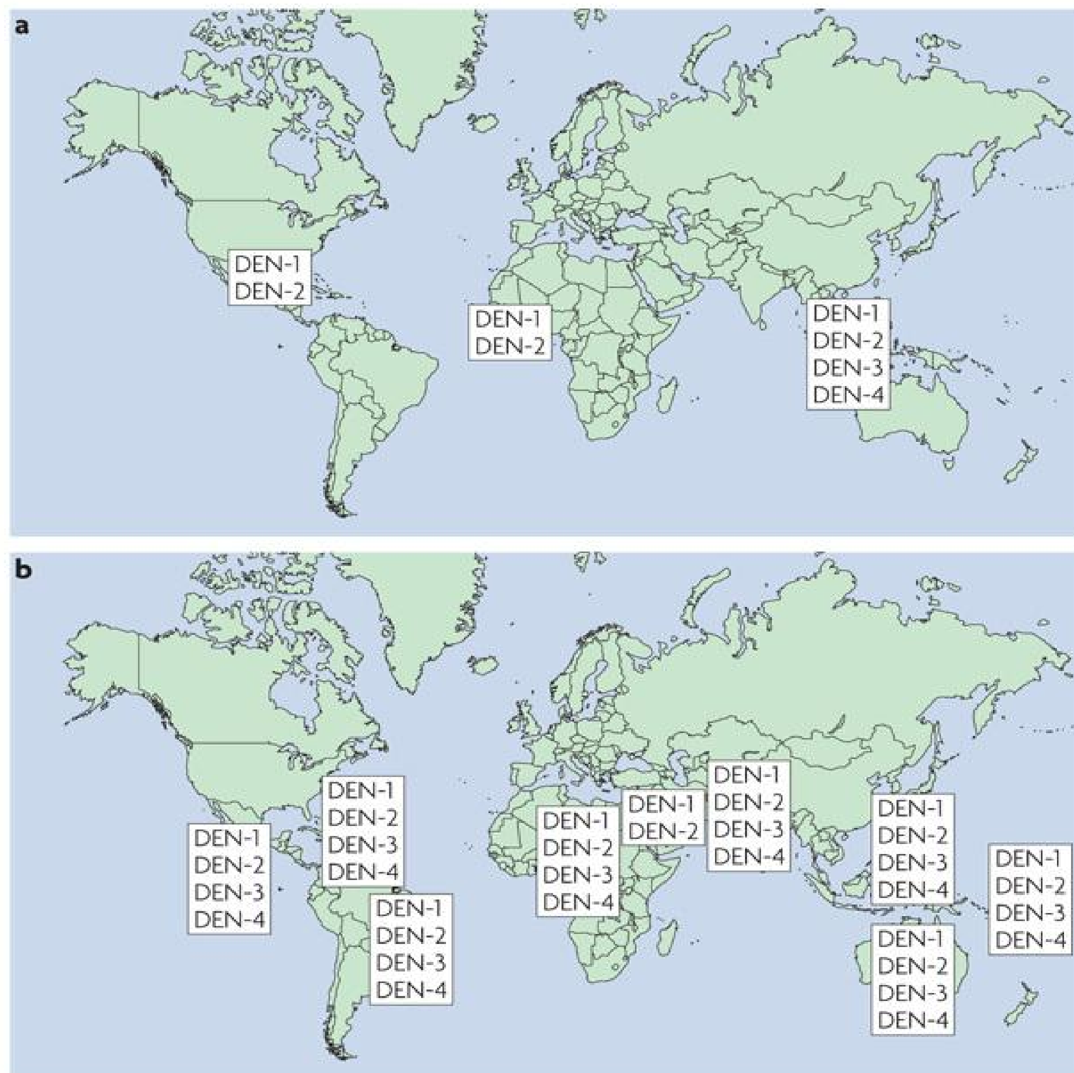

Figure 2.1: The change in distribution of dengue serotypes

The distribution of dengue serotypes in 1970 (a) and 2004 (b).
Source: Guzman *et al.,* 2010, Nature Publishing Group

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2.5 The Dengue Virus

2.5.1 Historical Background of Dengue Virus

The Dengue viruses were first isolated by Sabin and his Co-workers. They isolated a number of Dengue virus strains by inoculating infectious sera to human volunteers during World War II (Sabin, 1952) but 2 of the 4 known serotype were not established until the Philippine hemorrhagic fever in 1960 (Hammon *et al*., 1960).Sabin isolated two immunologically distinct but related viruses from the serum of clinically suspected dengue patients by sub-inoculation into 6 human volunteer, now referred to as Dengue-1 (DEN-1) and Dengue-2 (DEN-2). In 1956 Hammon and co-workers isolated two new serotypes of dengue virus designated now DEN-3 and DEN-4 as well as the previously recognized DEN-1 and DEN-2 during the epidemic hemorrhagic fever among children in the Philippines (Hammon *et al*., 1960). Dengue virus in the form of human sera is remarkably stable and remains infective for a period of 5 years if stored frozen in a dry ice chest or in the lyophilized state in ordinary refrigerator. An exposer of 0.47 second of UV light and 0.05% of formalin can inactivate the virus and destroy its immunogenicity. The dengue viruses can be preserved for several years at -70°C and after suitable lyophilization (Sabin, 1952).

2.5.2 Taxonomy

Dengue viruses are the important members of Flavivirus as well as arbovirus family. In 1984 the International Committee on Taxonomy of viruses (ICTV) approved a recommendation of the ICTV Togavirus study group to transfer the Flavivirus into a distinct newer family, the Flaviviridae.The family contains only one genus that includes prototype, dengue and other 67 members divided into 8 major antigenic complexes (Westaway *et al*., 1985; Calisher *et al.,* 1989). A serotype is a group of viruses classified together based on their antigens on the surface of the virus. These four subtypes (DEN-1, DEN-2, DEN-3, DEN- 4) are different strains of dengue virus that have 60-80% homology between each other. On the basis of nucleotide sequence variation of envelope E glycoprotein,4 serotype of dengue viruses further classified into multiple sub classes .Viruses belonging to each genetic subgroup within a serotype are designated genotype of the serotype (Lewis *et al*., 1993; Rico-Hesse, 1990). Within each genotype the nucleotide sequences of the variant species have at least a 99% sequence

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homology. Based on E protein gene DEN-2 has five distinctive genotype (Lewis *et al*., 1993) . Den-3 has 4 genotype (Lanciotti *et al*., 1994).DEN-4 has 2 genotypes (Mackow *et al*., 1987) and DEN-1 has 5 genotype (Rico-Hesse, 1990).

2.5.3 Virus Structure and Genomic Organization:

All members of the family Flaviviridae share common morphologic characteristics, genome structure, and replication and translation strategy. Mature dengue virion consist of a single stranded ribonucleic acid (RNA) genome surrounded by an approximately icosahedral or isometric neucleocapsid of about 30 nm diameter**.** The nucleocapsid is covered by a lipid envelope of 10 nm thickness derived from host cell membrane and contains the envelope and membrane proteins (Westaway *et al*., 1985). The complete virion measures about 50nm diameter. The virion has a density of about 1.23g/cm as measured by equilibrium centrifugation in deuterium oxide sucrose gradients and a sedimentation co-efficient of around 210s20w (Russel *et al*., 1980). Under electron microscope dengue virion appeared as spherical particles of approximately 50 nm diameter with 7 nm diameter small particles on the surface (Smith et al, in 1970)**.** The viral genome is approximately 11kb in size and a molecular weight of about $3.3\times10^3\times10^3$ (Stollar *et al.,*1966). The viral genome is infectious, has a messenger like positive polarity and can be translated in vitro (Ada *et al* 1959; Nimmanitya *et al*., 1969; Stollar.*et al*., 1966; Svitkin *et al*., 1984).

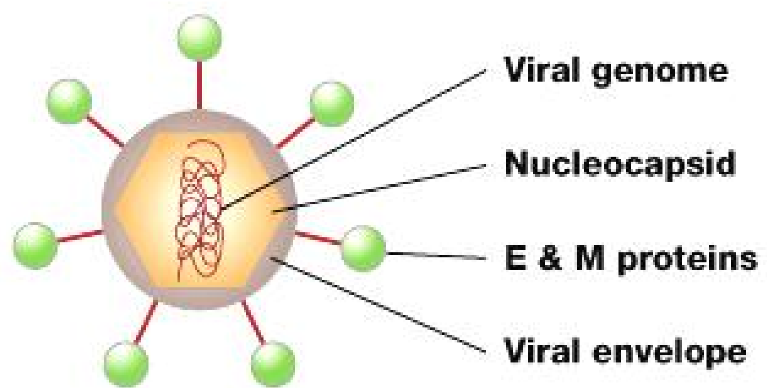

Figure 2.2: Dengue virus structure; Source: 2011, Nature Education

The 5´ end of viral RNA is capped but it lacks a poly (A) tail at the 3´ end. It contains a single open reading frame of about 10, 000 nucleotide, encoding three structural and seven non structural proteins (Cleaves & Dublin, 1979; Wengle *et al*., 1978).

The gene   order is 5´-C-pr M (M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3´**(Fig 2. 3)**

The mature virion contains 3 structural proteins: C, the nucleocapsid or core protein; a membrane-associated protein and an envelope protein E (Rice *et al*., 1985; Stollar *et al*., 1966). Immature intracellular virus contains a protein known as prM or (sometimes, per M) which is a precursor of M (Wengler & Wenglar, 1989; Shapiro *et al.*, 1972). The genes encoding the dengue virus structural proteins are located at the 5´ end of the genome and comprise slightly more than one fourth of the coding capacity of the virural RNA (Mason *et al*., 1987). The proteins are derived from a large, single precursor polypeptide or poly protein. (Zhao *et al*., 1986; Osatomi *et al*.,1988; Mason *et al*., 1987).

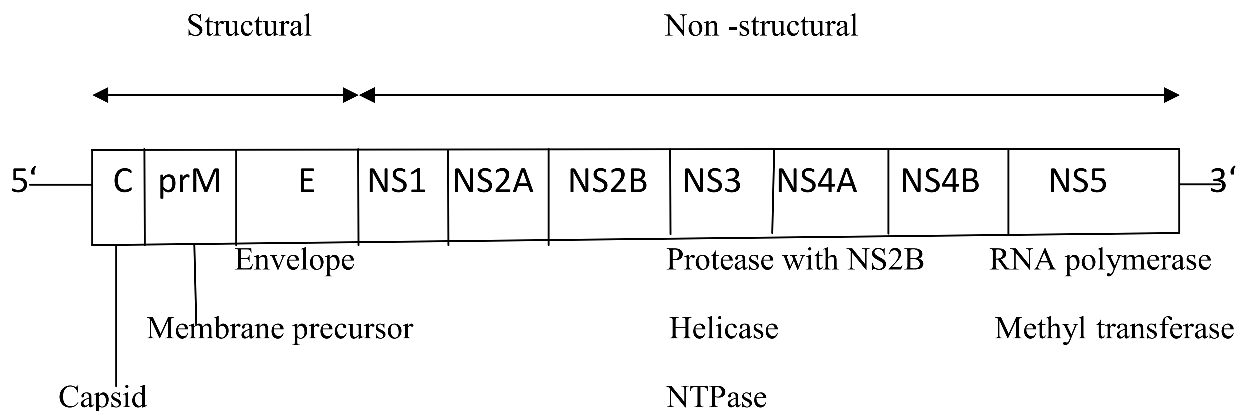

Fig 2.3: Dengue virus genome structure with the structural and non-structural genes.

The C protein is the first synthesized viral polypeptide during translation and is rich in lysine and arginine residue. This highly basic character probably enables it to interact with the virion RNA. The C protein lacks an N terminal, hydrophobic signal sequence suggesting non-membrane bound ribosome are the site of its synthesis. C protein posses a hydrophobic stretch of amino acid at its carboxy terminus and seems to act as a trans-membrane signal for the adjacent pr M which is the precursor of M protein. The domain also serves as a temporary anchor of C protein to a membrane at the replication site after cleavage probably by a host cell "signalase" (Markoff, 1989) at the N terminus of prM. During viral maturation specific proteolytic cleavage of prM precursor results in the formation of M protein. This cleavage occurs in acidic pregolgi vesicles and appears to facilitate virus release from the cells. (Zhao *et al.*, 1986; Mason *et al.*, 1987; Osatomi *et al.*, 1988; Hahn *et al.*, 1988; Deubel *et al.*, 1990). This terminal event of virion morphogenesis, which involves formation of M from prM, appears to be crucial (Randolph *et al.*, 1990). It results in increase viral infectivity and reorganization of viral surface structure (Wengler & Wengler, 1989). The role of M protein in mature virion is not known. The major structural envelope glycoprotein is E protein appears as a homotrimer on the surface of mature virion. It is involved in major biological function of the virion including cell tropism, acid catalyzed membrane fusion and induction of haemagglutination inhibiting, neutralizing and protective antibodies (Depress *et al.*, 1993). Seven non-structural (NS) viral proteins has been identified and mapped to the viral RNA. The first non-structural protein NSI , chemically a glycoprotein, the function of which in viral life cycle is unknown (Winkler, *et al.*, 1989) .NSI proteins are detected in high titer among patients with secondary dengue infection in contrast with primary infection (Kuno *et al*,. 1991). The NS2 coding region encodes 2 proteins NS2a and NS2b. NS2a is required for proteolytic cleavage of terminus of NS1 (Falgout *et al.*, 1989).

The function of NS2b is unknown in viral replication. NS3 is a component of viral RNA polymerase and involved in the post translational processing of the poly proteins. It shares sequence homology with trypsin like serine protease (Bazan & Fletterick, 1989; Cahour *et al.*, 1992). Very recently NS4a and NS4b non-structural proteins have been mapped like proteins encoded in the NS2 region on viral RNA but their role in viral replication is unknown (Preugschat *et al*, 1991). The NS5 protein is thought to be a viral RNA dependent RNA polymerase (Grun *et al*, 1987).

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2.5.4 The Life Cycle of Dengue Virus

The life cycle of dengue involve endocytosis via a cell surface receptor. The virus uncoats intracellular via a specific process. In the infectious form of the virus, the envelope protein lays flat on the surface of the virus, forming a smooth coat with icosahedral symmetry. However, when the virus is carried into the cell and into lisozome the acidic environment causes the protein to snap into a different shape, assembling into trimetric spike insert into the lysozomal membrane and cause the virus membrane to fuse with lysozome. This releases the RNA into the cell and infection starts. The DENV RNA genome is in the infected cell get translated by the host ribosome. The resulting poly protein is subsequently cleaved by cellular and viral proteases at specific recognition sites.

The viral nonstructural proteins use a negative-sense intermediate to replicate the positive-sense RNA genome, which then associates with capsid protein and is packaged into individual virions. Replication of all positive-stranded RNA viruses occurs in close association with virus-induced intracellular membrane structures. DENV also induces such extensive rearrangements of intracellular membranes, called replication complex (RC). These RCs seem to contain viral proteins, viral RNA and host cell factors. The subsequently formed immature virions are assembled by budding of newly formed nucleocapsids into the lumen of endoplasmic reticulum (ER), thereby acquiring a lipid bilayer envelope with the structural proteins prM and E.

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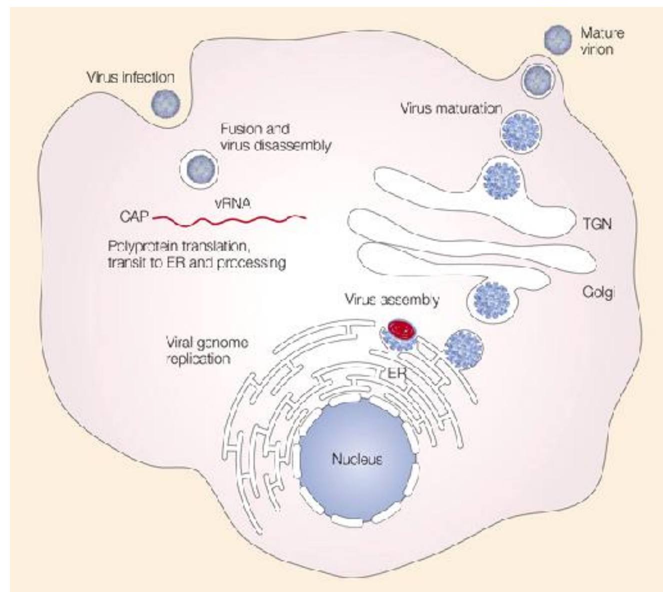

Figure 2.4: Dengue virus replication cycle

Source: Mukhopadhya *et al.,* 2005, Nature Publishing Group

The virions mature during transport through the acidic trans-Golgi network, where the prM proteins stabilize the E proteins to prevent conformational change. Before release of the virions from the host cell, the maturation process is completed when prM is cleaved into a soluble peptide and virion associated M by the cellular protease furin. Outside the cell, the virus particles encounter a neutral pH, which promotes dissociation of the peptides from the virus particles and generates mature, infectious virions. At this point the cycle repeats itself (Mukhopadhyay *et al*., 2005, Whitehead *et al*., 2007; Halstead *et al*., 2007).

2.6 Mosquito as a Vector of Dengue Transmission

2.6.1 The Vector

Grahan in 1903 suggested the transmission of dengue occur by mosquitoes. The actual transmission by a true vector *Ae. aegypti* was documented first by Bancroft and coworkers in 1906 which was confirmed later by other researchers. Siler *et al*., in 1926 successfully transmitted dengue through *Ae. aegypti* to human volunteers and reported first that an incubation period was required before the mosquitoes could transmit the virus. They also showed that the mosquitoes once infected remains infected for life long and can transmit virus for a very long time. In 1928 Schule observed successful transmission of dengue with *Ae. aegypti* in human volunteer and showed a minimum period of 8 days required before the infected mosquitoes could transmit the disease. Working in the Philippines, Simons *et al*., in 1931 successfully showed that like *Ae. aegypti*, *Ae. albopictus* could effectively transmit dengue virus in human, which was widely distributed in Manila. All of these reports based on experimental transmission of dengue virus on epidemiological grounds. The first recorded isolation of dengue virus from wild caught mosquitoes was done by Hammon *et al. in* 1960. They reported one isolate of dengue virus type 3 from *Ae. aegypti* and *C. tritaeniorynchus* in the Philippines and three isolate of DEN-2 from *Ae. aegypti* in Bangkok. Now it is well established that *Ae. aegypti* is the principal epidemic vector of dengue virus .

2.6.2 Transmission of Dengue by Vector Mosquito

The maintenance of dengue viruses between epidemics has not been clearly defined. A year round horizontal human-mosquito-human cycle may be considered. Vertical (Trans ovarian) transmission in vector is also an established way of maintenance. All four Dengue serotypes can be transmitted through trans-ovarian transmission by *Ae. albopictus*. The rate of such transmission also varies with different serotypes and strain of viruses. The highest rate of transmission was observed in type DEN-1 and lowest in DEN-3. *Ae. aegypti* is less efficient with trans-ovarian transmission and can transmit only dengue type 1 at a low rate (Rosen *et al*., 1983: Ahmed *et al*., 1997). Transmission of dengue is also influenced by high temperature that increase vector efficacy i.e. by increasing viral external replication in mosquitoes and by reduction of the incubation period in the vector (Watts *et al*., 1987; Koopman *et al.,* 1991). In an electron

microscopic study, it was revealed that replication of dengue viruses occurred in the mosquito cells having an active rough endoplasmic reticulum and the substantial replication was confined to cells of salivary glands, nervous tissue with lesser involvement of mid gut, homocytes, epidermal cells, fat body and foregut (Sriurairatan & Bharmarapravati, 1977).

2.6.3 Geographical Distribution of Dengue vector

Ae. aegypti is wide spread between 30° North latitude and 20° South Latitude in tropical and subtropical areas of the world (Halstead, 1984; Rico Hesse, 1990). More accurately it is distributed in the Southeast Asian, Western pacific, African and American countries lying between January (N)10°C and 10°C July (S) Isotherm (Knudsen *et al*, 1996). Any country where *Ae .aegypti* mosquitoes present should be considered as to be at potential risk of dengue infection (Gubler *et al*, 1996).

Ae. albopictus has been recognized as a secondary disease vector of dengue and DHF and is distributed in the urban, sub-urban and some rural environments in more than 100 countries (Knudsen,1996). *Ae. albopictus* is important in the maintenance of the virus. It is essentially a species of the Oriental and Indo-Malayan region which has spreaded and became established worldwide during the last 30 years (WHO, 1999).

Ae. aegypti and other vectors of dengue belong to the genus *Aedes* and sub-genus *Stegomyia*. *Ae. niveus* is the exception and zoonotic cycles of dengue virus transmission have been documented between monkey and *Ae. niveus* in Malaysia and Vietnam. *Ae. samoanus* and *Ae. fijiensis* are suspected as vectors in Samoa and Fiji respectively. They are the species belonging to the Finlaya subgroup and are leaf axial breeder. *Ae. aegypti* is most common in urban areas and their spreads to rural area reported recently. Urbanization tends to increase the number of its habitat. In semi-dried areas like India its population typically fluctuates with rainfall and water storage habit.Areas in Southeast Asia where rainfall is more than 200 cm *Ae. aegypti* population is more stable. Because of the traditional water storage practice in Indonesia, Myanmar and Thailand the density of *Aedes* is higher in semi-urban areas than in urban areas. In many areas *Ae. aegypti* and *Ae. albopictus* occur together but *Ae. aegypti* is the dominant species depending on the availability of the larval habitat and the extent of urbanization. Altitude is also an

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important factor in the distribution of the *Ae .aegypti*. In Southeast Asian countries it is limited between 1000 to 5000 meters above the sea level in distribution (Rudnick et al., 1967; Rudnick, 1974; WHO, 1993; WHO, 1999).

2.6.4 Distribution of Dengue Vector in Bangladesh

Out of 500 species of the Genus *Aedes*, there are 26 species of *Aedes* present in Bangladesh. Barraud (1934) found *Ae .aegypti* in artificial container in Dhaka and some other cities in Bangladesh during collection of mosquitoes in India. During mosquito collection in malaria control work in East Pakistan *Ae .aegpyti* was found in tree holes and banana stumps in Dhaka city (Nasiruddin, 1952). Aziz *et al*., 1967 also reported the presence of *Ae. albopictus* in Dhaka city in a study. In their report *Ae.aegypti* the classical vector of dengue was not found in mosquitoes collected from two localities of Dhaka city. Ameen & Moizuddin collected a large number of mosquitoes in Bangladesh in 1970. They reported 23 species of *Ae. aegypti* and *Ae. albopictus*, the population of which reached 2 peaks in the month of April and September. This two peak month corresponds with pre and post monsoon (Ameen & Moizuddin, 1973). Khan, 1980 found both *Ae. aegypti* and *Ae. albopictus* in different artificial containers in Dhaka city.

Khan and Ahmed (1986) made an ovitrap survey to detect the presence or absence of *Ae. aegypti* and *Ae. albopictus* in Dhaka city. They surveyed 23 areas of different socio-economic pattern of the population and out of 23, 22 were positive.The *Ae. aegypti* index was 16.2 and that of *Ae. albopictus* was 5.35. In another study, the container habitat mosquitoes were also reported in Bangladesh. The study was conducted in different places of Bangladesh including Dhaka city, where *Ae. albopictus* (Skuse) larvae was found the commonest container breeder (both artificial and natural) all over the country including both urban and rural areas and *Ae. aegypti* were found in artificial containers only in urban areas (Ahmed *et al*,.1990)

In 1997, Knudsen visited Bangladesh as a WHO consultant and made a survey work in an effort to assess the vector mosquito population of dengue. The survey was carried out in urban areas of both Dhaka and Chittagong City. The report of that survey indicated that the breteau index of Dhaka was 30.8 and that of 18. 2 in Chittagong (Knudsen, 1997). According to WHO (1995), if the bretau index exceeds 18, it is risky for community. The result of the study indicates that

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Dhaka was above the risk level of Dengue transmission by the vector mosquitoes (Knudsen, 1997). Ali *et al*., 2003 used conventional and spatial analytical tools to characterize patterns of transmission during a community-wide outbreak of dengue hemorrhagic fever in Dhaka, Bangladesh in 2000. Clusters of dengue illness and high-density vector populations were observed in a distinct sector of the city. They found a spatial association between dengue cluster and vector population. They made the disease –environment map which displays areas where transmission is more intense**.**

2.6.5 Bionomics of Dengue vector

A high degree of anthropophilia, multiple and interrupted blood feeding habits and urban location of breeding makes *Ae. aegypti* a highly efficient vector of dengue (Macdonald, 1956). The female mosquito feeds during the daytime with pick activity in the morning and late afternoon. Only the female bites for blood which she needs to mature her eggs.To find a host *Ae. aegypti* are attracted to chemical compounds that are emitted by mammals (Brien *et al.,* 2010). *Ae. albopictus* also feeds in daytime and has been shown to have a higher biting frequency than *Ae. aegypti* (Lim, 1979). But its role in epidemic transmission is yet uncertain. *Ae. aegypti* predominantly breeds in clean stored water in the artificial container, in indoors and in natural and artificial container, which trap rain water in outdoors of the dwelling places (Gould *et al*., 1968). The most dangerous areas are wet shower floors and toilet tanks, as they allow the mosquitoes to breed in the residence. Research has shown that certain chemicals emanating from bacteria in water containers stimulate the female mosquitoes to lay their eggs. They are particularly motivated to lay eggs in water containers that have the correct amounts of specific fatty acids associated with bacteria involved in the degradation of leaves and other organic matter in water. The chemicals associated with the microbial stew are far more stimulating to discerning female mosquitoes than plain or filtered water in which the bacteria once lived (News wise Inc, 2010). *Ae . albopictus* breeds both in the natural and artificial containers in outdoor. It means that *Ae .aegypti* are present mainly in urban areas. The artificial containers where the *Aedes* breeds includes flower base, broken water pots, discarded pots, tins, cans, coconut shells, motor tires and water buckets. The natural containers include tree holes, bamboo stump and leaf axils (WHO, 1999). *Ae . aegypti* has a short (50-100 yards) flight range (Reiter, 1995). They seldom disperse more than a few hundred yards from their place of birth (Sheppard, 1969). The

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mean distance travelled by *Ae. aegypti* is 56m and 35m for the males and females respectively and the maximum distance travelled is 160m by both sexes (Muir& Kay, 1998). Therefore the geographical dispersion of dengue viruses is largely by movement of viremic human beings. Yet crowded urban areas provide ideal opportunities for dengue transmission.

2.6.6 Genomics

The genome of *Ae. aegypti* species of mosquito was sequenced and analyzed by a consortium including scientists at The Institute for Genomic Research (now part of the J.Craig Venter Institute), the European Bioinformatics Institute, and the University of Notre Dame, and published in 2007. The effort in sequencing its DNA was intended to provide new avenues for research into insecticides and possible genetic modification to prevent the spread of virus. This was the second mosquito species to have its genome sequenced in full (the first was *Anopheles gambiae*). The published data include the 1.38 billion base pairs containing the insect's estimated 15, 419 protein encoding genes. The sequence indicates that the species diverged from *Drosophila melanogaster* (the common fruit fly) about 250 million years ago , and that the *Anopheles gambiae* and this species diverged about 150 million years ago (Kowalski, 2007, Nene *et al.*, 2007).

2.6.7 Life cycle of *Aedes aegypti*

Ae. aegypti completes development in 10 days at 26-30°C (Fig 2.9). Although the lifespan of an adult *Ae. aegypti* is two to four weeks depending on conditions (Zettle *et al*.,2010) the eggs can be viable for over a year in a dry state, which allows the mosquito to reemerge after a cold winter or dry spell (Mortimer, 2010).

2.6.7.1 Egg

The eggs of *Aedes* are laid in a batch but are not attached to each other. They differ from *Anopheles* by the absence of floats. Eggs of *Aedes* are laid on the moist surface at the water's edge and not on the water itself. When the eggs are first laid they are susceptible to desiccation and collapse and die if dried. When the embryo is fully developed, eggs can withstand desiccation and remain viable in the dried state for many months, depending on the species. The production of eggs resistant to desiccation makes *Aedes* species ideal colonizers of temporary

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collection of water, e.g. salt marshes, tree holes, etc. When the eggs are flooded, most of them hatch immediately, but some will remain dormant and hatch at the second or third flooding. The early emergence of larvae is essential if the life cycle is to be completed before the habitat dries up (Kettle, 1984; Kumar *et al*., 1995).

2.6.7.2 Larva

Larvae possess a siphon on the penultimate segment of the abdomen. The tracheae are continued into the siphon and the spiracles open at its tip. In *Aedes* the siphon is typically short. *Aedes* larvae may feed at the surface while respiring by twisting the abdomen to bring the mouthparts into contact with the surface film. They also feed on the bottom out of contact with the surface (Kettle, 1984).

2.6.7.3 Pupa

The head and thorax of the pupa are combined into a single division, the cephalothorax, which is joined posterior to a segmented abdomen. At rest the pupa floats at the water surface with the abdomen reflected under the cephalothoraxes. The pupa does not feed and is the ninth segment of the abdomen carries a pair of broad, flat plates, the paddles. The shape of the respiratory horn is tubular (Kettle, 1984).

2.6.7.4 Adult

Living adults can be readily recognized by the stance they adopt when resting on a flat surface. The culicine adult rests with its body angled and the abdomen directed back towards the surface on which it is resting. The proboscis, head and anterior part of the thorax from one line and the posterior part of the thorax and the abdomen another. In the female the palps are considerably shorter, usually about one quarter of the length of the proboscis. In the male the palp areas long the proboscis and taper distally, but the tapering is sometimes obscured by the development of tufts of hair on the distal segment (Kettle, 1984)

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Fig 2.5: Eggs of *Aedes aegypti* in clean stagnant water

Fig 2.6: Larva of *Aedes aegypti*

Fig 2.7: Pupa of *Aedes aegypti*

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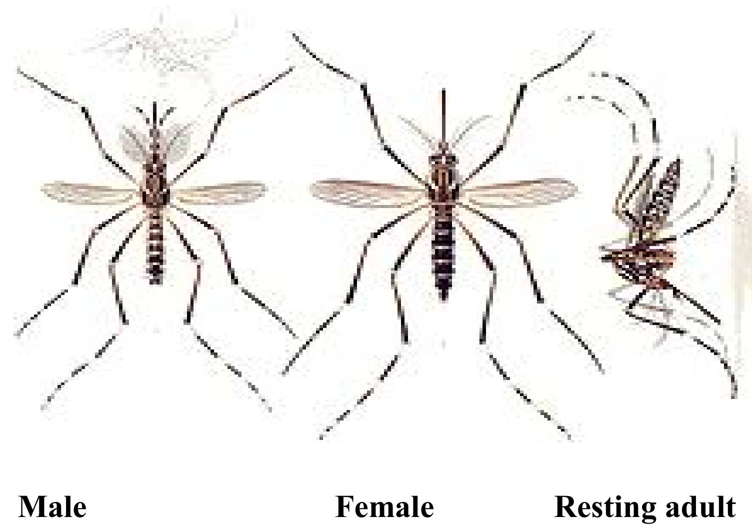

Fig 2.8: Differentiation between male and female *Ae .aegypti* and resting adult

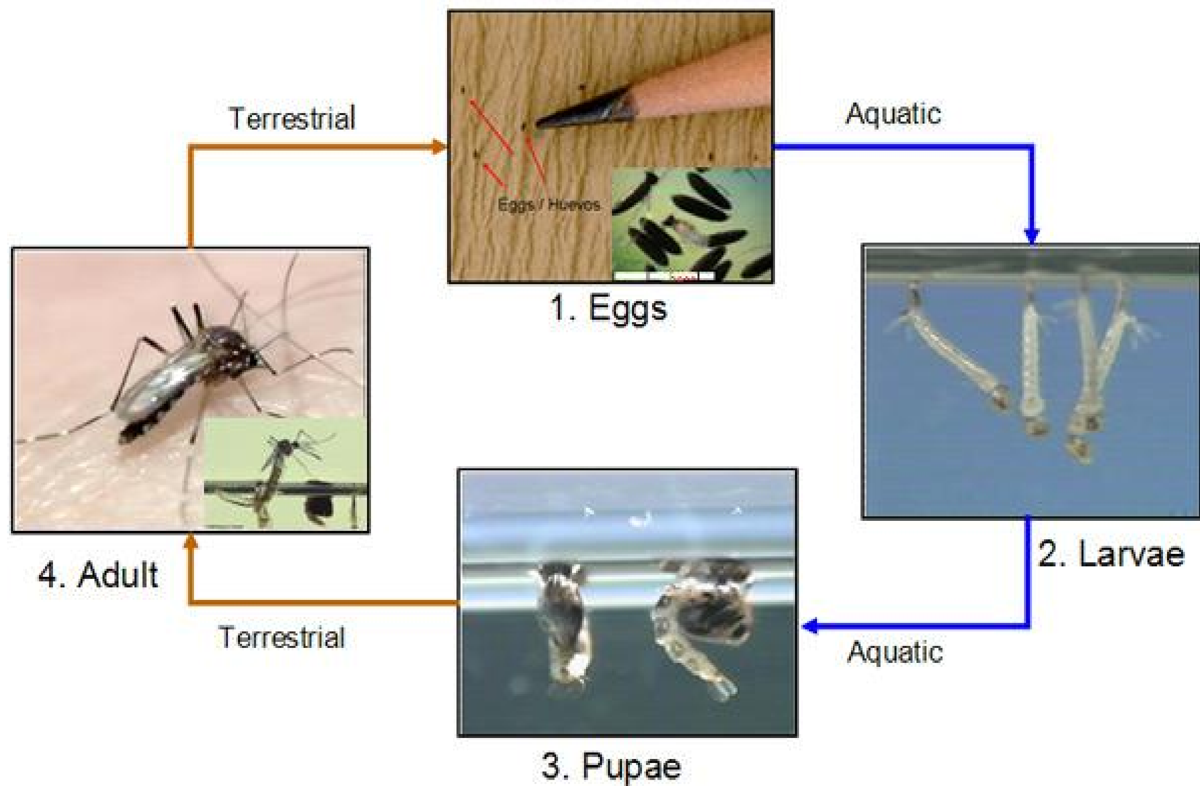

Fig 2.9: Life cycle of *Ae. aegypti.*

Egg to egg life cycle 10 days at 26-30°C and total life span 14-28 days. Eggs are draught resistant, after rainfall larvae hatched out within 2-3 hours; larvae take 4-5 days to become pupae, after 2-3 days pupae began to hatch as adults.

2.6.8 Identifying Characters and Comparison Between adult *Ae. aegypti* and *Ae. albopictus*

Adult *Ae. aegypti* is a smallish, dark mosquito with conspicuous white markings and banded legs; the proboscis is all black although the palps are white tipped; the scutum has a dorsal pattern of white scales in the form of 'lyre' with curved lateral and two central contrasting with the general covering of narrow dark scales; wings are dark scaled; hind legs femur pale scaled for basal three quarters with dark scales dorsally on apical two-thirds and ventrally on apical third, tibia dark but tarsi with pale basal bands on 1-4 and 5 all pale; abdominal tergites with median and lateral white scale patches or bands (possibly some white scales on apical margins), sternites predominantly pale scaled with sub apical bands on distal segments (Russel,1996).

In adult *Ae albopictus* the scutum, the dorsal portion of an insect's thoracic segments, is black alongside the characteristic white midline. The proboscis is dark colored, the upper surface of the end segment of the palps is covered in silvery scales. On the side of the thorax, the scutellum, and the abdomen there are numerous spots covered in white-silvery scales. Such white-silvery scales can also be found on the tarsus and femur particularly on the hind legs that are commonly suspended in the air. The transparent wings have white spots on the base of the Costas (Huang, 1968; Belkin, 1962). In Asia, Asian tiger mosquito *Ae. albopictus* can be mistaken for other members of the subgenus *Stegomyia*, particularly the yellow fever mosquito *Ae. aegypti* (the most prevalent species in the tropics and subtropics), because both species display a similar black and white pattern. A single silvery –white line of tight scales begins between the eyes of *Ae. albopictus* and continues down the dorsal side of the thorax. This characteristic marking is easiest and surest way to identify the *Ae .albopictus* (Huang, 1968, 1969).

2.6.9 Adaptation Ability of *Ae. aegypti*

Ae. aegypti is a highly domesticated mosquito which can complete its entire life cycle within the confines of a single human dwelling. The female lays its eggs in small containers, such as flower

vases, water storage jars and other containers holding water in houses. It will also lay eggs in small amounts of peri domestic water which collects in tires, plastic containers and other debris associated with human settlement. When the embryo inside the egg of *Ae. aegypti* has developed to a certain stage the egg becomes resistant to desiccation. It may then enter diapauses in which it can remain for about a year. When the eggs are flooded they hatch and the larvae commence their development immediately. This ability of *Ae. aegypti* to produce diapausing eggs enables the species to survive in areas with prolonged dry season while the rapid hatching of the eggs on flooding and the speedy development of the immature stages are adaptations to breeding in temporary collections of water.Adult *Ae. aegypti* emerging from breeding sites indoors can complete their cycle without going outside. Swarming is not an essential component of mating. In domestic female blood feeding presents no problem because the female is strongly anthropophilic and feeds readily on human inhabitants of the dwelling. The blood-fed female rests in the house while maturing her ovaries and then deposits her eggs in domestic water containers. The cycle than complete. So *Ae. aegypti* had readily adapted to laboratory colonization. *Ae. aegypti* produces diapausing eggs made it easy to disseminate materials widely throughout the world (Kettle, D.S. 1984).

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Fig 2.10: Adult *Aedes aegypti* , the principal dengue vector (the yellow fever mosquito)

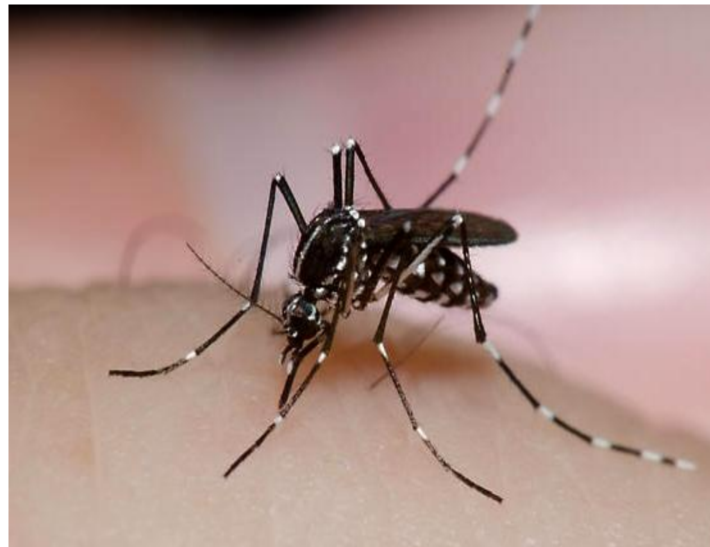

Fig 2.11: Adult *Aedes albopictus*, the secondary dengue vector (Asian tiger mosquito)

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2.7 Human as host of Dengue Virus

2.7.1 Clinical Presentation

Dengue has been known by any of the pseudonyms like Break bone fever, Dandy fever, Dengue, Bouquet fever, Giraffi fever, Polka fever or the 5 days or 7 days fever (Sabin, 1959). Depending on their age and immunological condition of the host, the clinical spectrum of dengue virus infection ranging from asymptomatic infection to undifferentiated febrile illness (Viral syndrome), Dengue fever (DF), Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) (Nimmannitya,1993; WHO,1997).

2.7.1.1 Undifferentiated fever

Infants and children during the first exposure of dengue virus develop simple fever which may indistinguishable from other viral infections and sometimes associated maculopapular rash during fever or during defervasence are classified in this category (WHO, 1993).

2.7.1.2 Dengue fever

Clinical manifestations of dengue fever is an acute biphasic fever of abrupt onset with high fever, headache, retro-orbital pain, particularly on eye movements and eye pressure patient may describe as “Fire is coming out of my eyes”. Generalized bony pain with anorexia, nausea, vomiting and prostration follows initial symptoms. Sometimes it becomes an incapacitating disease with sever muscle and joint pain and some named as “Break bone fever”, particularly in adults and occasionally with unusual hemorrhage (Siler et al., 1926: Simons et al., 1931 and Sabin, 1952, Kaunter *et al.,* 1997).

Dengue fever is most common in adults and older children. The incubation period in host is usually 2-7 days from the time of mosquito bite and may ranges from 2-14 days. Usually the patient experiences fever ranges between 39°C-40°c (100°F-106°F) and the fever lasts approximately 5-7 days. Sometimes the fever may follow a biphasic course (Saddle-back appearance). Some described that patients develop generalized macular rash, which balances on pressure during the first 24 to 48 hours of fever. Desquamation of rash may occur and lasts for 1-5 days. At the end of febrile phase the generalized rash fades and localized clusters of petechiae

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may appear over the dorsum of the feet, hands, arms and on the legs. ((WHO, 1997; Nimaannitya, 1993; 1996, Hayes & Gubler1992; Monath & Heinz, 1996, (Nimmannitya *et al.,* 1993; 1996; Hanchel &Putnak, 1990; Balaya *et al.,* 1969; Kuberski *et al.,* 1977, Hayes & Gubler , 1992). Dengue fever can be confirmed by study of dengue antibody response and virus isolation (Nimmannitya, 1993).

Fig 2.12: Typical Dengue patient's skin rash

2.7.1.3 Dengue Hemorrhagic fever:

Dengue hemorrhagic fever is very common under the age 15. Most studies showed that highest incidence occurs in the 5-9 years age group (Gubler *et al.,* 1978, Rojanapithayakorn, 1998). During some epidemics it was found that DHF was also common in adult age group (Sharma *et al.,* 2005, Kaur *et al.,* 1997, Kouri *et al.,* 1983) and also in infants (Lan *et al.,* 1998). The incubation period of DHF is unknown but is probably similar to that of DF. It is thought that following an incubation period of 4-6 days the illness begins abruptly with high fever accompanied by facial flush and headache. Typically the dengue hemorrhagic fever begins abruptly with acute onset of high continuous fever lasting for 2-7 days in most cases.

Occasionally the temperature may be as high as 40°C-41°C with febrile convulsion particularly in infants (Nimmanitya, 1996). The course of fever commonly accompanied with severe

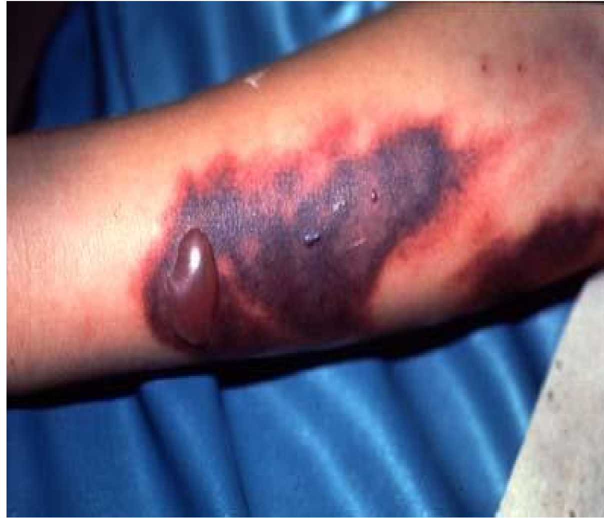

Fig 2.13: Dengue Hemorrhagic lesion

anorexia, nausea, vomiting, generalized abdominal pain or with epigastric discomfort and tenderness at the right costal margin. During the first few days the illness resembles classical dengue fever but a maculopapular rash is less common (Nimmannitya ,1993). Sometimes a rash may appear early in the course of illness. This is the commonly demonstrated hemorrhagic diathesis scattered on the extremities, face, axillae and trunk, appears usually by the 3rd day. The mortality rate of DHF ranges from 1-30% depending on availability of supportive care (Nimmannitya, 1993, 1996; Kaunter *et al.,* 1997). Serological tests and viral isolation from blood during the febrile phase of illness can confirm clinical diagnosis.

2.7.1.4 Dengue Shock Syndrome (DSS)

Dengue shock syndrome is the terminal severe stage of DHF which is manifested by hypovolamic shock due to plasma leakage. In severe cases of DHF, shock ensues and the patient may die within 24-48 hours. The dengue shock syndrome is the most severe form of DHF characterized by hypotension where pulse pressure is less than or equal to 20 mm of Hg regardless of pressure level or hypotension with cold clammy extremities and restless or

profound shock. During the course of DHF, the critical stage starts at the end of febrile phage usually ranged between 2-7 days when a rapid fall in temperature often accompany the varying degree of circulatory disturbances. The frequent complaint before the onset of shock is acute abdominal pain. The progression of shock is rapid and 50% of patients with profound shock die without proper treatment. The most patient remains conscious almost to the terminal stage and the duration of shock is short. Sometimes patients may die or recovers rapidly after volume replacement therapy (Monath & Heniz, 1996, Nimmannitya, 1996 and WHO, 1997).

2.7.2 Pathogenesis of Dengue

The pathogenesis of DHF and DSS is still controversial. Two theories, which are not mutually exclusive, are frequently cited to explain the pathogenic changes that occur in DHF and DSS. The most commonly accepted is known as the secondary -infection or immune enhancement hypothesis which implies that patients experiencing a second infection with a heterologous dengue virus serotype have a significantly higher risk for developing DHF and DSS. Pre existing heterologous dengue antibody recognizes the infecting virus and forms an antigen-antibody complex, which is then bound to and internalized by immunoglobulin Fc receptors on the cell membrane of leukocytes, especially macrophages. Because the antibody is heterologous, however, the virus is not neutralized and is free to replicate once inside the macrophage. Thus, it hypothesized that prior infection, through a process known as antibody-dependent enhancement (ADE), enhances the infection and replication of dengue virus in cells of the mononuclear cell lineage. It is thought that these cells produce and secrete vasoactive mediators in response to dengue infection, which causes increased vascular permeability leading to hypovolemia and shock (Brandt *et al.,* 1982; Halstead, 1988; Morens *et al*., 1987).

The other hypothesis assumes that dengue viruses, like all animal viruses, vary and change genetically as a result of selection pressures as they replicate in humans and /or mosquitoes and that there are some virus strains that have greater epidemic potential (Gubler *et al*., 1988 and Rosen, 1977).Phenotypic expression of genetic changes in the virus genome may include increased virus replication and viremia, severity of disease (virulence), and epidemic potential.

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Antibody-dependent Enhancement can be narrated as follows (Fig 2.14) -

- Formation of immune complexes between dengue virus and non-neutralizing antibodies
- Mononuclear phagocytes infected through their Fc receptors by immune complexes
- Severe disease results by suppression of innate immune response secondary to ADE
- Release of inflammatory cytokines and chemokines
- Enhanced disease

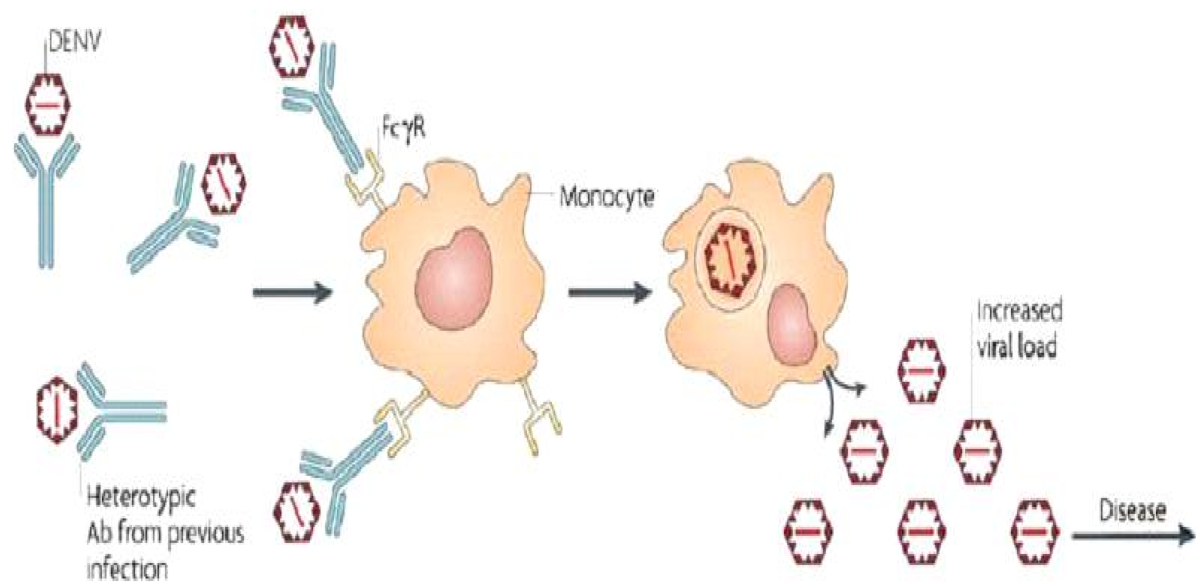

Fig 2.14: Antibody–dependent Enhancement

2.7.3 Serologic Diagnosis

Five basic serologic tests have been routinely used for diagnosis of dengue infection; haemagglutination–inhibition (HI), complement fixation (CF), neutralization test (NT), immunoglobulin M (IgM) capture enzyme-linked immuno sorbent assay (MAC-ELISA), and indirect immunoglobulin IgG ELISA (Gubler *et al.,* 1995 , Guzman and Kouri, 1999, Vorndam, 1997).

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2.7.3.1 Enzyme linked immunosorbent assay (ELISA):

The acquired immune response to infection with dengue virus consists of the production of IgM and IgG antibodies primarily directed against the virus envelope proteins. The immune response varies depending on whether the individual has a primary or a secondary infection .A primary infection with dengue is characterized by a slow and low titre antibody response. IgM antibody is the first immunoglobulin isotype to appear. Anti-dengue IgG at low titre detectable at the end of the first week of illness, increasing slowly thereafter. In contrast, during a secondary infection (a dengue infection in a host had been previously infected by a dengue virus or other flavivirus) antibody titres rise extremely rapidly and antibody reacts broadly with many flavivirus.High levels of IgG are detectable even in the acute phase and they rise dramatically over the following 2 weeks.According to the Pan American Health Organization (PAHO) guidelines (PAHO,1994), IgM antibody is detectable by day 5 of illness in 80% of all dengue cases , and by day 6-10 of illness in 93-99% of cases , and may then remain detectable for more than 90 days. IgM antibody -capture enzyme -linked immunosorbant assay (MAC-ELISA) has become an important tool in the routine diagnosis of dengue. (Innis *et al*., 1989).It is especially useful for hospitalized patients, because with other flavivirus infections such as yellow fever , Japanese encephalitis etc,the response is generally more specific in this method,but it cannot be used to identify the dengue virus serotype.This method usually takes two or more weeks. IgM capture ELISA (MAC ELISA) can measure dengue specific IgM and even in cases where single specimen is available, detection of anti-dengue IgM indicate the diagnosis of recent dengue infection in both primary and secondary dengue infections. MAC-ELISA is based on detecting the dengue specific IgM antibodies in the test serum by capturing them out of solution using anti-human IgM that was previously bound to the solid phase. An indirect IgG ELISA has been developed and can be used to differentiate primary and secondary dengue infection. The anti-dengue IgM develops early than IgG in primary infection. Because the association of sequential infection and DHF, it is important to classify fever to differentiate primary from secondary infection. In the case of sequential dengue infection the antibody response to the initial infecting virus type may exceed to the current infecting type (Halstead *et al*., 1984).

A number of rapid serological (Immunocromatographic test) kits are available now for diagnosis of dengue infections. They produce result as quick as 15 minutes (Palmer *et al*., 1999; Vaughn *et*

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al., 1998; Sang *et al.,* 1998). Unfortunately, the accuracy of most of these tests is unknown because proper validation studies have not been done.

2.7.4 Virus Isolation

Four isolation systems have routinely been used for dengue viruses: intracerebral inoculation of 1-to 3- day old baby mice, the use of mammalian cell cultures (primarily LLC-MK2 cells), Intrathoracic inoculation of adult mosquitoes, and the use of mosquito cell cultures (Gubler and Sather, 1988; Guzman and Kouri, 1999, Vorndam and Kuno, 1997).

2.7.4.1 Baby mice

Although all four dengue serotypes were initially isolated from human serum by using baby mice (Hammon et al., 1960, Sabin, 1952), this method is very time-consuming, slow, and expensive. Moreover, because of the low sensitivity of the method, many wild-type viruses cannot be isolated with baby mice. Those that are isolated frequently require numerous passages to adapt the viruses to growth in mice. This method is no longer recommended for isolation of dengue viruses, but some laboratories continue to use it (Gubler and Trent, 1994).

2.7.4.2 Mammalian cell culture

Mammalian cell cultures have many of the same disadvantages as baby mice for isolation of dengue viruses- they are expensive, slow, and insensitive (Gubler and Sather, 1988; Guzman and Kouri, 1996; Vorndam and Kuno, 1997). As with isolation systems that use baby mice, viruses that are isolated frequently require many passages before a consistent cytopathic effect can be observed in the infected cultures. Although the use of this method continues in some laboratories, it is not recommended (Gubler and Trent, 1994; Vorndam and Kuno, 1997).

2.7.4.3 Mosquito inoculation

Mosquito inoculation is the most sensitive method for dengue virus isolation (Gubler and Sather, 1988; Rosen and Gubler, 1974). Isolation rates of up to 100% of serology confirmed dengue infections are not uncommon, and this is the only method sensitive enough for routine successful virologic confirmation of fatal DHF and DSS cases (Gublar and Sather, 1988; Gubler *et al.,*1979; Sumarmo *et al*.,1983; Vaughn *et al.,* 1997). Moreover there are many endemic dengue virus

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strains that can be recovered only by this method (Gubler and Sather, 1988; Gubler *et al.,* 1978, Gubler *et al.,* 1986).

Four mosquito species have been used for virus isolation, *Ae. aegypti*, *Ae. albopictus*, *Toxorhynchities amboinensis*, and *T. splendens*. Male and female mosquitoes are equally susceptible; dengue viruses generally replicates to high titers in as little as 4 to 5 days, depending on the temperature of incubation. Dengue viruses replicate in most mosquito tissues, including the brain. A recent variation on this method involves intracerebral inoculation of larval and adult *Toxorhynchities* mosquitoes (Lam *et al*., 1986, Thet-Win, 1982). However, these modifications neither increase sensitivity nor provide other advantages over intra thoracic inoculation (Rosen and Gubler, 1974). Virus detection in the mosquito, regardless of the species, is generally performed by the direct fluorescent-antibody DFA test on mosquito tissues, usually brain or salivary glands (Gubler and Sather, 1988; Gubler *et al*., 1979, Kuberski and Rosen, 1977). From Srisuphanunt *et al.,* 2007 this can imply that ELISA can be an alternative tool for epidemiological surveillance for dengue in mosquitoes.

The mosquito inoculation technique has the disadvantages of being labor- intensive and requiring an insectaria to produce large numbers of mosquitoes for inoculation. Also, unless strict safety precautions are maintained, the chance of laboratory infections increases, although this risk can be eliminated by using male *Aedes* mosquitoes or non biting *Toxorhynchites* species for inoculation (Gubler and Sather, 1988; Rosen and Gubler, 1974)

2.7.4.4 Mosquito cell culture

Mosquito cell cultures are the most recent addition to dengue virus isolation methodology (Gubler and Sather,1988, Gubler *et al*., 1979, Gubler *et al*., 1984; Igarashi,1978; Kuno *et al* .,1985; Tesh, 1979). Three cell lines of comparable sensitivity are most frequently used (Kuno *et al*; 1985). The first cell line developed, and still the most widely used, is the C6/36 clone of *Aedes albopictus* cells (Igarashi, 1978). The use of these cell lines has provided a rapid, sensitive and economical method for dengue virus isolation. Moreover, many serum specimens can be processed easily, making the method ideal for routine virologic surveillance (Gubler *et al.,* 1984). However, this system is less sensitive than mosquito inoculation (Gubler and Sather,

1988). Dengue antigen can be detected in infected-cell cultures by DFA (Direct Florescent Antibody) or IFA (Immuno Florescent Antibody) tests with the conjugates used for mosquito tissues (Gubler *et al.,* 1984). Use of the mosquito lines is the method of choice for routine virologic surveillance.

2.7.4.5 Virus Identification

The method of choice for dengue virus identification is IFA with serotype specific monoclonal antibodies produced in tissue culture or mouse ascetic fluids and an anti-mouse Immunoglobulin G–fluorescein isothiocyanate conjugate. This test can be easily performed with infected cell cultures, mosquito brain or tissue squashes, mouse brain squashes, or even on formalin-fixed tissue embedded in paraffin and sectioned for histopathologic testing It is simple and reliable and is the most rapid method. Moreover, it allows the detection of multiple viruses in patients with concurrent infections with more than one serotype (Gubler and Sather1988, Gubler *et al.,* 1988, Guzman and Kouri, 1996; Henchal *et al.*, 1982; Laille *et al*., 1991; Hall *et al.*, 1991).

2.7.5 New Diagnostic Technology –Reverse Transcriptase Polymerase Chain Reaction (RT- PCR)

In recent year, Reverse transcriptase Polymerase Chain Reaction (RT-PCR) has been developed for a number of RNA viruses. It has the potential to revolutionize laboratory diagnosis. For Dengue RT-PCR provides a rapid serotype- specific diagnosis. The method is rapid, sensitive, simple and reproducible if properly controlled and can be used to detect viral RNA in human clinical samples, autopsy tissues, or mosquitoes (Deuble 1997, Guzman and Kouri, 1999 , Lanciotti *et al.,*1992, Vorndam and Kuno,1997).

2.7.5.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands of millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the

method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA templet is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations. A basic PCR set up requires several components and reagents, Pavlova *et al.,* 2004. These components include:

- DNA template that contains the DNA region (target) to be amplified.
- Two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target.
- Taq polymerase or another DNA polymerase with a temperature optimum at around 70 °C.
- Deoxynucleoside triphosphates (d NTPs , nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand.
- Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- Divalent cations , magnesium or manganese ions
- Monovalent cation, potassium ions.

The PCR is commonly carried out in a reaction volume of 10–200 µl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration.

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three. The cycling is often preceded by a single temperature step (called **hold**) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers (Sharkey *et al*., 1994).

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2.7.5.2 Reverse Transcription PCR (RT-PCR)

For amplifying DNA from RNA, Reverse transcriptase reverse transcribes RNA into cDNA, which is then amplified by PCR. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites. If the genomic DNA sequence of a gene is known, RT-PCR can be used to map the location of exons and introns in the gene.

Nested PCR

Increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.

The PCR assay routinely used by some laboratories for the identification of dengue virus is the nested RT-PCR assay developed by Lanciotti (Lanciotti *et al*., 1992). This comprises a two step PCR reaction involving an initial reverse transcription and amplification step using universal dengue primers targeting a region of the virus genome (C-pr M) followed by a second amplification that is serotype specific. The products of these reactions are separated by electrophoresis on an agarose gel, and the different–sized bands observed are compared with a standard marker for the relative molecular mass of nucleic acids. Dengue serotypes are identified by the size of their band.

Lanciotti *et al*., 1992 reported on the development and application of a rapid assay for detecting and typing dengue viruses. Oligonucleotide consensus primers were designed to anneal to any of the four dengue virus types and amplified a 511-bp product in a reverse transcriptase–polymerase chain reaction (PCR). First, they produced a cDNA copy of a portion of the viral

genome in a reverse transcriptase reaction in the presence of primer D2 and then carried out a standard PCR (35 cycles of heat denaturation, annealing, and primer extension) with the addition of primer D1. The resulting double –stranded DNA product of the RT-PCR was typed by two methods: dot blot hybridization of the 511-bp amplified product to dengue virus type- specific probes or a second round of PCR amplification (nested PCR) with type–specific primers, yielding DNA products the unique sizes of which were diagnostic for each dengue virus serotype. The accumulated data demonstrated that dengue viruses can be accurately detected and typed from viremic human serum samples.

Four primer pairs were selected on the basis of the published sequence data of four dengue virus serotypes so that each unique target sequence size could be amplified for each serotype by polymerase chain reaction (Morita *et al*., 1991).The procedure consists of i) RNA preparation ii) reverse transcription, and iii) polymerase chain reaction, all of which could be completed within 2 h in a single tube for each specimen. The amplified sequenced size revealed by ethidium bromide stained agarose gel electrophoresis was unique for each serotype, using infected culture fluid of isolates from dengue fever or DHF patients in Thailand, Indonesia, and the Philippines as well as from prototype viruses, thus facilitating simultaneous identification and typing.

A simple and sensitive procedure of reverse transcriptase polymerase chain reaction (RT-PCR) was developed previously such that all 4 serotypes of dengue viruses could be detected and their serotypes identified simultaneously in a single-step procedure. Maneekarn et *al*., 1993 compared the RT-PCR with a conventional immuneperoxidase (PAP) staining method for the identification of dengue viruses isolated from patient sera. Thirty –two out of the 66 serum specimens tested (48.5%) were positive for dengue viruses. Of these, 5 were type 1(DEN-1), 25 were type 2 (DEN-2) and 2 contained both Den -1 and DEN-2. All cultures that were positive by PAP method were also positive by RT-PCR and vice versa. Thus, the results obtained by RT-PCR were in good agreement with those by PAP.

Eva Harris, T. Guy Roberts *et al*., 1998 rapidly detected and typed the dengue virus in clinical samples and mosquitoes. Assays based on reverse transcriptase (RT-PCR) amplification of dengue viral RNA can offer a rapid, sensitive, and specific approach to the typing of dengue viruses. They designed single-tube, a plasmid base internal control that produces a uniquely

sized product RT-PCR procedure. This single-tube RT-PCR procedure was used to type dengue viruses during the 1995 and 1997-1998 outbreaks in Nicaragua. In addition, an extraction procedure that permits the sensitive detection of viral RNA in pools of up to 50 mosquitoes without PCR inhibition or RNA degradation was developed. This assay should serve as a practical tool for use in countries where dengue fever is endemic, in conjunction with classical methods for surveillance and epidemiology of dengue viruses.

Virologic surveillance for dengue through the detection of the prevalent serotype (s) circulating in the human population during inter and intra epidemic periods constitutes a reliable sentinel systems for dengue outbreaks. Vincent *et al.,*1998 applied a rapid and sensitive, semi-nested, reverse transcription–polymerase chain reaction (RT-PCR) assay using nonstructural protein 3 gene primers for the type –specific –detection of dengue viruses in artificially infected and in field caught adult *Aedes* mosquitoes. In a prospective field study conducted from April 1995 to July 1996, female *Ae.aegypti* and *Ae. albopictus* mosquitoes were caught from selected dengue sensitive areas in Singapore and assayed by RT-PCR. Approximately 20% of 309 mosquito pools were positive for dengue virus. Infected *Ae aegypti* were detected as early as six weeks before the start of dengue outbreaks in 1995 and 1996. Virologic surveillance by RT-PCR for detecting dengue virus-infected *Aedes* mosquitoes in the field may serve as an early warning monitoring system for dengue outbreaks.

Pankhong *et al.,* 2002 developed in-house RNA extraction and RT-PCR reagent kits for the molecular sero typing of dengue viruses in field caught *Aedes* mosquitoes. Mosquitoes that showed positive results by ELISA or IFA were selected for the identification of dengue viruses in order to predict the distribution of the four dengue serotypes. Total RNA was extracted from one whole mosquito as well as from one dissected mosquito by guanidinium thiocyanate denaturation and isopropanol precipitation. The extracted RNA was amplified by their in-house RT-PCR reagents specific for each dengue serotype under optimized condition. These results indicated that most of the dengue viruses were located in the head and thorax rather than in the abdomen.

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2.8 Dengue Research in Bangladesh

Dengue was first reported as "Dacca fever" in Bangladesh in 1964 by Aziz and his colleagues. Subsequent reports suggested that dengue fever may have been occurring sporadically in Bangladesh from 1964 to 1999 (Aziz, 1967; Gaidamorich, 1980; Islam, 1982; Khan *et al.,* 1986; Amin, 1999; Alam, 2000; Hossain *et al.,* 2003).

An outbreak of an acute febrile illness clinically suspected as dengue and DHF occurred in and around Dhaka city during the summer of 1999 and serological evidence of dengue virus infection was found in the majority of the cases (Alam, 2000). The first epidemic of dengue was reported in the capital city Dhaka in the year 2000 (Rahman *et al.,* 2002 & Aziz, 2002). Since then the disease has shown an annual occurrence in all major cities of the country. During January 2000-December 2007, Bangladesh recorded a total of 22245 cases and 233 deaths (1.04%). Of these, Dhaka accounted for 20115 cases and 181 deaths (0.9%) (Choudhury *et al*., 2008). During 1982-83, an entomological survey conducted in Dhaka City revealed the presence of vector mosquito but the population density of the vector was below the optimal level for propagation of epidemic outbreaks. Thus *Ae. aegypti* index was 16.2 and that of *Ae. albopictus* was 5.35 (Khan & Ahmed,1986). Result of a mosquito survey carried out in Dhaka and Chittagong in 1997 indicated the Breteau Index in Dhaka 30.8 and in Chittagong 18.2 which was higher than previous index (Knudsen, 1997). Chowdhury, *et al*., 2000 revealed the vector position in Dhaka city. The overall Breteau Index (BI) was 22.6 (range from 0.0 to 94 in different city ward). Out of 90 wards of Dhaka City, 46 wards were above 20 BI. The health department of Bangladesh government also reported a high BI for Dhaka City BI-50. The increased number of vector mosquitoes resulted in increased number of cases and finally caused the outbreak in the year 2000. The number of dengue cases increased in the year 2000 not only in Dhaka city but also in other cities, e.g. Chittagong , Khulna, Barishal and Rajshahi (Yunus *et al.,* 2001, Ahmed *et al*., 2001).

The seasonal prevalence of *Ae. aegypti* and *Ae.albopictus* was studied in Dhaka city in the year 2001-2002 (Ahmed *et al.,* 2007). They collected the larvae of mosquitoes from indoor and outdoor in the urban areas of Dhaka city and found both the species were active in both dry and wet seasons with a peak in July, when the rain fall was the highest. From September to April, the

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larval population level remained low and the reduction of the population during winter was related to the low rainfall.

Container breeding mosquitoes in Dhaka city, was studied by Bashar *et al*., 2006. It was found that clay jar, pot, pitcher, bucket, gallon jar etc, cemented tanks artificial ponds, bathtubs, plastic tank, tin tank, water reservoir, drum, flower vase, tin pot, jerry can, aluminum jar/pot/ container etc are important indoor containers as mosquito breeding places. It was suggested to reduce the availability of water reservoir to control container breeding mosquitoes in Dhaka city. In recent years both the population of vector and that of human have increased alarmingly in Dhaka city, thereby increasing the likely hood of epidemic DHF if and when dengue viruses with epidemic potentials are introduced.

Unfortunately there is still no vaccine or specific therapy for dengue and the technology required for laboratory diagnosis are lacking in Bangladesh. Options for mosquito control are also limited. As such, extensive research on Virology, Immunology and Epidemiology is essential to identify risk factors of DHF and to recommend easy, affordable, sensitive and specific laboratory methods for the diagnosis of dengue infection.

A recent outbreak of dengue in Bangladesh was marked by many fatal complications. As clinical virulence varies among the genotypes of dengue virus, a study was conducted to investigate the molecular genotypes of dengue in Bangladesh by Aziz *et al*., 2002. RT-PCR was used to determine viral genotypes using oligonucleotide generic primers that produce a 511 bp product. The resulting product was typed by nested PCR with strain- specific primers, yielding 482 (DEN-1), 119 (DEN-2), 290 (DEN-3) and 392 (DEN-4), visualized on UV transilluminator after electrophoresis on 2% agarose gel stained with etidium bromide. Of 45 clinically diagnosed dengue patients DEN -3 was detected in all except 2 patients who were infected with DEN2. The study suggested the predominance of DEN- 3 infection with occasional co- infection with other types, during the recent outbreak of dengue in Bangladesh.

The first dengue virus isolation by mosquito inoculation and c6/36 cell culture in Bangladesh was reported (Pervin *et al*., 2003). The dengue virus isolation rate is higher by the mosquito inoculation than the cell culture inoculation technique. It was revealed from the study that, for

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routine surveillance of dengue infection, isolation of virus by mosquito inoculation is a more sensitive, economical, easy and sustainable method.

Dengue virus was isolated from 44 clinically suspected cases of dengue fever by mosquito inoculation technique using *Ae. aegypti* mosquito in the department of virology, BSMMU in the year 2000 (Pervin *et al*., 2002). Identification of dengue virus was done by direct fluorescent antibody technique using FITC conjugated anti-dengue monoclonal antibody and serotyping was carried out by indirect fluorescent antibody technique using serotype specific monoclonal anti–dengue antibody. The dengue virus isolation rate was 41.9%. The result of serotyping revealed circulation of all four serotypes of dengue virus in Dhaka, Bangladesh and DEN-3 was the predominant (70.5%) serotype during the outbreak of 2000. The mosquito inoculation technique is an easy, economical and sensitive method for dengue virus isolation and is recommended for routine virological surveillance in laboratories of Bangladesh where sophisticated facilities are limited.

During the febrile illness epidemic in Bangladesh in 2002, 58 people died out of the 6,132 affected. Two hundred hospitalized patients were analyzed clinically, serologically and virologically to determine the features of this dengue infection (Islam *et al*., 2006). Eight dengue virus strains were isolated, representing the first dengue virus isolation in the country, and all of the strains were dengue virus type-3 (DEN-3). Sequence data for the envelope gene of the DEN-3 Bangladeshi isolates were used in a phylogenetic comparison with DEN-3 from other countries. A phylogenetic analysis revealed that all 8 strains of DEN-3 were clustered within a well supported independent sub-cluster of genotype II and were closely related to the Thai isolates from the 1990s. Therefore, it is likely that the currently circulating DEN-3 viruses entered Bangladesh from neighboring countries.

Choudhury *et al.*, 2008 modeled the monthly number of dengue fever (DF) cases in Dhaka, Bangladesh, and forecast the dengue incidence using time series analysis. Seasonal Autoregressive Integrated Moving Average (SARIMA) models have been developed on the monthly data collected from January 2000 to October 2007 and validated using the data from September 2006 to October 2007.

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CHAPTER 3 *METHODS & MATERIALS*

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3.1 Bionomics study of *Ae. aegypti* in Dhaka city

The Bionomics study component of this research was done in a small range which basically helped us for collection of mosquito samples for molecular studies and other laboratory analysis. *Ae. aegypti* and *Ae. albopictus* were recognized as the principal and the secondary disease vector for the disease dengue and DHF, respectively. Though *Ae. albopictus* also transmits dengue in Bangladersh our searches were focused on the Bionomics study of the principal dengue vector *Ae. aegypti's* . Since *Ae.aegypti* is a highly domesticated mosquito which can complete its entire life cycle within the confines of a single human dwelling and can readily be adapted to laboratory colonization, we have chosen this vector for field collection. *Ae. aegypti* produces diapausing eggs making it easy to preserve for a long time. With a small number of volunteers it was easy and convenient for us to collect the indoor resting adults and monitor the biting rates on regular basis.

3.1.1 Mosquito surveys

Dhaka is the capital and main city of Bangladesh located at 23°42′ 0" N. 90°22' 30"E, covering an area of 815.485 km² (315sq miles). The population of Dhaka, currently 13 million people, is projected to 20 million by the year 2025 (Wikipedia, 2014). Dhaka is the largest city in Bangladesh and the eleventh most populous city of the world (The World Gazetteer, July 16, 2012). The Dhaka region was chosen as the study area because of its relative high incidence of DF between 2000-2009. Indoor resting adults and larval mosquito surveys were carried out in five representative dengue prevalent areas in Dhaka city from July 2009- June 2010. In 2003, a comprehensive household level mosquito survey was conducted by Ali, *et al.,* where clusters of dengue illness and high density vector populations were observed in a distinct sector of the city.They found a spatial association between dengue clusters and dengue populations and prepared Disease-environment map which displays areas where transmission is most intense. We used that map to select five high risk areas where the dengue cases and the vector population were comparatively high. The areas were: a) Dhaka University Campus; b) Shegunbagicha; c) Dhanmondi Residential Area; d) Rampura and e) Mirpur (Fig 3.1).

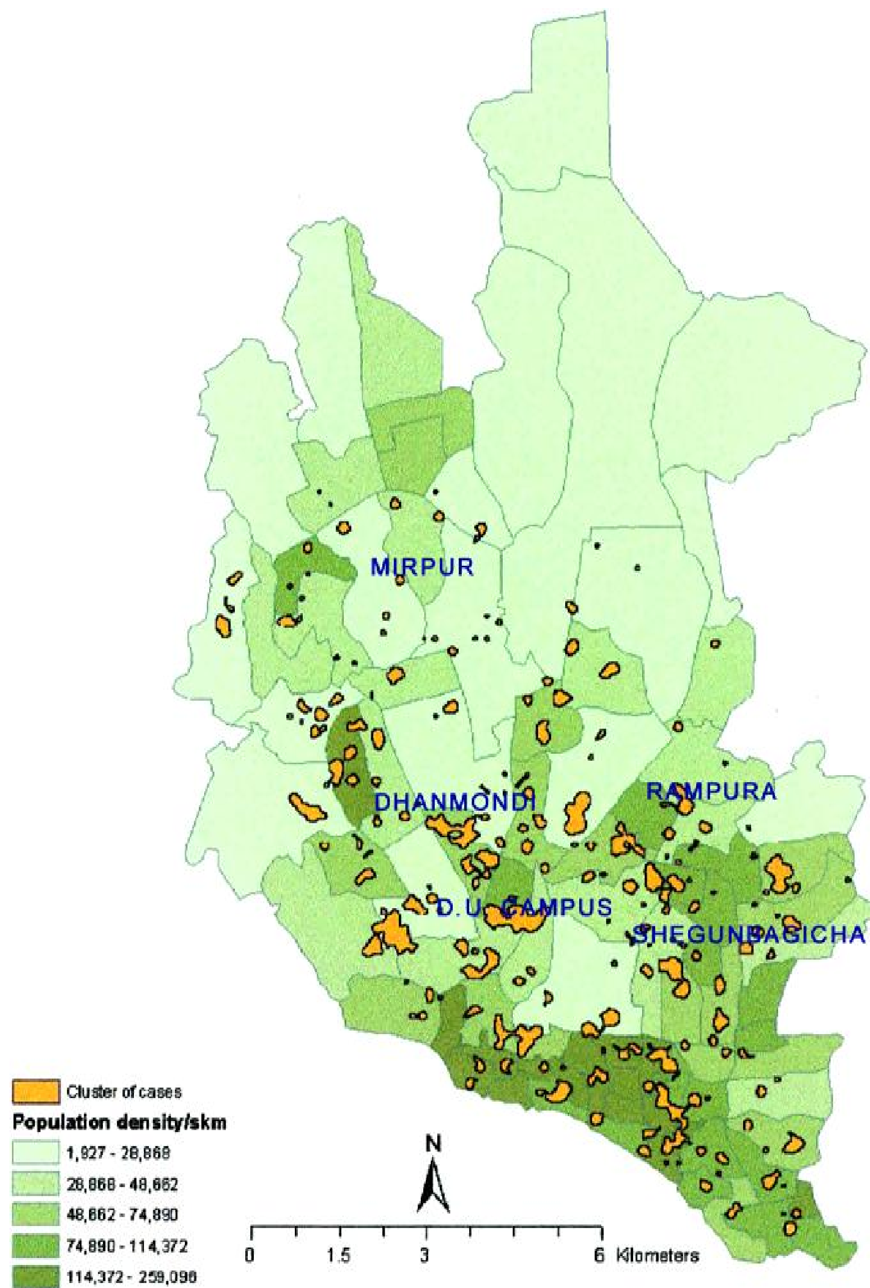

Fig 3.1: Disease-environment map showing Dengue prevalent areas in Dhaka City, (Ali, *et al.,* 2003)**; five selected experimental areas marked by name**

The observations and inferences were made under two main headings:

1) Breeding habitat of *Ae. aegypti*

2) Seasonal prevalence of larval and adult *Ae. aegypti* population by observing Breteau Index, Container Index and Indoor resting adult mosquitoes biting rate and Probable relation to disease outbreak.

3.1.2 Larval collection

For larval survey the entomological indices: House Index (HI); Container Index (CI) and Breteau Index (BI) were used for measuring the larval population (Sharma *et al*., 2005).

$$\text{House Index} = \frac{\text{No. of houses positive (larvae)} \times 100}{\text{No. of houses inspected}}$$

$$\text{Container Index} = \frac{\text{No. of containers positive} \times 100}{\text{No. of containers inspected}}$$

$$\text{Breteau Index} = \frac{\text{No. of containers positive} \times 100}{\text{No. of house inspected}}$$

In each survey, all potential mosquito breeding containers were examined for mosquito breeding. Small containers were emptied into white pan and the contents were examined for larvae and pupae. Large containers which could not be emptied or aspirated were carefully examined, using flashlight necessary, and a sample of larvae and pupae was collected. Larvae were reared to adult stage for species identification. Routine collection and observation were made twice in each area per month from during study period. All five representative areas were visited at 15 days interval (twice a month), collection was made 10 houses per day in each area and a total of 250 houses in each study area were visited during one year study period for larvae collection.

3.1.3 Adult collection

Ae. aegypti is known to bite diurnally and there are two peaks of biting activity (McClelland, 1959, 1960). The biting activity of female mosquitoes had been monitored to observe the seasonal prevalence of Adult *Ae. aegypti*. The biting rate is expressed as the number of female mosquitoes caught per man per hour. Indoor resting *Aedes* adults were collected before sunset and after sunrise from the above mentioned spots by the help of aspirator to analyses the mosquitoes behavior, seasonal fluctuation, biting rates and to preserve them for further investigation.

The collections were made in the morning after sunrise between 7-9 am and in the afternoon before sunset between 4-6 pm at an interval of 15 days. Each collection consisted of 30 min in each house. The collections were made 4 houses per day in each area. So, a total of $(2\times4) = 8$ houses were visited in an area per month, and a total $8\times 12 = 96$ houses of an study area were visited in during one year of study period for biting adult collection. A volunteer setting inside a dark corner of a room and all mosquitoes were collected by a special glass aspirator which landed on his bare arms (Fig 3.2-3.3) and feet within these 30 minutes time period. The specimens were taken to the laboratory and checked for sex and for species.The total number of female *Aedes* species counted in each morning or evening collection and biting rate per man per hour had been counted, recorded and preserved separately for each area.

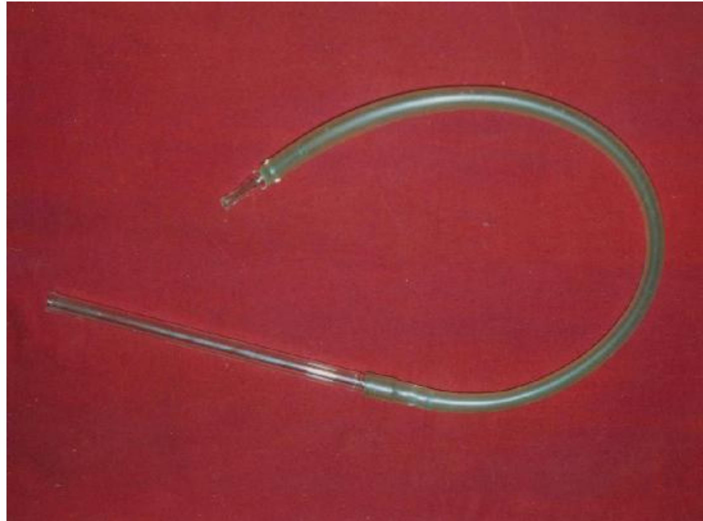

Fig 3.2: Special glass Aspirator for adult mosquito collection

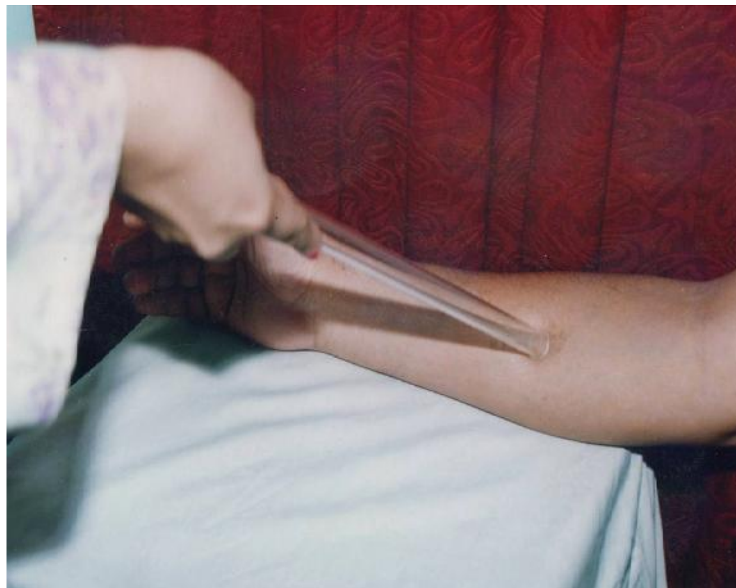

Fig 3.3: Indoor resting adult *Aedes* collection when landed on a human volunteer

3.2 Molecular Analysis of Dengue virus

The following laboratory procedures were used for molecular analysis: **(a)** Laboratory infected mosquitoes were prepared **(b)** Dengue virus in lab reared mosquitoes were detected by **indirect dengue Ag-capture ELISA**. Detection and typing of dengue virus RNA by reverse transcriptase polymerase chain reaction **(RT-PCR)** were done in **lab reared mosquitoes** and in **dengue patients clinical samples** and finally Detection and typing of dengue viral RNA were done in **field–caught mosquito specimens** by Reverse Transcriptase polymerase chain reaction **(RT-PCR)**

3.2.1 Preparation of Laboratory Infected Mosquitoe through Inoculation of Dengue Virus

3.2.1.1 Setting up of Mosquito Insectaria (Fig 3.4):

Materials required to rear *Ae. aegypti* mosquito colony are described in annexure-1.

3.2.1.1.1 Collection of Mosquito Eggs:

For egg collection, six water filled earthen containers had been placed in different location of Dhaka University campus. After 7-10 days, larvae collected from those containers and maintained in lab till adult emergence.Only *Ae .aegypti* adults were identified and kept for colonization.The male *Aedes* mosquitoes were maintained by cotton soaked sucrose solution and females were blood fed 2-3 days after emerging. Strips of *Aedes aegypti*'s eggs were collected by placing the wet blotting paper inside the mosquito cage and preserved**.** The *Aedes* colony was maintained further from these egg strips.

3.2.1.1.2 Hatching of Eggs and Rearing of Larvae:

The stripe of *Ae.aegypti* eggs was immersed under half liter of water in a bowl kept at room temperature (26±2° C)**.** Within 2-3 hours the larvae hatched out. The strip of egg was kept under water for another 2-3 days for maximum hatching out of larvae. On the next day the 1st instars larvae were transferred with a wide mouth dropper to another bowel of fresh water. The room temperature was kept within 26±2°C. "Cerelac" baby food solution was used as larval food. Feeding was given on the second day. 1-2 drops of Cerelac suspension was added to the bowl

with 1st instars larvae by dropper.To avoid fungal contamination, the larvae were transferred regularly from one bowl to another and the water was changed every day before the supply of food. Larvae took 4-5 days to become a pupa. The pupae were collected immediately and kept in an 80 ml plastic glass half filled with water and were placed in the cages**.** After 2/3 days the pupae began to emerge. The males always emerged earlier than females.

3.2.1.1.3 Adult Feeding:

Both the adult male and female *Ae. aegypti* were kept in a 18" × 18"×18" mosquito rearing cages**.** A piece of cotton soaked with 10% sugar solution was placed in a petri dish inside the cage and were supplied daily to the adults. The male mosquitoes usually take sugar feed**.** During first 2-3 days females also took the sugar feed. Then pigeon blood meal was supplied during day time usually in the morning to the females for the development of eggs. The pigeon was tied at their legs and wings and after removing the feathers of its thorax it was kept on the top of the cage. Every day 1-hour blood meal was given till they started to lay eggs, which allowed the females to suck blood to their full content.The RT was 26±2°C, RH 75-85%.

3.2.1.1.4 Egg Laying:

Female begin to lay eggs within 72 hours of blood meal. The eggs were laid singly on a wet blotting paper placed around the inner surface of a black cup because the females prefer to lay eggs on the dark, wet and moist place. When the moist blotting paper strip became full of eggs, it was removed and placed in a dry place to dry the egg strip for preservation. After labeling, the dry strip was kept in a glass jar after properly dated used as desecrators. The jar was kept in a place free of mites and ants. The eggs were preserved in this condition for about 4-6 months.

3.2.1.1.5 Inoculation of Mosquito with Patient's Sera:

For inoculation of mosquito with patient's serum, 1-5 days old *Aedes* mosquitoes were used. Therefore separate cages were used for test mosquitoes. A mother cage was maintained for collection of eggs for continual production of new mosquitoes for experiments and also for maintenance of colony (Fig 3.5).

Fig 3.4: Mosquito Insectaria in Zoological garden, DU

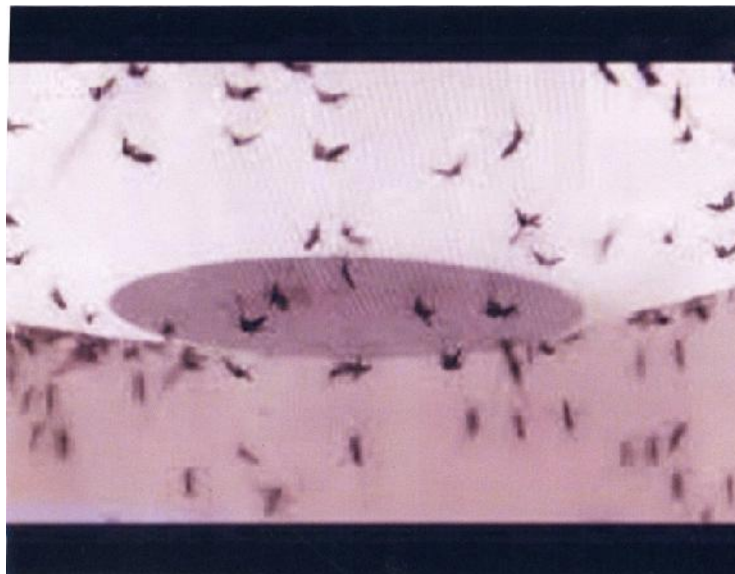

Fig 3.5: Mosquito colony inside a cage

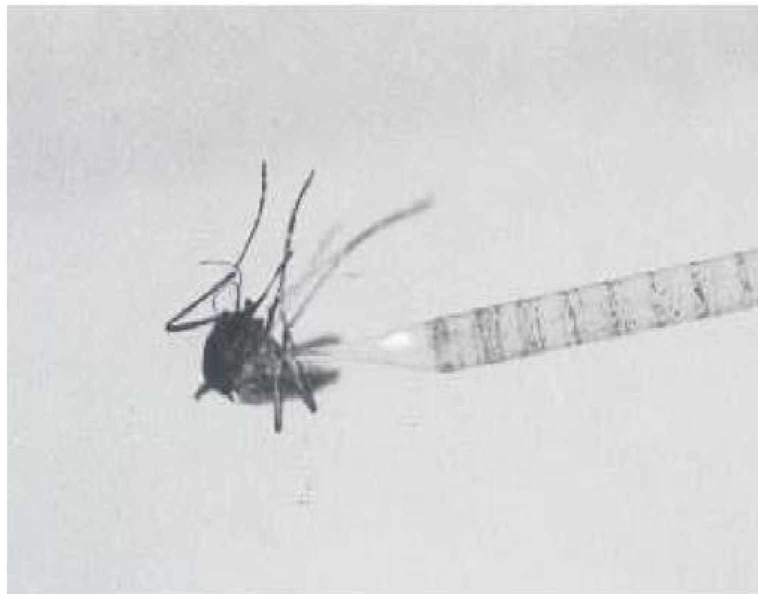

Fig 3.6: Pierced capillary needle in the thorax of mosquito

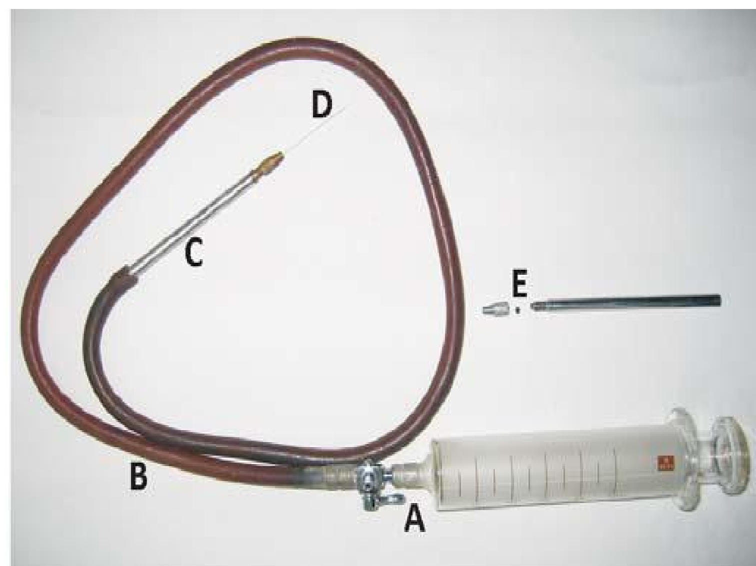

Fig 3.7: Parts of inoculation apparatus. A-Three way leur lock

B-Latex tubing; C-Metal needle holder; D-Glass capillary and E-Rubber washer

3.2.2 Intra Thoracic Inoculation of Dengue Virus

3.2.2.1 Preparation of Materials Used in Mosquito Inoculation Technique:

Mosquito inoculation technique described by Mourya *et al.*, 2007 was followed for Intra thoracic inoculation of dengue virus.Materials required for mosquito inoculation technique are shown in Annex-2, Fig-3.6-3.7. Inoculation needle used for infecting mosquitoes is prepared from capillary tubing normally measuring about 60 mm in length with approximately 0.5 mm inner and 1 mm outer diameter. Preparation of the inoculation needle involves heating at the middle of the capillary tube over a micro-burner and by drawing apart the two inoculation needles are obtained. The tapering tip of each inoculation needle thus prepared is broken off at an appropriate point to leave a sharp tip with fine bore. To graduate the capillary for calculating the precise amount of inoculums, the untapered portion of the needle is marked at 1 mm distance with a rubber stamp specially prepared for the purpose or with any marking device/pen**.** The calibration permits the inoculation of the required volume of inoculums by observing the length through which the inoculums is moved. These capillaries (D) are attached to a fabricated tubular metal needle holder(C). At the other end of the needle holder, latex tubing (B) is slid over which is connected to a three-way leur lock (A) fixed to the syringes. The diameter of the needle and small tapering end make it difficult to draw the fluid in. This requires considerable pressure in order to overcome the resistance offered at the fine tip of the inoculation needle. A 5ml metal luer lock numbered standard lab glass syringe (graduation0.5ml) (Top glass syringe manufacturing Co.Pvt Ltd, India) had been used for this purpose. The three-way leur lock is turned on so that the needle is connected to the syringe. The plunger of the syringe was then withdrawn. Enough care was taken to prevent the inoculums from traversing the entire length of the inoculation needle and entering the needle holder.

3.2.2.2 Collection of Patient's Serum

For this study serum sample was collected from thirty clinically suspected patients of dengue fever seeking treatment and diagnosis facility in Labaid Hospital, Dhaka during an outbreak of dengue from November, 2010 to January, 2011. All patients were enrolled on the basis of specific selection criteria with fever above 100°F and within five days from the onset and of all

ages and sexes with any two or more of the symptoms described in the cases definition of DF and DHF by WHO. Blood sample was drawn after one week onset of fever. Dengue cases were confirmed on the basis of patient's IgM and IgG status by serological test MAC-ELISA (Diagnostic blood report shown in Table-4.15, in Chapter 4). With aseptic precaution 5 ml of venous blood was collected and was immediately transferred to a sterile vacutainer. After separation (centrifuge 1000 rpm, 2 minutes at room temperature), the serum was transferred to labeled ependroff tube in duplicate. RNA was extracted from one tube on the day of blood collection and was stored at -70°C until RT-PCR. Another serum containing tube was stored at -70°C till inoculation into the mosquitoes.

3.2.2.3 Calculation for the amount of inoculums injected:

Manufacturer's specification of glass capillary tube is 0.7 to1.0 mm diameter with approximately wall thickness of 0.2mm. Measuring inner diameters of 100 lengths of tubing Rosen and Gubler (1974) gave a mean value of 0.469±0.002mm.

Using $V=\pi r^2 h$

Where $\pi=3.1416$

$h=1$ mm

$r^2 =(0.469/2)^2$

$V =3.1416\times (0.469/2)^2$

$=3.1416\times 0.055\times 1$

$=0.173\text{mm}^3$

$V =0.17\mu l$ or 0.00017ml

3.2.2.4 Inoculation Procedure:

Adult *Aedes* mosquitoes (1-5 days) old were removed with an aspirator from the rearing cages and transferred them into glass test tubes stopper with cotton. Then the tube with mosquitoes was

placed in a beaker filled with small pieces of wet ice for 10-20 minutes to immobilize the mosquitoes.

3.2.2.5 Preparation of the Serum Inoculums:

As patient's sera appeared to be toxic for *Aedes* mosquitoes, the serum of the patient was first diluted in 1-5 dilution using diluents made with PBS, 0.5% Gelatin and 5% inactivated fetal calf serum with pH 7.4 (Rosen & Gubler, 1974). List of reagents required are shown in Annexure-3. The composition of PBS and PBS diluents is described in Annexure-4 and 5. Preparation of the inoculums is described in Annexure-6.

3.2.2.6 Inoculation Technique:

Method described by Rosen and Gubler (1974) was followed for mosquito micro inoculation. The capillary needle was filled by immersing the tip of the needle in the inoculums. Before inoculation, the mosquitoes were immobilized by confining to glass test tubes on ice bath. These were then transferred on a filter paper disc placed on the stages of the compound microscope. Mosquitoes remain immobile for about half a minute, which is sufficient to manipulate them for inoculation. The inoculation method employs compressed air by pushing the plunger of the syringe to force the inoculums through the glass inoculation needle. The inoculation needle was introduced inside the mosquito thorax by piercing the membranous area, just anterior to the mesenteron below the spiracle of female mosquitoes. After inoculation of 0.17µl of inoculums, mosquitoes were kept in the plastic glasses provided with cotton pad soaked in 10% sucrose solution to feed and held for incubation in the insectaria at 28 ± 1°c with high relative humidity (80±5%) for 10-14 days. The plastic glasses were covered with a piece of fine net. The net was fixed at the edge of the glass with a rubber band. They were then individually separated by pools. About fifty mosquitoes were inoculated per patient serum.The sample number and the date of inoculation was marked on the glass with a marker pen.

Fresh cotton pad soaked with saturated sugar solution was placed on the net on the top of the container. Every day the cotton pad was wetted and after every 3-4 days the pad was changed and replaced with another fresh cotton pad soaked with sugar solution. In case of any growth the pad was immediately replaced. The glass containing inoculated mosquitoes was placed in the

deep chamber of the refrigerator to kill the survivors on day 12-14. Dead mosquitoes were removed from the glass with a forceps and were dissected into two parts for two different future analyses: the heads were then tested by ELISA & the thoraxes by RT-PCR. Per patient serum twenty inoculated mosquitoe heads were kept in one ependroff tube (which was called a head pool) and their thoraxes (which was called a thorax pool) were kept in another ependroff tube. This way 30 individual head and thorax pools of mosquitoes inoculated by 30 patient blood serum were labeled in different eppendroff tubes and preserved in -70°C for future use.

3.3 Detection of Dengue Virus by Indirect Dengue Ag-Capture ELISA

To test and compare ELISA and RT-PCR method for the virologic surveillance of dengue virus-infected *Aedes* mosquitoes lab inoculated mosquito's heads pools were tested by ELISA and their thorax pools by RT-PCR.

3.3.1 Collection of Materials for ELISA

Antigen capture ELISA based methods using specific monoclonal antibodies (MAB) s were followed. Goat-anti-human IgG, Goat-anti mouse IgG-HRP conjugated and mouse flavivirus specific monoclonal antibodies (4G2) were obtained from Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA and Department of Virology, the US Army Medical Components (AFRIMS), Thailand respectively.

3.3.2 Preparation of Mosquitoes for ELISA:

The adult dengue inoculated *Ae. aegypti* female head pool containing tubes were triturated separately with 1 ml plastic mosquito tissue grinders in a solution of 20% acetone-extracted normal human serum in PBS, pH 7.4, 0.05% Tween-20 and 0.02% NaN_3 in PBS with pH 7.4. The suspensions produced were stored at -70°C until the experiment was done. The procedure described by Sithiprasasna (Sithiprasasna *et.al,.*1994) was adopted to optimize detection of antigen in this study.

3.3.3 ELISA Procedure

ELISA plates (96-well U-bottom polystyrene plates, Titertek. Flow) were sensitized coated with an anti-human goat globulin, diluted 1:800 in 0.1M carbonate buffer, pH 9.0 by absorbing

100µl/well at 24°C for 4h. Plates were stored at 4°C overnight. Just prior to use in the assay and between each of the incubations that followed, the sensitized plates were washed six times, each time with 200µl/well of 0.01M PBS, pH7.4, containing 0.05% Tween-20 (PBS-TW). To block non-specific protein binding, wells were flooded with 1% casein in PBS-TW at 24°C for 2 h. The polyclonal anti-flavivirus human IgG capture antibody was then diluted 1:100 in PBS, added to the wells (50µl /well) and incubated at 24°C for 2 h. The test samples were then added to duplicate wells (50µl/ well) and incubated at 4°C overnight. Mouse anti-flavivirus monoclonal-detector antibody (4G2), diluted 1:1000 in 20% acetone- extracted normal human serum in PBS pH7.4, was then added to all wells (50µl/well) and incubated at 24°C for 2 h. Goat anti-mouse IgG conjugated to Horse-Reddish Peroxidase was diluted 1:1000 in 20% acetone-extracted normal human serum in PBS and incubated in the well at 24°C for 2 h. A small quantity (67 µl) of the substrate solution 5 mg O-phenyline- diamine diluted in 10 ml citrate phosphate buffer, pH 5.0 and 33µl of 3% hydrogen peroxide were finally added to each well and incubated for 10 min in darkness. The reaction was stopped by addition of 50 µl of 4M H_2SO_4/well and the absorbance was read at 492 nm. All optical density (OD) values were read directly from a Dynatech MR600 reader to a microcomputer, and the data were stored using Lotus 1-2-3 spreadsheet software. Specimens were considered positive if their OD values were greater than the mean value plus three standard deviation (S.D) of the negative controls. Negative controls consisted of suspensions of triturated mosquitoes of the same stage and species as that place in the test wells, and of a number equal to the maximum number of specimens in test. Positive controls consisted of various serial dilutions of the virus seed.

Fig 3.8: Assembly for Identification of dengue virus by

Indirect dengue Ag-capture ELISA

3.4 Molecular Detection and Typing of Dengue Virus by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The nested RT-PCR assay developed by Lanciotti (Lanciotti *et al*., 1992) was followed which comprises a two step PCR reaction involving an initial reverse transcription and amplification step using universal dengue primers targeting a region of the virus genome (C-pr M) followed by a second amplification that is serotype specific. The products of these reactions are separated by electrophoresis on an agarose gel, and the different–sized bands observed are compared with a standard marker for the relative molecular mass of nucleic acids. Dengue serotypes are identified by the size of their band. Prototype DEN-1, DEN-2, DEN-3 and DEN-4 strains were collected from the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand through scientific collaboration agreement with Labaid Hospital, Dhaka.

3.4.1 RNA Extraction

3.4.1.1 RNA Extraction from Mosquito Thorax:

RNA was extracted from 30 pools of artificially infected mosquitos' thoraxes which were macerated in phosphate buffer saline (PBS) and clarified by centrifugation. Viral RNA was extracted using a commercial reagent, TRIZOL LS (Gibco BRL, Maryland, USA). The reagent is a monophasic solution of phenol and guanidine isothyocyanate and has been modified for the single step RNA isolation method developed by Chomezynski and Sacchi, 1987. 750 µl of TRIZOL LS were added to each ependroff tube containing 250 µl of sample and then mixed several times through a pipette to promote lysis of the cells in the sample. The homogenized sample was incubated for 5 minutes at 15-30°C to permit the complete dissociation of nucleoprotein complexes.200 µl of chloroform were added to this solution and shaken vigorously for 15 seconds and then incubated at 15-30 °C for 2-15 minutes. The sample was centrifuged at 12000 rpm for 20 minutes at 2-8°C. Following centrifugation, the mixture was separated into a lower red phenol-chloroform phase, an interface, and a colorless upper aqueous phase; RNA is found only in the aqueous phase. The aqueous phase was transferred to a clean tube and RNA was precipitated by mixing with isopropyl alcohol. After incubation at15-30°C for 10 minutes, the sample was centrifuged at 12000rpm for 15 minutes at 2-8°C. The supernatant was discarded and the RNA pellet was washed with 75% ethanol and centrifuged at 7, 500rpm for 10 minutes at

2-8°C. Finally, the RNA pellet was air dried for 5-10 minutes and dissolved in RNAase free water and was stored at -70°C for further analysis.

3.4.1.2 RNA Extraction from Dengue Patient's serum:

Extraction of RNA from dengue patient's serum was done on the day of blood collection. 750 µl of TRIZOL LS were added to each 250 µl of serum samples and then single step RNA isolation method was followed as described above. Extracted RNA was stored at -70°C for further analysis by RT-PCR.

3.4.2 Sequence of Oligonucleotide Primers Used to Amplify and Type Dengue Viruses

Dengue virus genetic primers & Sequence	Size in bp of amplified DNA product
D1 (5'TCA ATA TGC TGA AAC GCG CGA GAA ACC G 3')	511
D2 (5' TTG CAC CAA CAG TCA ATG TCT TCA GGT TC 3')	511
The type- specific primers & Sequence	
TS1 (5' CGT CTC AGT GAT CCG GGG G 3')	482 (D1 & TS1)
TS2 (5' CGC CAC AAG GGC CAT GAA CAG 3')	119 (D1 & TS2)
TS3 (5'TAA CAT CAT CAT GAG ACA GAG C 3')	290 (D1 & TS3)
TS4 (5'CTC TGT TGT CTT AAA CAA GAG A 3')	389 (D1 & TS4)

Primers were purchased commercially from Gibco -BRL, USA.

3.4.3 Reverse Transcription and Amplification of RNA

Target sequence of the virus RNA was converted to a complementary DNA copy (cDNA) using reverse transcriptase (RT) and the dengue virus downstream consensus primer (D2), homologous to the genomic RNA of the four serotypes, prior to enzymatic DNA amplification. Subsequently, Taq polymerase amplification of resulting cDNA was performed using the upstream dengue virus consensus primer (D1).

Step-1: Twelve micro liters of extracted RNA were added to the 13µl of RT mixture.

RT mixture

5× RT buffer

(250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$)

1.25mM of each dNTP's (Gibco BRL, Maryland, USA)

25 pmol D2 primer

200 units of Superscript II and

20 units of RNase inhibitor (Promega, Madison , USA).

The cDNA was synthesized by incubating this mixture at 37°C for 60 minutes, followed by enzyme inactivation at 100°C for 10 minutes.

Step-2: The first round of PCR was carried out using 50µl volume. Twelve micro liters of cDNA were added to 38 µl of the PCR mixture.

The PCR mixture

10 × PCR buffer (50 mM KCl; 10 mM Tris-HCl-pH 9.0; 1.5 mM $MgCl_2$ and 0.1% Triton X-100)(Promega, Madison, USA)

2.5 mM of each d NTP'S (Gibco BRL, Maryland, USA)

12 pmol D1 primer and 1 unit *Taq* DNA polymerase (Promega, Madison,USA).

The reaction was carried out in a Techne (Duxford Cambridge, UK) thermal cycler at 94°C for 5 minutes of initial denaturation followed by 35 cycles of denaturation (94°C for 1 minutes) primer annealing (55°C for 1.5 minutes) primer extension (72°C for 2.5 minutes) followed by final extension at 72°C for 7 minutes.

Fig 3.9: Placing a strip of eight PCR tubes, into the PCR machine

3.4.4 Typing of Dengue Virus by nested PCR

A second amplification reaction was performed with 1.0µl of the amplified product of the first amplification reaction. The reaction mixture contained all the components described for the first amplification reaction with one exception: the primer D2 was replaced with the dengue virus type- specific primers TS1, TS2, TS3 and TS4. After initial denaturation at 94°C for 5 minutes, the samples were subjected to 30 cycles of denaturation at 94° C for 1 minute, primer annealing at 58°C for 1 minute and primer extension at 72°C for 2 minutes, followed by final extension at 72°C for 7 minutes. Fifteen micro liters of the amplicon were subjected to ethidium bromide stained 2.0% agarose gel (Sigma, Missouri, USA) electrophoresis and the size of resulting DNA band was characterized for each dengue virus type. 482 bp (Den-1), 119 bp (Den-2), 290 bp (Den-3) and 389 bp (Den-4).

3.4.5 Detection and Typing of Dengue viral RNA in Patient Serum by nested RT-PCR

Extracted RNA from 30 patient serum samples stored at -70°C were thawed and 12µl from each pool were added to the 13 µl of RT mixture. Target sequence of the virus RNA was converted to cDNA using reverse transcriptase (RT) and the dengue virus downstream consensus primer (D2). The cDNA was synthesized by incubating the mixture. The first round of PCR was carried out in 50 µl reaction volume .Twelve (12)µl of cDNA were added to 38 µl of the PCR mixture. Amplification was performed using the upstream dengue virus consensus primer (D1). The reaction was carried in gene cycler with following PCR program that mentioned:

Initial denaturation 94°C for 5 minutes, Denaturation 94°C for 1 minutes (35 cycles), Primer annealing 55°C for 1.5 minutes , Primer extension 72°C for 2.5 minutes & Final extension at 72°C for 7 minutes.

A second amplification reaction was performed with 1.0µl of the amplified product of the first amplification reaction. D2 was replaced with the dengue virus type-specific primers TS1, TS2, TS3 and TS4. After initial denaturation at 94°C for 1 minute, primer annealing at 58°C for 1 minute and primer extension at 72°C for 2 minutes, followed by final extension at 72°C for 7 minutes .Fifteen micro liters of the amplicon were subjected to ethidium bromide stained 2.0% agarose gel (Sigma, Missouri, USA) electrophoresis and the size of resulting DNA band was characterized for each dengue virus type , 482 bp (Den-1), 119 bp (Den-2), 290bp (Den-3) and 389 bp (Den-4).

3.4.6 Detection and Typing of Dengue Viral RNA in Field Caught Mosquito Specimens by nested RT-PCR

Adult *Ae. aegypti* mosquitoes were captured while landing on human baits or resting inside houses between 15days intervals from September-November, 2010; September–November, 2011 and July-December, 2012 from five dengue prevalent locations in Dhaka city. During the total 12 months study period of three calendar years, total 1251 female *Ae .aegypti* in 188 pools (ranging 1-13 mosquitoes per pool) had been collected.The mosquito pools were marked with date and place and processed separately in eppendroff tube. In each tube mosquitoes were

triturated in 0.6 ml cold MEM with 2% FBS and antibiotics, centrifuged at 14,000 rpm for 3 minutes at 4°C. 250 µl of samples were transferred for RNA extraction and remaining stored at -70°c for future study. RNA was extracted from 188 pools of field caught mosquitoes. The RNA extraction, amplification and virus typing by the nested PCR procedures that we followed for analyzing viral RNA in lab inoculated mosquito and clinical specimens were adopted for field caught mosquito specimens.

3.4.7 Agarose Gel Electrophoresis:

Materials required for agarose gel electrophoresis are described in Annexure -11.

Agarose gels are cast by melting the agarose in the presence of the desired buffer until a clear, transparent solution is achieved. The melted solution is then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose. When an electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates toward the anode.

3.4.7.1 Preparation of Gel:

Sufficient electrophoresis buffer were prepared to fill the electrophoresis tank and to prepare the gel, than added the correct amount of powdered agarose to a measured quantity of electrophoresis buffer in an Erlenmeyer flask, heated the slurry in a microwave oven until the agarose dissolves. Cooled the solution to 60°C and ethidium bromide to a final concentration of 0.5µg/ml added and mix thoroughly.Horizontal slab gels were poured on a glass plate or plastic tray that can be installed on a platform in the electrophoresis tank. 0.5-1 mm comb was positioned above the plate so that a complete well is formed when the agarose was added, then the gel was allowed to set.

3.4.7.2 Electrophoresis Procedure:

Electrophoresis was carried out with the gel submerged just beneath the surface of the buffer. The samples of DNA were mixed with the desired gel-loading buffer and the mixture was loaded into the slots of the submerged gel using a disposable micro pipette. Marker DNAs of known size was loaded into slots on the left sides of the gel. The lid of gel tank was closed and attached

the electrical leads so that the DNA migrated towards the anode (red lead). Applied a voltage of 3V/cm (measured as the distance between the electrodes) . To obtain maximum resolution of DNA fragments agarose gels should was run no more than 5V/cm at room temperature.

Bubbles generated at the anode and the cathode (due to electrophoresis) and within a few minutes the bromophenol blue migrated from the wells into the body of gel, run the gel until the bromophenol blue and xylene cyanol FF had migrated the appropriate distance through the gel. The presence of ethidium bromide allowed the gel to be examined by ultraviolet illumination at any stage during electrophoresis. Turned off the electric current and removed the lid from the gel tank. The gel was examined by UV light and photographed. Ethidium bromide was present in the gel and electrophoresis buffer to make visualization easy.

CHAPTER 4 *RESULTS*

Pages 71 - 121

4.1 Bionomics Study of Field Caught *Ae.aegypti* in Dhaka city

4.1.1 Breeding Habitat of *Ae. aegypti*

In Bionomics study container breeding habitat of *Ae. aegypti* has been observed from July, 2009 - June, 2010 in five dengue prevalent areas of Dhaka city as mentioned in methods and materials chapter. Eggs, larvae and pupae of *Ae. aegypti* were found in 85.15% of artificial containers and only 14.85% of natural containers. The observation about types of containers found positive for *Ae. aegypti* larvae inside or outside the households are summarized in Table-4.1 and in figure 4.1.

Empty flower pots, flower pot's saucers**,** flower-vases and glass jars with rooted plants which kept in city dwellers houses or student hostels for recreation or kept backyard carelessly caused water-logging and created the excellent places for *Ae. aegypti* to breed. Within 141 *Aedes* larvae positive containers 38.29% of larval presence (6.38%+14.89%+7.09%+9.93%) has been found in pot gardening objects.Cemented water reservoirs in under constructed residences were also good containers for *Aedes* breeding, in our observation 15.60% of larvae positive containers were the cemented reservoirs. Bucket, jars, tin, pot, plastic bowls & bottles, jerry cans and discarded appliance that carelessly scattered on the back yards and streets has been found as good habitat for *Aedes* larvae and almost 7.09% of larvae positive containers were scattered appliances. Man-made earthen containers like clay jars and pitchers were also preferable sites for *Aedes* to breed and 7.1% of those broken or thrown away clay jars or pitchers have been found to have larvae inside. Dumped tires containing rain water were good place for *Aedes* breeding and 7.09% of larvae positive containers were these tires that containing rain water. Dhaka city's water supply was inadequate in many places including these five survey areas. People usually stored water in large drums and these water storage drums were found ideal breeding ground of *Aedes* mosquitoes as we found 7.80% contained *Ae. aegypti* larvae. 2.18% larvae have been found in AC/Refrigerator drip pans during the observation period.

Cut bamboo stumps have been found as good man made natural containers.There were cut stumps used to make the finch of garden or field in many areas of Dhaka city, many of which contained water and 10.60% of positive containers were these bamboo stumps which contained larvae inside.Thrown away coconut shells and leaf axils or tree holes were also the natural water container and found to be the good places for *Aedes* breeding (4.25% of total positive containers).

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Table 4.1: Types of containers found positive (+ve) for *Ae.aegypti* larvae inside and outside the households from five sampling sites (Shegunbagicha-S, Rampura-R, Dhaka University Campus-DUC, Dhanmondi Residential Area-D and Mirpur-M) of Dhaka City

Types of container	S (+ve)	R (+ve)	D.U.C (+ve)	D (+ve)	M (+ve)	Total (+ve)	% of (+ve)
1. Clay jar, Pots and Pitchers	3	2	0	2	3	10	7.1
2. Unused Bucket, Gallon, Jars, Pots, Cans	0	1	1	1	3	6	4.25
3. Bamboo Stumps	1	1	7	6	0	15	10.6
4. AC/Refrigerator Drip Pan	1	1	0	1	1	4	2.18
5. Flower -vase	2	2	0	3	3	10	7.09
6. Coconut Shell	0	2	0	0	0	2	1.42
7. Drums	1	2	2	2	4	11	7.80
8. Discarded Appliances	1	1	0	0	2	4	2.84
9. Tyre	2	2	0	3	3	10	7.09
10. Tree Holes/Leafaxils	0	0	2	1	0	3	2.83
11. Glass Jar With Rooted Plants	2	3	4	2	3	14	9.93
12. Flower Pot Saucers	6	5	4	3	3	21	14.89
13. Cemented Water Reservoir Tank	5	4	3	5	5	22	15.60
14. Empty flower pots	1	2	2	1	3	9	6.38
Total	**25**	**28**	**25**	**30**	**33**	**141**	**100**

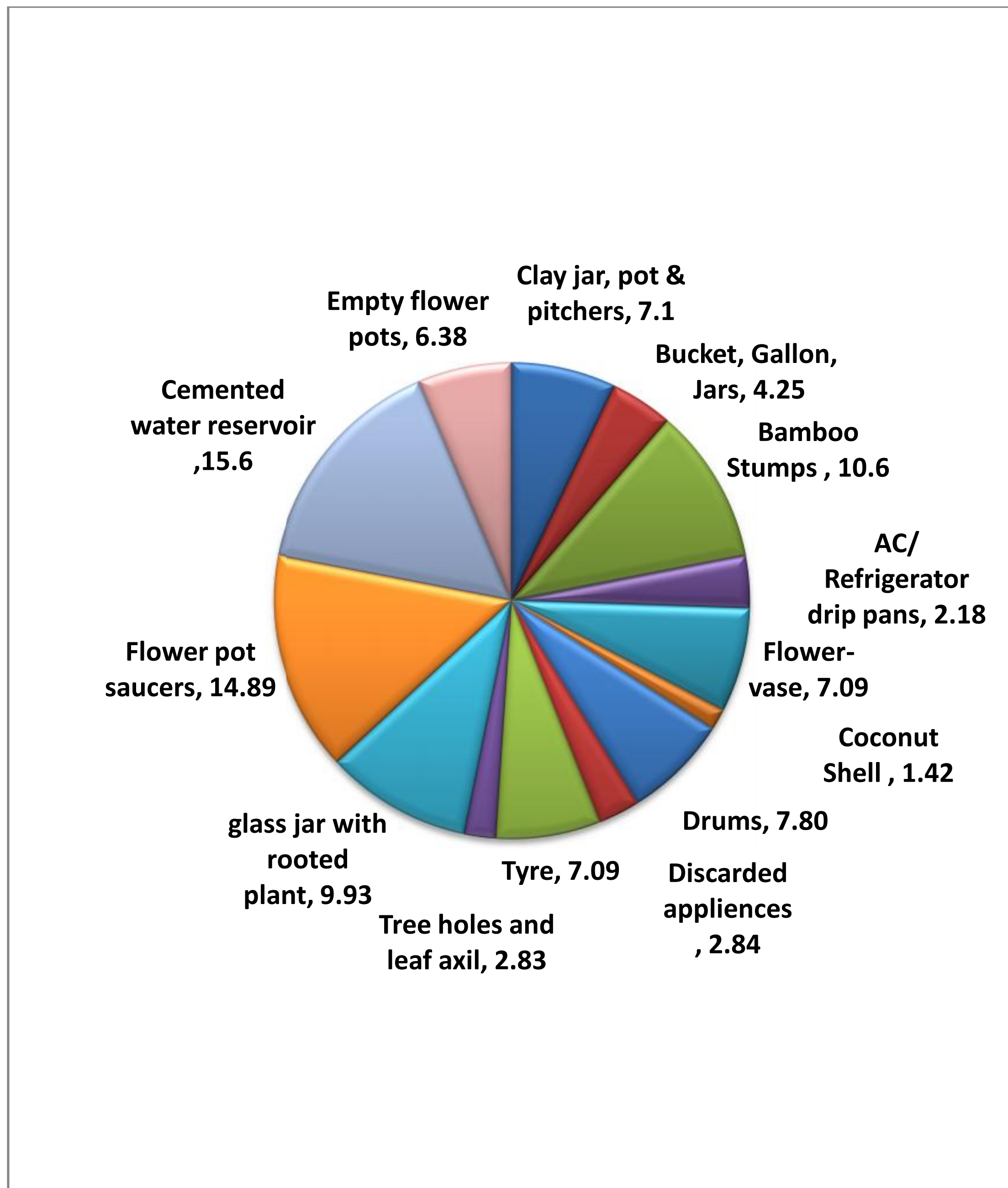

Figure 4.1: Pie diagram representing the types of containers and their percentages (%) found positive for presence of *Ae. aegypti* larvae from five sampling sites of Dhaka city

In figure 4.2.a and 4.2.b picture of some represented container breeding habitats of *Ae. aegypti* during field collection are shown.

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A. Flower tub saucer containing stagnant water **B. Glass jar containing rooted plant**

C. Bucket **E. Discarded backyard applience**

Fig: 4.2.a. Different container breeding habitats of *Ae. aegypti*

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F. Coconut shells **G. Tires**

H. Leaf axils **I. Bamboo stumps**

Fig 4.2.b: Different container breeding habitats of *Ae. aegypti*

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4.1.2 Seasonal Prevalence of Larval and Adult *Ae. aegypti* Population

For larval survey the entomological indices: House Index (HI); Container Index (CI) and Breteau Index (BI) were used for measuring the larval population. All five selected areas were visited at 15 days interval (twice a month) and collections were made 10 houses per day in each area. A total of 250 houses of each study area were visited during the study period (July, 2009-June, 2010) for larvae collection.

4.1.2.1 *Ae. aegypti* population on the basis of container positivity (CI) for the presence of larvae

A total of 141 (Rampura =28, Shegunbagicha=25, D.U Campus=25, Dhanmondi R/A= 30, Mirpur=33) containers found positive for *Ae.aegypti* mosquito larvae out of total 3773 containers checked (Rampura=793, Shegunbagicha=746, D.U Campus=728, Dhanmondi R/A=632, Mirpur=874). There were 3.53%, 3.35%, 3.45%, 4.75% & 3.78% of the larvae positive containers found in Rampura, Shegunbagicha, D.U Campus, Dhanmondi R/A and Mirpur area, respectively. On an average 3.74% of containers were found as *Ae aegypti* larvae positive. Highest number of positive containers (4.75%) were found in Dhanmondi R/A as shown in Table 4.2.

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Table 4.2: Percentages of mosquito population on the basis of positive containers for presence of *Ae .aegypti* larvae in five sampling sites of Dhaka city

Areas	TCC	TCP	CI
Rampura	793	28	3.53%
Shegunbagicha	746	25	3.35%
D.U Campus	728	25	3.45%
Dhanmondi R/A	632	30	4.75%
Mirpur	874	33	3.78%
Total	3773	141	3.74%

TCC=Total Container Checked, TCP=Total Container Positive

$$CI = \frac{\text{Number of container positive}}{\text{Number of container inspected}} \times 100$$

4.1.2.2 Seasonal Prevalence of *Ae. aegypti* larvae on the basis of larval Breteau Index (BI)

During the study period, total 1250 houses were visited and *Ae. aegypti* mosquito larvae were found in 141 containers. Breteau Index was calculated as measuring index of larval population.

$$\text{Breteau Index} = \frac{\text{No. of containers positive}}{\text{No. of house inspected}} \times 100$$

$= (141 \div 1250) \times 100 = 11.28$

Combined data on the seasonal prevalence of *Ae. aegypti* larvae in all five selected dengue prevalent areas are shown in Table 4.3 and Figure 4.3. In our study overall BI result was 11.28. But in yearly observation it varied season to season and also area to area. Highest number of positive containers was found in the month of September.

Mosquito population usually rises in the month of June-July after the rainfall. But monsoon was quite late in year 2009. Usually rainy season starts from mid June in Bangladesh but in year 2009 after hot long & dry summer the monsoon came at the mid of July. There was heavy rain fall in the month of August, 2009. In our study we observed that post monsoon was the most suitable period for larval growth. The Breteau Index more than 20 was considered as the risk level for transmission of Dengue. Our larva survey showed the Breteau Index of 20-40 during August to October, 2009 in all five selected areas of Dhaka city. That was well above the risk level for dengue virus transmission by the vector mosquitoes. From mid November 2009 –February 2010 the BI was around 10, from March to June, 2010 BI dropped to 0 (zero) in almost all the five spots and remained zero for 4 months (March-June, 2010). Cold wave passed all over the country from mid December, 2009 to mid January, 2010 and no rainfall was seen until the end of June, 2010 in Dhaka city. Country experienced scattered rainfall at the end of June, 2010. BI value 10 was found again in all the five spots in the first week of July, 2010. In our observation winter and draught interrupted the mosquito's larval growth.

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Table 4.3: Seasonal prevalence of *Ae.aegypti* larvae from five sampling sites of Dhaka city (Shegunbagicha-S, Rampura-R, Dhaka University Campus-DUC, Dhanmondi Residential Area-D and Mirpur-M) on the basis of larval Breteau Index (BI)

Date	R	S	DUC	D	M
2.7.09	10	10	0	10	20
17.7.09	10	10	10	10	20
1.8.09	20	20	20	20	20
16.8.09	20	20	20	20	20
1.9.09	30	30	30	30	30
16.9.09	40	40	40	40	40
2.10.09	40	40	30	40	40
17.10.09	20	20	20	20	40
1.11.09	10	20	10	20	20
16.11.09	10	10	10	10	20
2.12.09	10	10	10	10	10
17.12.09	0	10	10	10	10
1.1.10	10	0	10	10	10
16.1.10	10	0	0	10	0
1.2.10	10	0	0	10	10
15.2.10	10	0	10	10	10
2.3.10	10	0	10	0	0
17.3.10	0	0	0	0	0
1.4.10	0	0	0	0	0
16.4.10	0	0	0	0	0
2.5.10	0	0	0	0	0
17.5.10	0	0	0	0	0
1.6.10	0	0	0	0	10
16.6.10	0	0	0	0	10
1.7.10	10	10	1 0	10	10

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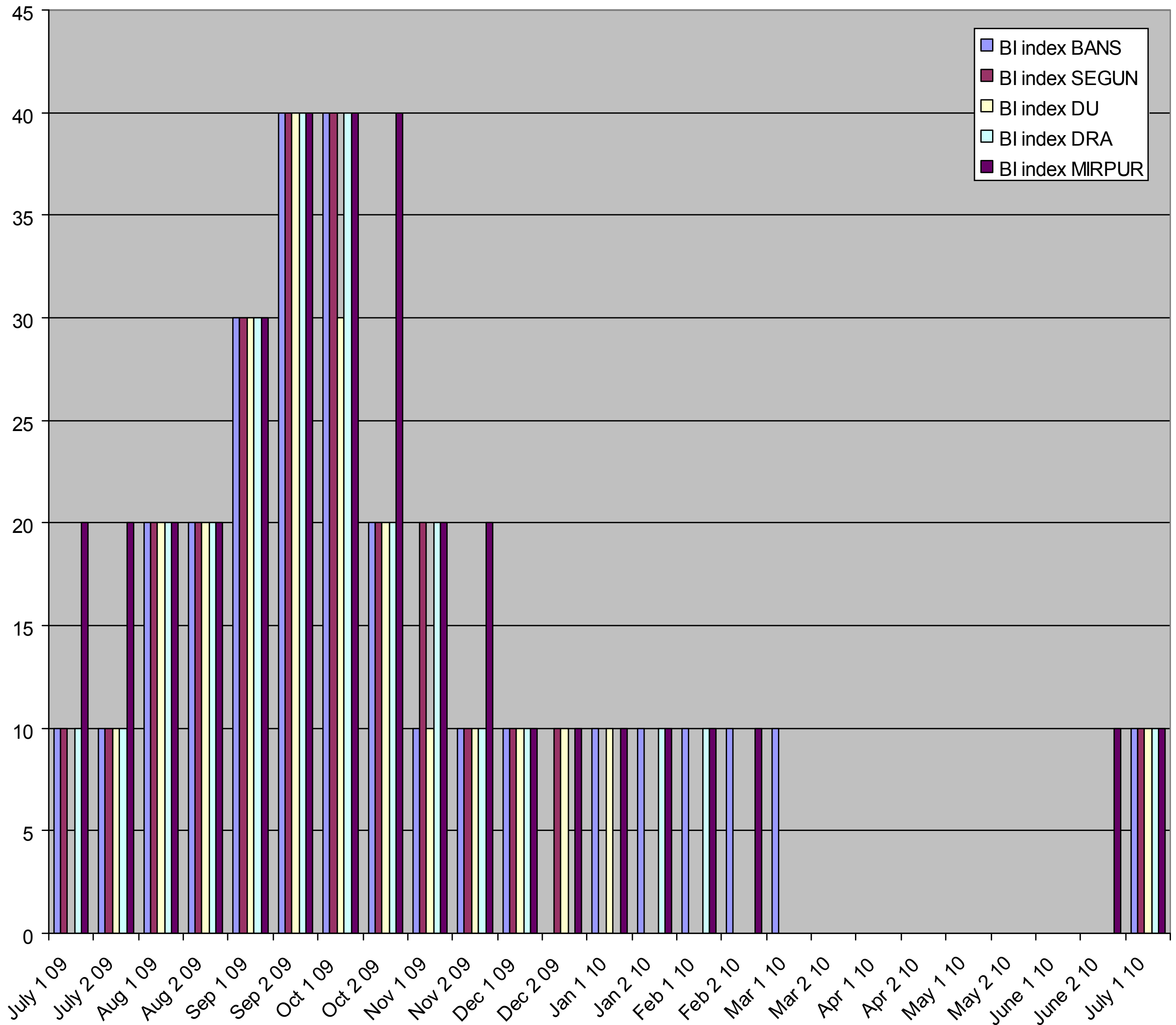

Sample collection Periods (15 days Interval)

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Fig 4.3: Seasonal prevalence of *Ae. aegypti* larvae from five sampling sites of Dhaka city

4.1.2.1 Seasonal prevalence of *Ae.aegypti* larvae in individual study area

Seasonal prevalence of *Ae. Aegypti* larvae in individual sampling area (Rampura, Shegunbagicha, D.U Campus, Dhanmondi R/A and Mirpur) were calculated on the basis of Container Index and Breteau Index. Resuls are shown in Tables (4.4, 4.5, 4.6, 4.7 and 4.8) and Figures (4.4, 4.5, 4.6, 4.7 and 4.8).

Large numbers of larvae population were observed in Rampura from August – Mid October and the highest population found around mid September –first week of October. From mid March to end of the June almost no larval population had been seen in this area. Small number of population had seen around the rest of the months (July-February). Collection data of seasonal prevalence of *Ae. aegypti* larvae in Shegunbagicha have been summarized in table 4.5 and fig 4.5 where the highest population also seen in September to first week of October. From December - mid June almost no larval population had seen . Rest of the months showed moderate larval population.Table 4.6 shows the summarized data from Dhaka university campus where peak larval population has seen in whole month of September. Larval population very small (almost zero) from January-June which gave us impression that winter, draught and hot summer interrupted the larval growth.

In Dhanmondi (Table and fig4.7) the larval population had seen from Mid June-Mid February and the peak population had seen in month of September. From March-May very small population (0) had been recorded.Though the inhabitants of this area were in high income groups, they made favoubable conditions for *Aedes* growth. Many individual houses in this area had gardens in front of them which were surrounded by finches made by bamboo stumps.As it was in the centre of capital it is demanding place for living ,many multistoried buildings were found under construction, temporay water reservoir tanks and drums in these underconstructed buildings caused water logging and favourable condition for *Aedes* growth. In Mirpur (Table and fig 4.8) the highest larvae population found mid September-mid October. Larvae found mid June-January. This was also the

fast growing and over populated portion of Dhaka city like Dhanmondi. City dwellers life style made these places favourable habitat for *Ae.aegypti*.

Table 4.4: Seasonal prevalence *Ae.aegypti* larvae in Rampura, Dhaka on basis of Container Index (CI) & Breteau Index (BI)

Date	TCC (793)	TCP (28)	CI	BI
2.7.09	35	1	2.85	10
17.7.09	32	1	3.125	10
1.8.09	33	2	6.06	20
16.8.09	32	2	6.25	20
1.9.09	34	3	8.82	30
16.9.09	35	4	11.43	40
2.10.09	33	4	12.12	40
17.10.09	30	2	6.66	20
1.11.09	29	1	3.45	10
16.11.09	26	1	3.85	10
2.12.09	28	1	3.57	10
17.12.09	27	0	0	0
1.1.10	28	1	3.57	10
16.1.10	26	1	3.85	10
1.2.10	29	1	3.45	10
15.2.10	32	1	3.025	10
2.3.10	33	1	3.03	10
17.3.10	35	0	0	0
1.4.10	37	0	0	0
16.4.10	29	0	0	0

2.5.10	30	0	0	0
17.5.10	32	0	0	0
1.6.10	35	0	0	0
16.6.10	38	0	0	0
1.7.10	35	1	2.88	10

Table 4.5: Seasonal prevalence of *Ae. aegypti* larvae in <u>Shegunbagicha, Dhaka</u> on basis of container Index (CI) & Breteau Index (BI)

Date	TCC (746)	TCP(25)	CI	BI
1.7.09	30	1	2.85	10
16.7.09	31	1	3.125	10
31.7.09	30	2	6.06	20
15.8.09	32	2	6.25	20
31.8.09	30	3	8.82	30
15.9.09	29	4	11.43	40
1.10.09	31	4	12.12	40
16.10.09	32	2	6.66	20
31.10.09	31	2	3.45	20
15.11.09	31	1	3.85	10
1.12.09	29	1	3.57	10
16.12.09	29	1	3.75	10
31.12.09	28	0	0	0
15.1.10	29	0	0	0
31.1.10	28	0	0	0
14.2.10	30	0	0	0
1.3.10	28	0	0	0
15.3.10	29	0	0	0
1.4.10	30	0	0	0

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16.4.10	31	0	0	0
1.5.10	30	0	0	0
16.5.10	31	0	0	0
31.5.10	29	0	0	0
15.6.10	28	0	0	0
30.6.10	30	1	2.88	10

Table 4.6: Seasonal prevalence of *Ae. aegypti* larvae in <u>Dhaka University Campus</u> on the basis of Container Index (CI) & Breteau Index (BI)

Date	TCC (728)	TCP(25)	CI	BI
1.7.09	30	0	0	0
16.7.09	30	1	3.33	10
31.7.09	32	2	6.25	20
15.8.09	30	2	6.66	20
31.8.09	28	3	10.71	30
15.9.09	25	4	16	40
1.10.09	27	3	11.11	30
16.10.09	26	2	7.64	20
31.10.09	26	1	3.846	10
15.11.09	28	1	3.57	10
1.12.09	29	1	3.45	10
16.12.09	28	1	3.57	10
31.12.09	28	1	3.57	10
15.1.10	26	0	0	0
31.1.10	29	0	0	0
14.2.10	32	1	0	10
1.3.10	25	1	0	10
15.3.10	32	0	0	0
1.4.10	29	0	0	0

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16.4.10	31	0	0	0
1.5.10	33	0	0	0
16.5.10	31	0	0	0
31.5.10	30	0	0	0
15.6.10	33	0	0	0
30.6.10	30	1	3.33	10

Table 4.7: Seasonal prevalence of *Ae.aegypti* larvae in Dhanmondi R/A, Dhaka on basis of Container Index (CI) & Breteau Index (BI)

Date	TCC(632)	TCP(30)	CI	BI
3.7.09	33	1	3.03	10
18.7.09	35	1	2.86	10
2.8.09	33	2	6.06	20
17.8.09	30	2	6.67	20
1.9.09	34	3	11.33	30
17.9.09	35	4	8.75	40
2.10.09	32	4	8	40
17.10.09	30	2	6.66	20
1.11.09	29	2	6.9	20
17.11.09	26	1	3.85	10
2.12.09	28	1	3.57	10
17.12.09	15	1	6.66	10
1.1.10	15	1	6.66	10
17.1.10	26	1	3.85	10
2.2.10	29	1	3.45	10
17.2.10	15	1	6.66	10
3.3.10	25	0	0	0
18.3.10	17	0	0	0

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2.4.10	16	0	0	0
17.4.10	18	0	0	0
2.5.10	20	0	0	0
17.5.10	18	0	0	0
2.6.10	20	0	0	0
17.6.10	18	1	5.55	1
3.7.10	35	1	2.86	10

Table 4.8: Seasonal prevalence of *Ae. aegypti* larvae in Mirpur, Dhaka on basis of Container Index (CI) &Breteau Index (BI)

Date	TCC(874)	TCP(33)	CI	BI
3.7.09	39	2	5.13	20
18.7.09	36	2	5.56	20
2.8.09	38	2	5.26	20
17.8.09	38	2	5.26	20
1.9.09	37	3	8.11	30
17.9.09	36	4	9	40
2.10.09	37	4	10.82	40
17.10.09	35	4	11.43	40
1.11.09	37	2	5.41	20
17.11.09	32	2	6.25	20
2.12.09	34	1	2.94	10
17.12.09	33	1	3.03	10
1.1.10	33	1	3.03	10
17.1.10	35	1	2.86	10
2.2.10	38	1	2.63	10
17.2.10	32	0	0	0
3.3.10	33	0	0	0

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18.3.10	35	0	0	0
2.4.10	37	0	0	0
17.4.10	29	0	0	0
2.5.10	30	0	0	0
17.5.10	32	0	0	0
2.6.10	35	0	0	0
17.6.10	38	1	2.63	10
3.7.10	35	1	2.86	10

Figure 4.4: Graphical representation of seasonal prevalence of *Ae. aegypti* larvae in Rampura , Dhaka

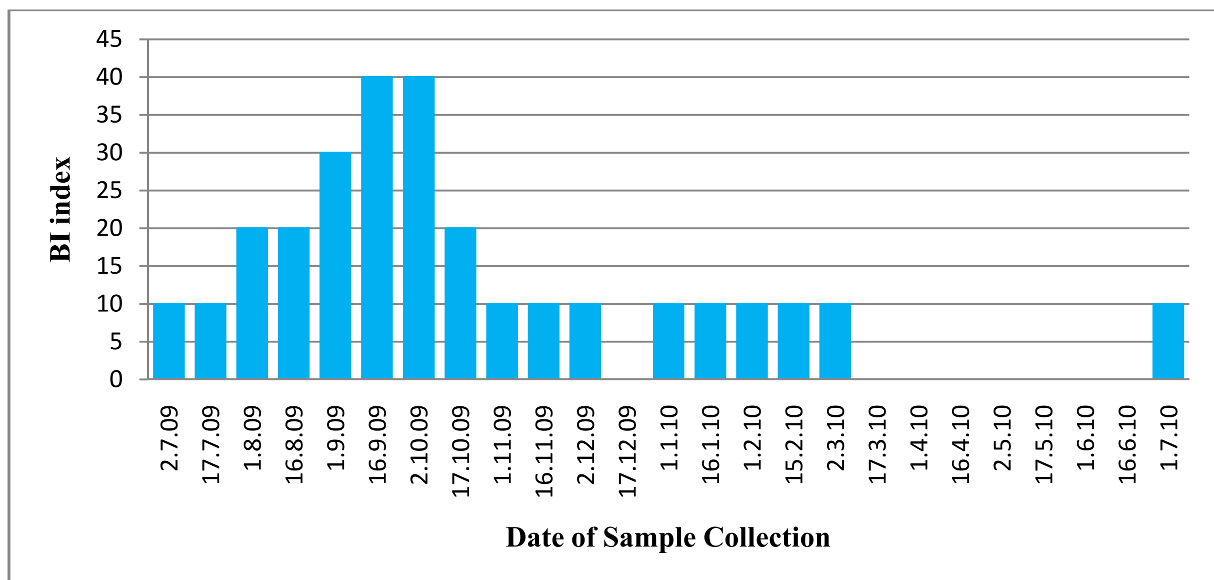

Figure 4.5: Graphical representation of seasonal prevalence of *Ae. aegypti* larvae in Shegunbagicha , Dhaka

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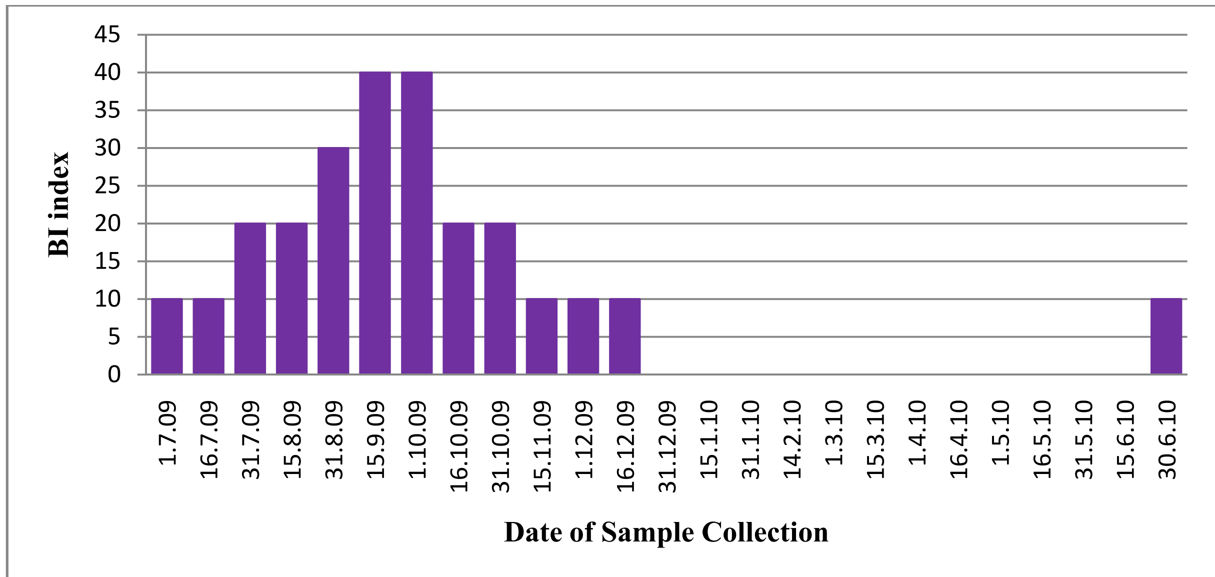


Figure 4.6: Graphical representation of *Ae. aegypti* larvae in <u>Dhaka University Campus</u>

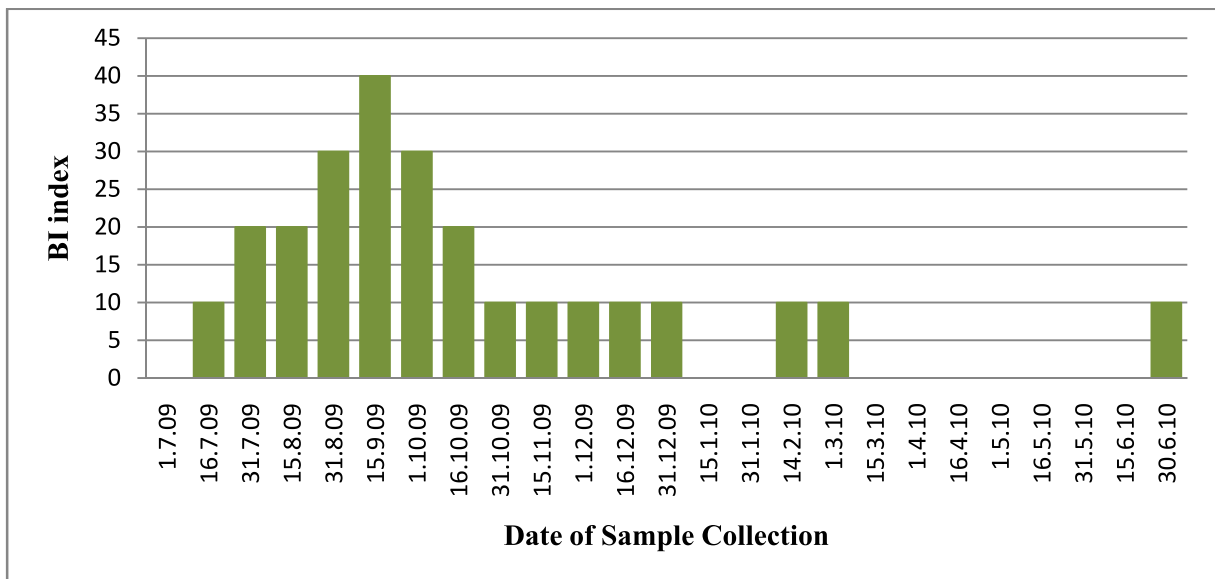


Figure 4.7: Graphical representation of *Ae.aegypti* larvae in <u>Dhanmondi R/A, Dhaka</u>

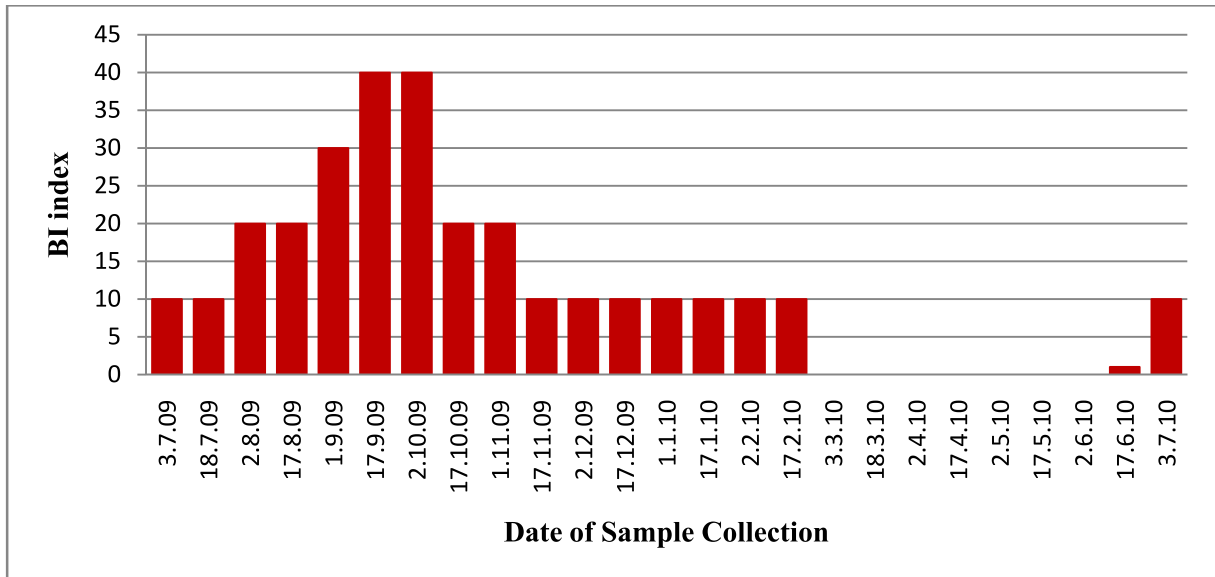
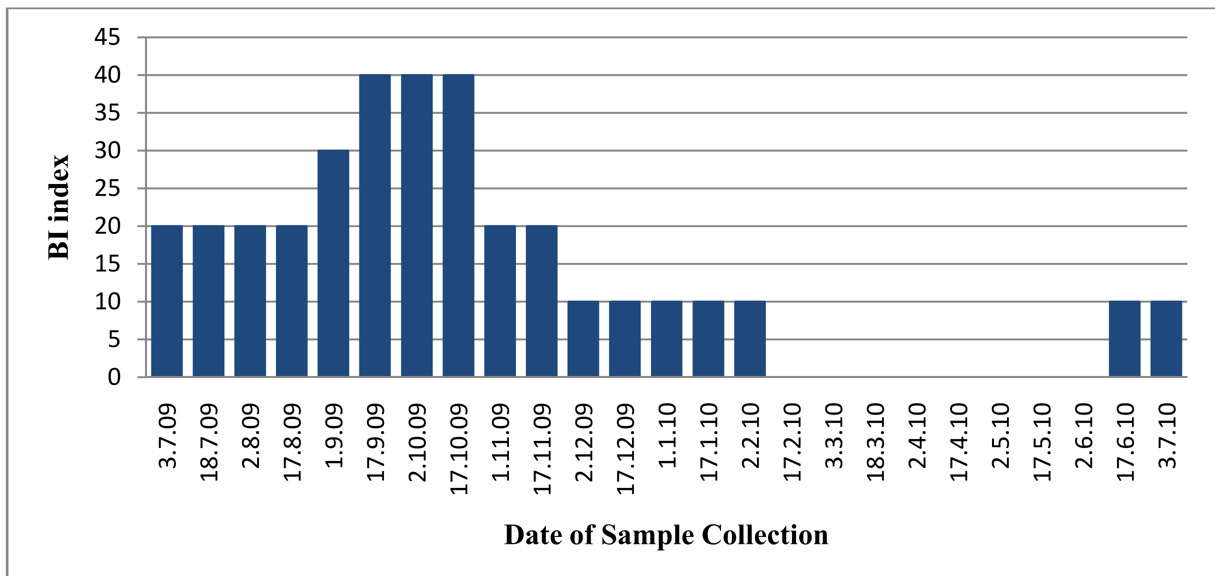


Figure 4.8: Graphical representation of *Ae. aegypti* larvae in <u>Mirpur, Dhaka</u>



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4.1.2.4 Seasonal Prevalence of Adult *Ae. aegypti*

The survey result of adult mosquito population is calculated and expressed as adult biting rate.Combined data on seasonal prevalence of adult *Ae. aegypti* in all five study areas are shown in Table-4.9. The combined data is also being shown Fig 4.9 as line diagram. The Indoor resting adult population start rising from August and the peak mosquito population were found during September-November, which was consistent with the larvae survey. Adult population dropped from the starting of December, and few mosquitoes found until the next monsoon. Last week of December, 2009 to second week of January 2010 the mean temperature of Dhaka city was around 10-12°C. Interruption of transmission occurred during winter but transmission may have occurred throughout the year in some areas, peaking in the monsoon season.

Table 4.9: Seasonal prevalence of adult *Ae . aegypti* in five sampling areas of Dhaka city from July ,09 –June, 10 on the basis of Mosquito Biting rate (Man/Hour). (Shegunbagicha-S, Rampura-R, Dhaka University Campus-DUC, Dhanmondi Residential Area-D and Mirpur-M)

Date	R	S	DUC	D	M
2.7.09	2.5	0	0	1.5	1.5
17.7.09	6	2	2	3.5	2.5
1.8.09	8	3	4	4	4
16.8.09	8	3.5	5.5	6.5	6
1.9.09	9.5	4	7.5	13	11
16.9.09	10. 5	6.5	11	11.5	15.1

2.10.09	16	11	16.5	10.5	14.5
17.10.09	13.5	8.5	14.5	11	10.5
1.11.09	9	6	8.5	6	9.5
16.11.09	10	6	6	5.5	8
2.12.09	5	2.5	3.5	2	3.5
17.12.09	3.5	2.5	2.5	1.5	3.5
1.1.10	2.5	2	1.5	1	1.5
16.1.10	2	0.5	1	0.5	1.5
1.2.10	1.5	0	0.5	0	1
15.2.10	2	0.5	0.5	0	3
2.3.10	1.5	0	2	1	2.5
17.3.10	1.5	0	2	1.5	2
1.4.10	1	0	1.5	1.5	1.5
16.4.10	0	0	1	1	1
2.5.10	0	0	0.5	0.5	0
17.5.10	0	0	0	0	0
1.6.10	0	0	0	0	0
16.6.10	0	0	0	0	0
1.7.10	0	0	0	0	0
Total	**112.5**	**58.5**	**92.0**	**84.5**	**104**

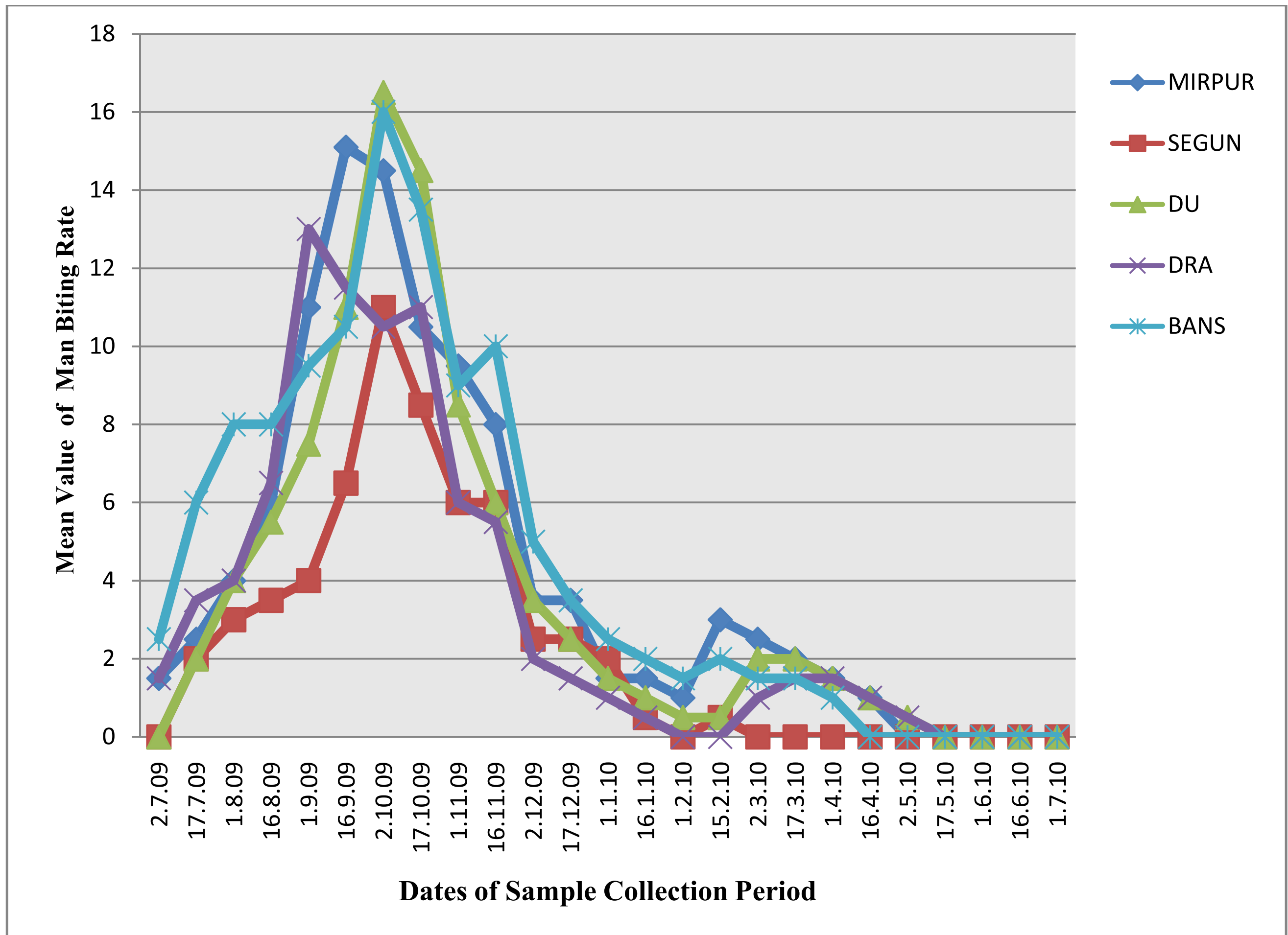

Figure 4.9: Graphical representation of adult *Ae . aegypti* in five sampling areas of Dhaka city from July, 09 - June, 10 on the basis of Mosquito Biting Rate (Man/Hour). **(Shegunbagicha-SHEGUN, Rampura, Banasri-BANS, Dhaka University Campus-DU, Dhanmondi Residential Area- DRA and Mirpur-M)**

4.1.2.5 Seasonal prevalence of *Ae.aegypti* Adults in individual study area

Seasonal prevalence of adult *Ae. Aegypti* in individual study area (Rampura, Shegunbagicha, D.U Campus, Dhanmondi R/A and Mirpur) were calculated and expressed as adult biting rate. Results are shown in Tables (4.10, 4.11, 4.12, 4.13 and 4.14). Data on individual area are also shown in line diagram in Figures (4.10, 4.11, 4.12, 4.13 and 4.14).

Table 4.10: Seasonal prevalence of adult *Ae. aegypti* in Rampura, Dhaka on basis of mosquito biting rate (Man/Hour)

Date	After sunrise	Before sunset	Mean
2.7.09	2	3	2.5
17.7.09	5	7	6
1.8.09	7	9	8
16.8.09	7	9	8
1.9.09	9	10	9.5
16.9.09	10	11	10. 5
2.10.09	15	17	16
17.10.09	12	15	13.5
1.11.09	8	10	9
16.11.09	10	10	10
2.12.09	5	5	5
17.12.09	3	4	3.5
1.1.10	2	3	2.5
16.1.10	2	2	2
1.2.10	2	1	1.5
15.2.10	2	2	2
2.3.10	1	2	1.5
17.3.10	1	2	1.5
1.4.10	1	1	1
16.4.10	0	0	0
2.5.10	0	0	0
17.5.10	0	0	0
1.6.10	0	0	0
16.6.10	0	0	0
1.7.10	0	0	0

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Total	104	123	112.5

Table 4.11: Seasonal prevalence of adult *Ae. aegypti* in Shegunbagicha, Dhaka on basis of mosquito biting rate (Man/Hour)

Date	After sunrise	Before sunset	Mean
1.7.09	0	0	0
16.7.09	2	2	2
31.7.09	2	4	3
15.8.09	2	5	3.5
31.8.09	3	5	4
15.9.09	5	8	6.5
1.10.09	10	12	11
16.10.09	8	9	8.5
31.10.09	5	7	6
15.11.09	5	7	6
1.12.09	3	2	2.5
16.12.09	2	3	2.5
31.12.09	2	2	2
15.1.10	0	1	0.5
31.1.10	0	0	0
14.2.10	1	0	0.5
1.3.10	0	0	0
15.3.10	0	0	0
1.4.10	0	0	0
16.4.10	0	0	0
1.5.10	0	0	0
16.5.10	0	0	0
31.5.10	0	0	0
15.6.10	0	0	0

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30.6.10	0	0	0
Total	50	67	58.5

Table 4.12: Seasonal prevalence of adult *Ae. aegypti* in Dhaka Univ.Campus on the basis of mosquito biting rate (Man/Hour)

Date	After sunrise	Before sunset	Mean
1.7.09	0	0	0
16.7.09	2	2	2
31.7.09	4	4	4
15.8.09	6	5	5.5
31.8.09	8	7	7.5
15.9.09	10	12	11
1.10.09	15	18	16.5
16.10.09	13	16	14.5
31.10.09	8	9	8.5
15.11.09	5	7	6
1.12.09	3	4	3.5
16.12.09	2	3	2.5
31.12.09	1	2	1.5
15.1.10	1	1	1
31.1.10	0	1	0.5
14.2.10	1	0	0.5
1.3.10	0	2	2
15.3.10	2	2	2
1.4.10	1	2	1.5
16.4.10	1	1	1
1.5.10	1	0	0.5
16.5.10	0	0	0
31.5.10	0	0	0

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15.6.10	0	0	0
30.6.10	0	0	0
Total	84	98	92.0

Table 4.13: Seasonal prevalence of adult *Ae. aegypti* in Dhanmondi , Dhaka on basis of mosquito biting rate (Man/Hour)

Date	after sunrise	before sunset	Mean
3.7.09	2	3	1.5
18.7.09	3	4	3.5
2.8.09	4	4	4
17.8.09	7	6	6.5
1.9.09	12	14	13
17.9.09	10	13	11.5
2.10.09	10	11	10.5
17.10.09	9	13	11
1.11.09	5	7	6
17.11.09	5	6	5.5
2.12.09	2	2	2
17.12.09	2	1	1.5
1.1.10	1	1	1
17.1.10	0	1	0.5
2.2.10	0	0	0
17.2.10	0	0	0
3.3.10	0	2	1
18.3.10	2	1	1.5
2.4.10	2	1	1.5
17.4.10	1	1	1
2.5.10	1	0	0.5
17.5.10	0	0	0

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2.6.10	0	0	0
17.6.10	0	0	0
3.7.10	0	0	0
Total	78	91	84.5

Table 4.14: Seasonal prevalence of adult *Ae. aegypti* in Mirpur, Dhaka on basis of mosquito biting rate (Man/Hour)

Date	After sunrise	Before sunset	Mean
3.7.09	2	1	1.5
18.7.09	3	2	2.5
2.8.09	2	4	4
17.8.09	3	7	6
1.9.09	10	12	11
17.9.09	14	17	15.1
2.10.09	13	16	14.5
17.10.09	10	11	10.5
1.11.09	9	10	9.5
17.11.09	7	9	8
2.12.09	3	4	3.5
17.12.09	3	4	3.5
1.1.10	1	2	1.5
17.1.10	1	2	1.5
2.2.10	1	1	1
17.2.10	2	4	3
3.3.10	3	2	2.5
18.3.10	2	2	2
2.4.10	2	1	1.5
17.4.10	1	1	1
2.5.10	0	0	0

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17.5.10	0	0	0
2.6.10	0	0	0
17.6.10	0	0	0
3.7.10	0	0	0
Total	92	112	104

Figure 4.10: Graphical representation of Seasonal prevalence of *Ae.aeqypti* adults in Rampura, Dhaka on basis of biting rate

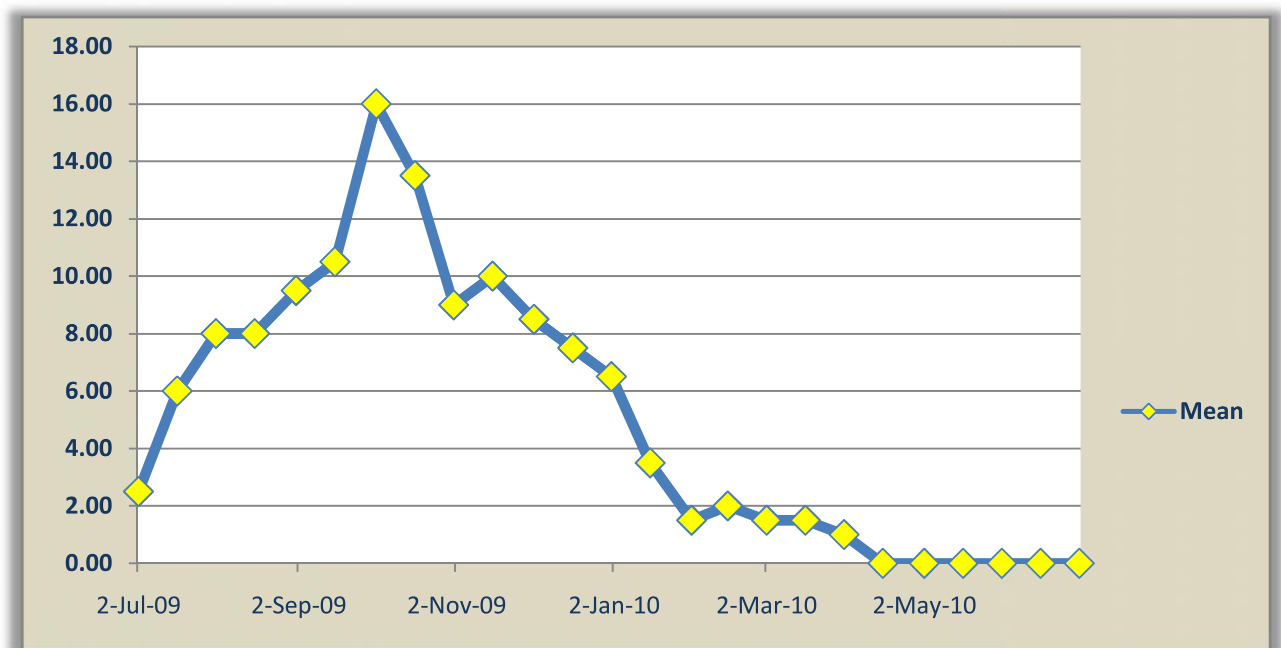

Figure 4.11: Graphical representation of Seasonal prevalence of *Ae. aegypti* adults in Shegunbagicha, Dhaka on basis of biting rate

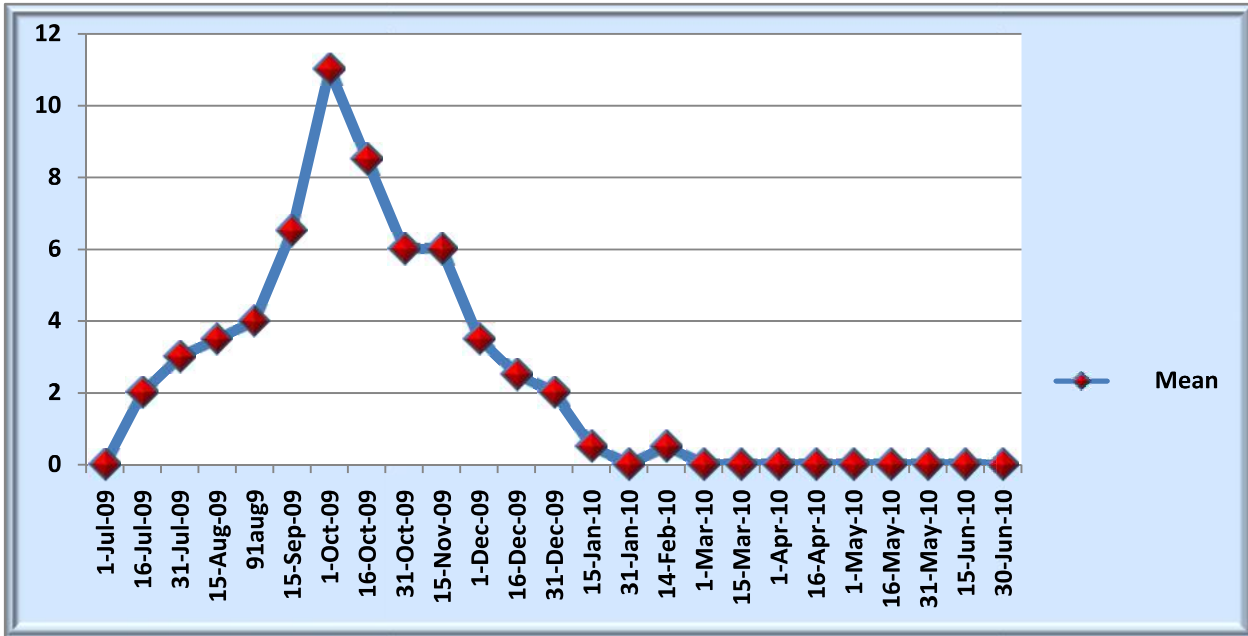

Figure 4.12: Graphical representation of Seasonal prevalence of *Ae. aegypti* adults in <u>Dhaka Univ. Campus , Dhaka</u> on basis of biting rate

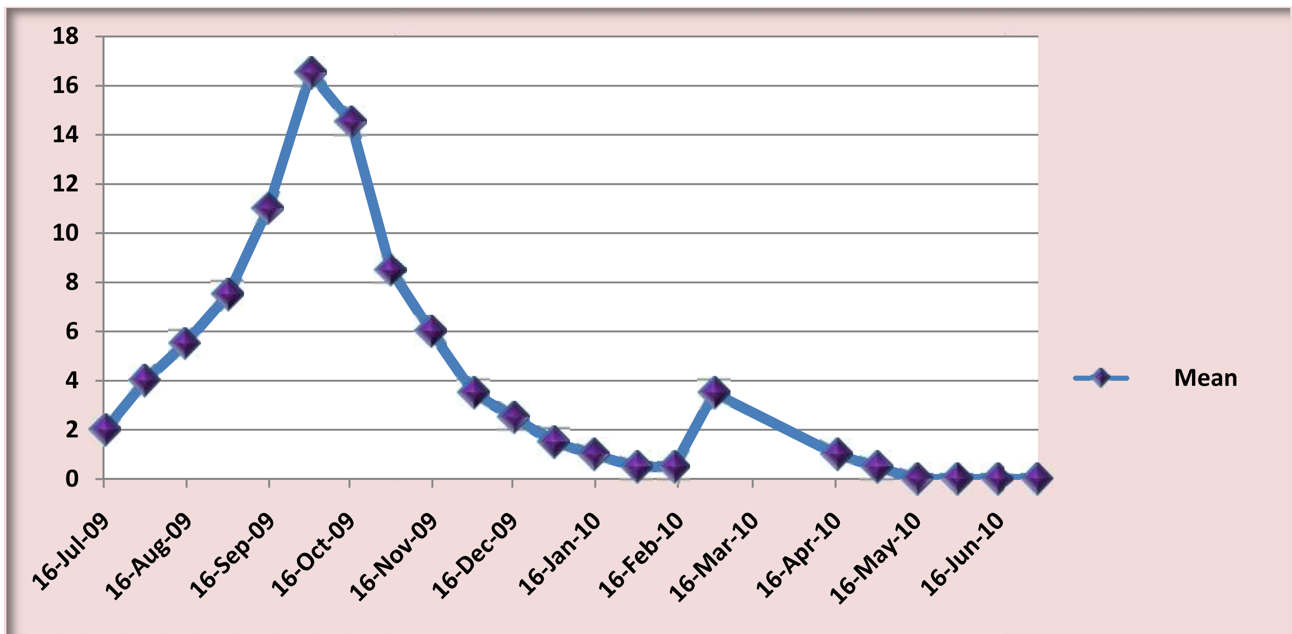

Figure 4.13: Graphical representation of Seasonal prevalence of *Ae. aegypti* adults in <u>Dhanmondi, Dhaka</u> on basis of biting rate

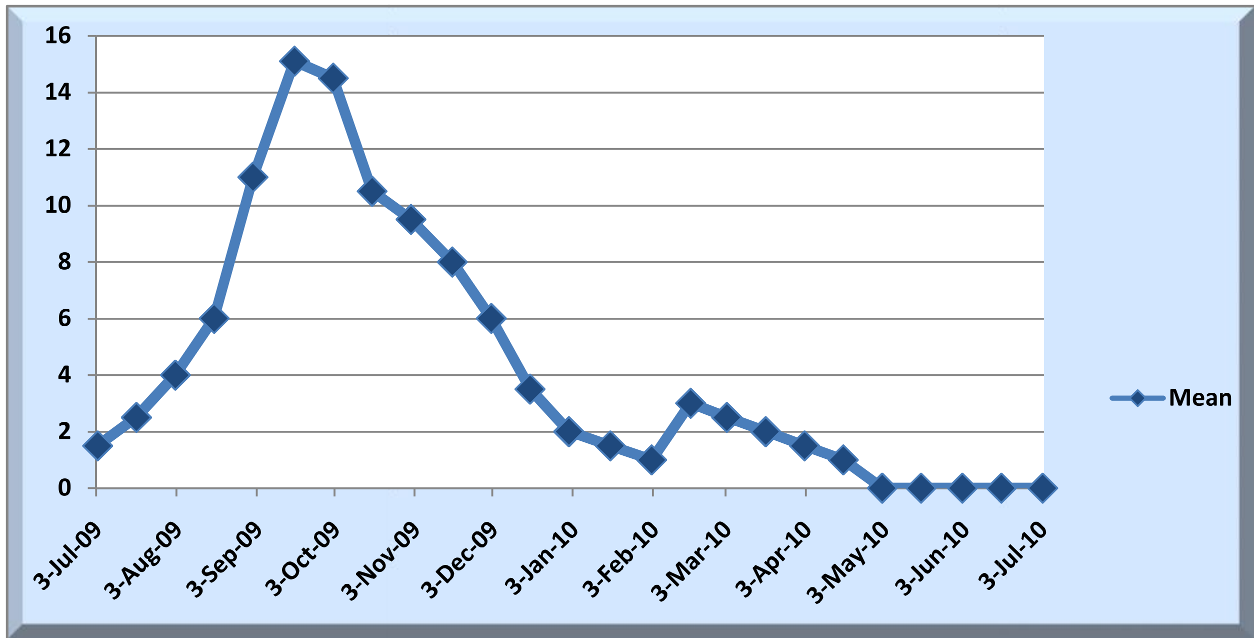

Figure 4.14: Graphical representation of Seasonal prevalence of *Ae . aegypti* adults in <u>Mirpur, Dhaka</u> on basis of biting rate

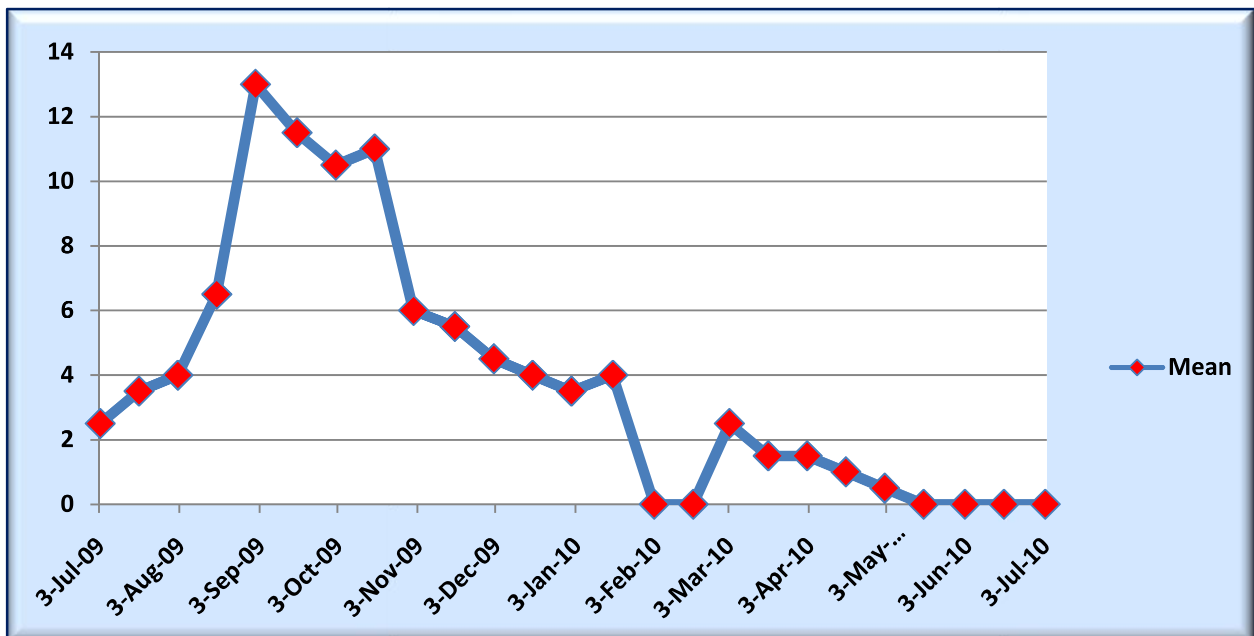

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Table and fig 4.10 shows the collection data of man biting *Ae. aegypti* mosquitoes from Rampura where the peak biting season found from September-mid of November. In Shegunbagicha the peak biting season found from mid September to mid November (Table and fig 4.11).Table and figure 4.12 summerize the adult *Ae. aegypti*'s seasonal data from Dhaka University campus, where the month September and October showed the peak man biting rates. In Dhanmondi Residential area the adult *Aedes* biting rate found from July-May, (Table and fig-4.13) where from mid August-mid November the peak biting activities had been seen. Table and fig 4.14 shows the collection data of man biting *Ae. aegypti* mosquitoes from Mirpur where the peak biting season found from mid August - mid of November.

4.2 Development of RT-PCR based molecular method for rapid detection and identification of Dengue virus in field caught vector specimens

4.2.1 Detection of dengue virus in laboratory reared mosquitoes by indirect dengue Ag-capture ELISA

During the peak hospitalized period (November-December, 2010) blood samples were collected from thirty dengue patients and serum were used for mosquito inoculation. Peak hospitalization period was found consistent with the adult mosquitoe peak biting period. Dengue cases were confirmed on the basis of patient's IgG and IgM status as described in diagnostic blood report shown in Table- 4.15. Among 30 patients, 10 were anti-dengue antibody IgM +ve and 22 were anti-dengue antibody IgG +ve. All represented dengue infection. Two patients were found positive for both IgG and IgM, indicating the presence of secondary dengue infection.

To prepare the dengue infected mosquitoes, patient serum had been intrathoracically inoculated into the lab reared mosquito. Mosquitoes were then dissected and 30 patient serum inoculated

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mosquitoe head pools were tested by ELISA for the presence of dengue virus. Out of 30 lab infected head pools of mosquitoes, only 10 were found positive for the presence of dengue virus tested by ELISA. Specimens were considered positive if their OD values were greater than the mean value plus three S.D. of negative control. An average of 33.33% (10 of 30) of inoculated *Ae. aegypti* mosquito tested were positive for dengue virus infection by ELISA. Fig 4.15 represents the results of dengue virus detection in lab inoculated mosquitoes by ELISA method.

Table 4.15: Anti-Dengue IgM & IgG Antibody in 30 Dengue patients' blood serum

Patient SL.No	Date of Collection	Bill No	Sex	Age	Dengue IgM	Dengue IgG
01.	20-11-2010	G16861	Male	77	-ve	+ve
02.	29-11-2010	G18896	Male	55	-ve	+ve
03.	27-11-2010	G18231	Male	53	+ve	-ve
04.	27-11-2010	G18264	Female	44	-ve	+ve
05.	27-11-2010	G18392	Male	26	+ve	-ve
06.	24-11-2010	G17639	Female	2	+ve	-ve
07.	28-11-2010	G18534	Female	11	-ve	+ve
08.	27-11-2010	T65673	Female	1y,9m	+ve	-ve
09.	29-11-2010	T66234	Male	24	-ve	+ve
10.	20-11-2010	T63670	Female	4y,3m	+ve	-ve
11.	29-11--2010	M34109	Female	11m	+ve	-ve
12.	29-11-2010	L27656	Male	47	-ve	+ve
13.	29-11-2010	L27525	Male	31	-ve	+ve
14.	27-11-2010	L26691	Female	33	+ve	-ve

15.	27-11-2010	M33518	Female	12	-ve	+ve
16.	25-11-2010	T64980	Male	29	+ve	+ve
17.	25-11-2010	Y37310	Female	21	-ve	+ve
18.	24-11-2010	L25865	Female	35	+ve	+ve
19.	21-11-2010	L24575	Female	50	-ve	+ve
20.	1-12-2010	L28172	Female	42	-ve	+ve
21.	8-12-2010	L30716	Female	65	-ve	+ve
22.	8-12-2010	G20883	Female	46	-ve	+ve
23.	8-12-2010	L30743	Male	36	-ve	+ve
24.	6-12-2010	L30054	Female	60	-ve	+ve
25.	4-12-2010	G19856	Male	32	+ve	-ve
26.	4-12-2010	G19916	Female	31	-ve	+ve
27.	5-12-2010	L29342	Male	2	-ve	+ve
28.	5-12-2011	T67857	Female	33	-ve	+ve
29.	01-1-2011	G25608	Male	30	-ve	+ve
30.	01-01-2011	G25448	Female	11	-ve	+ve

1 2 3 4 5 6 7 8 9 10 11 12

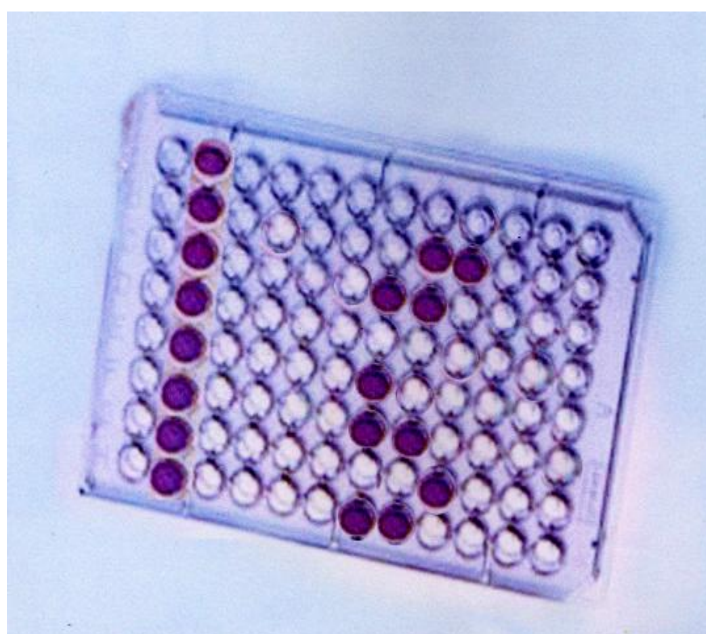

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Fig 4.15: Detection of dengue virus in laboratory reared mosquitoes by indirect ELISA.

In this photograph, wells in column-1 represents negative controls, column-2 positive controls, column 3-6 lab reared uninfected 30 mosquito samples, column 7-10 patient serum inoculated 30 mosquito samples. Well 7(3), 7(5), 7(6), 7(8), 8(2), 8(3), 8(6), 8(8), 9(2), 9(7) showed positive for Dengue virus antibody.

4.2.2 Molecular detection of dengue virus in laboratory inoculated mosquito specimens and patient's serum samples by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

4.2.2.1 Detection of Dengue virus RNA in lab inoculated mosquito specimens

Thirty thorax pools of laboratory inoculated mosquitoes were tested for the presence of viral RNA by RT-PCR. Nine out of thirty thorax pool tested were found positive for dengue virus. All the positive samples represented Den-3 serotype by showing a DNA band at around 290 bp on 2% agarose gel. No co-infection was found in inoculated mosquito specimens. Photograph of agarose gel electrophoresis of RT-PCR experiment is shown in Figures 4.16, 4.17 & 4.18. Figure 4.16 represents mosquito thorax pool sample no 1-10, where samples 3, 5, 6, 8 & 10 showed positivity for dengue virus DNA product. Figure 4.17 represents mosquito thorax pool sample no 11-20, where samples 11, 14, 16 & 18 showed positivity for dengue virus DNA product. Figure 4.18 represents mosquito thorax pool sample no 21-30, where no sample showed dengue virus DNA. Lane M in each gel represents 100 bp ladder marker.

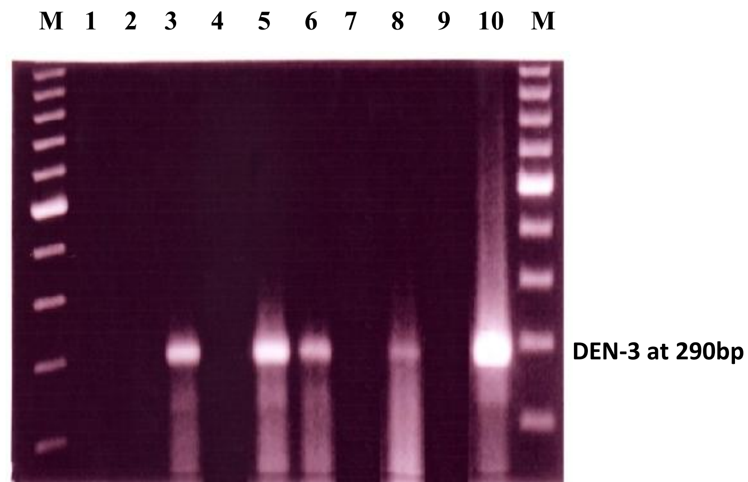

FIG 4.16: Gel-1: Dengue virus RNA detection by RT-PCR method in lab inoculated mosquitoe specimens, Lane M represent the 100bp marker (lowest band shows 200bp), Lane 1-10 represent mosquito sample no 1-10, in which sample no 3, 5.6, 8 &10 showed dengue +ve RNA. All +ve bands indicate the presence of DEN-3 at 290bp

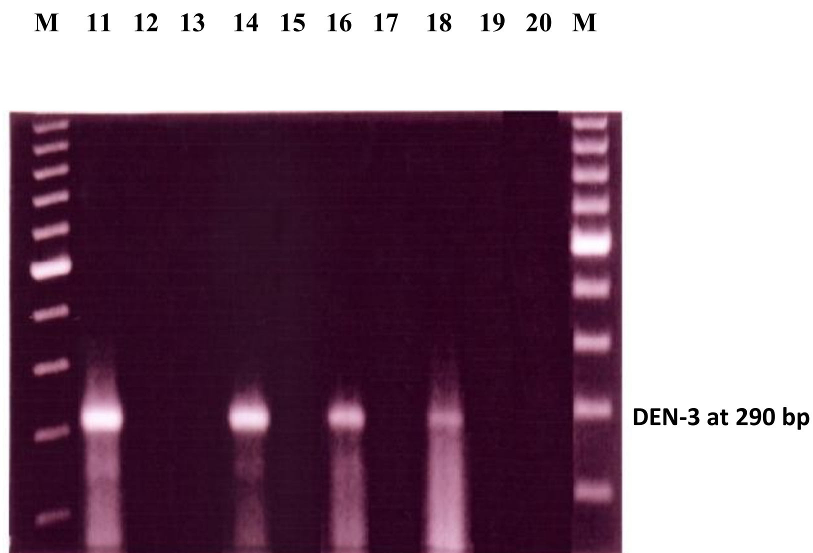

Fig 4.17: Gel-2: Dengue virus RNA detection by RT-PCR Method in lab inoculated mosquitoes, Lane M represent the 100bp marker (lowest band shows 200bp), Lane 11-20 represent mosquito sample no11-20, in which sample no11,14,16 & 18 showed dengue +ve RNA. All +ve bands indicate the presence of DEN-3 at 290bp

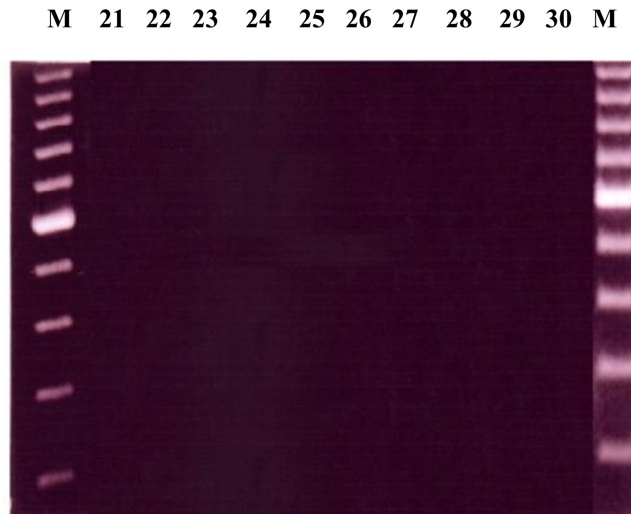

Fig 4.18: Gel-3: Molecular detection of Dengue virus by RT-PCR in lab inoculated mosquitoes Lane M-Marker, Lane 21-30 represent mosquito samples (sample no.21-30)**;** No specimen showed Dengue virus RNA by RT-PCR

4.2.2.2 Detection of Dengue Virus RNA in Patients Samples

Thirty serum samples collected from dengue patient's were tested for the detection of dengue viral RNA by RT-PCR. Nine out of thirty patient's sample tested were found positive for dengue viral RNA. All the positive samples represent Den-3 serotype by showing a DNA band at around 290 bp on 2% agarose gel except sample 16 & 18, where another band was noticed at around 100 bp indicating co- infection with **Den-3.** Figure 4.19 represents serum sample no 1-15, of which samples 3, 5, 6, 8, 10, 11 & 14 shows positivity for dengue virus RNA. Figure 4.20 represents serum sample no 16-20, of which samples 16 & 18 shows positivity for dengue virus RNA. Lane M in each gel represents 100 bp ladder marker.

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M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

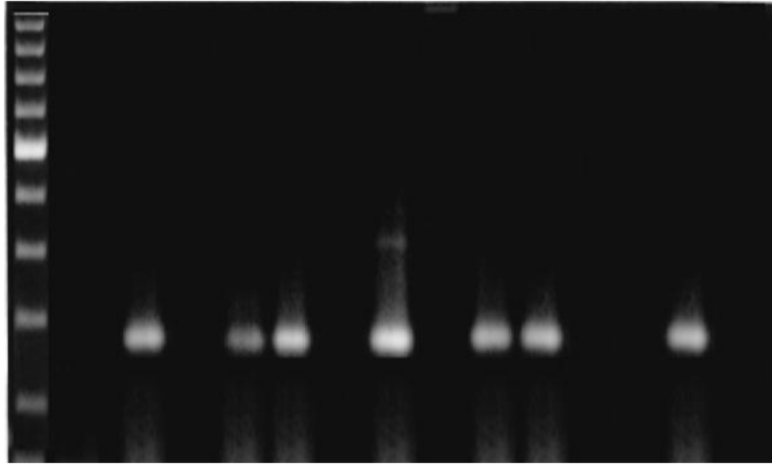

Fig 4.19 : Gel-4: Detection of Dengue Viral RNA in Patient Serum Sample by RT-PCR, Lane M represent the 100bp marker (lowest band shows 100bp), Lanes 1-15 represent sample no1-15, of which sample no 3, 5, 6, 8 ,10,11 & 14showed dengue +ve RNA . All +ve bands indicated the presence of DEN-3 at 290 bp

M 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

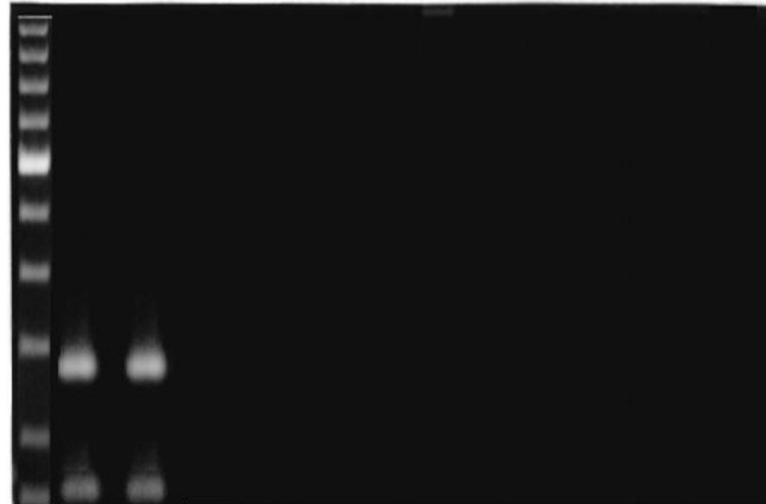

Fig 4.20: Gel 5: Detection of Dengue Viral RNA in Patient Sample by RT-PCR ; Lane M represent the 100bp marker (lowest band shows 100bp), Lanes 16-30 represent sample no16-30, of which sample 16 and 18 showed dengue +ve RNA. Positive bands at 290 and 119 bp indicate co-infection of Den-3 and Den-2

Table 4.16: Comparison of ELISA and RT-PCR Method for detecting dengue virus in Patient samples and Lab inoculated mosquitoes

SL. No	RT-PCR Result in dengue patient serum	ELISA result in lab infected mosquitoes	RT-PCR Result in lab infected mosquitoes
01	-ve	-ve	-ve
02	-ve	-ve	-ve
03	+ve	+ve	+ve
04	-ve	-ve	-ve
05	+ve	+ve	+ve

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06	+ve	+ve	+ve
07	-ve	-ve	-ve
08	+ve	+ve	+ve
09	-ve	-ve	-ve
10	+ve	+ve	+ve
11	+ve	+ve	+ve
12	-ve	-ve	-ve
13	-ve	-ve	-ve
14	+ve	+ve	+ve
15	-ve	-ve	-ve
16	+ve	+ve	+ve
17	-ve	-ve	-ve
18	+ve	+ve	+ve
19	-ve	-ve	-ve
20	-ve	-ve	-ve
21	-ve	-ve	-ve
22	-ve	-ve	-ve
23	-ve	-ve	-ve
24	-ve	-ve	-ve
25	-ve	+ve	-ve
26	-ve	-ve	-ve
27	-ve	-ve	-ve
28	-ve	-ve	-ve
29	-ve	-ve	-ve
30	-ve	-ve	-ve
Total +ve	09	10	09
Total -ve	21	20	21
GrandTotal	30	30	30

4.2.2.3 Comparison of ELISA and RT-PCR Methods for detection of dengue virus in patient serum and laboratory inoculated mosquitoes

Result of dengue virus detection in patient serum and lab inoculated mosquito pools by ELISA and RT-PCR methods has been summerised and compared in table 4.16. Patient serum samples and their corresponding inoculated mosquito pools had shown similar result by RT-PCR and ELISA individually. Out of thirty samples tested nine were found positive for dengue viral RNA in both groups. Out of thirty samples tested ten were found positive for dengue viral antibody in both groups. In both patient serum and inoculated mosquito groups, 10 samples were positive by ELISA

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but 9 were positive by RT-PCR. Patient samples having serial number 25 and its corresponding inoculated mosquito sample was found positive by ELISA but negative by RT-PCR. In remaining 20 patient serum and their mosquito samples no viral RNA could be detected by RT-PCR. RT-PCR showed 290bp band in all 9 inoculated mosquito samples indicating the presence of Den-3. But same RT-PCR method showed 290bp band in 7 samples with one additional band of 119 bp in remaining two patient samples indicating co-infection. The sensitivity of RT-PCR as compared to ELISA was 90% for detecting dengue virus in inoculated mosquito samples.

4.2.3 Detection of dengue viral RNA by RT-PCR in field-caught mosquito specimens

In a prospective field study adult *Ae. aegypti* mosquitoes were captured during September-November, 2010; September–November, 2011 and July-December, 2012 from five dengue prevalent locations in Dhaka city as mentioned before. A total of 188 (35+40+113) mosquitoe pools containing (315+362+574) 1251 *Ae. aegypti* females (ranging 1-12 mosquitoes per pool) were collected and processed for detection of dengue viral RNA by RT-PCR. Of these 9 pools were found positive for dengue viral RNA. One positive pool was collected during October 2010, two during September-October 2011 and six during September-October, 2012. In Table 4.17, result of dengue viral RNA detection in field caught *Aedes aegypti* in year 2010 has been

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summarized. In that year, only one dengue positive mosquito could be detected in a pool collected from Dhanmondi R/A during the month of October. Remaining 34 pools were found negative for dengue virus RNA. In Table 4.18 result of dengue viral RNA detection in field caught *Aedes aegypti* in year 2011 has been summarized. Two mosquito pools was found dengue RNA positive, one from Dhanmondi R/A in mid October and another one from Mirpur area at the end of September. Other 38 mosquito pools found negative for dengue virus RNA. In Table 4.19 dengue viral RNA detection of field caught *Aedes aegypti* in year 2012 has been summarized. Highest number (six) of dengue positive mosquito pools were detected in 2012 where collection of 113 mosquito pools was made for six months (July-December) during *Aedes* peak season. Four positive pools (2+2) were detected in mosquito collected from Rampura and Mirpur area. From Rampua one positive pool found in first week and another from mid of the month October. From Mirpur two positive pools had been detected, one during first week and another during mid of October, 2012. One positive pool was collected from each of Dhanmondi R/Aand Dhaka University Campus at the end of September. Remaining 107 mosquitoe pools were found negative for dengue virus RNA by RT-PCR.

Table 4.17: Summary of the result of the detection of dengue viral RNA in mosquitoe pools collected from five sampling areas of Dhaka city during the year 2010

Date	Rampura			Dhanmondi R/A			Mirpur			Shegunbagicha			D.U .Campus		
	+P	**PT**	**TM**	**+P**	**PT**	**TM**	**+P**	**PT**	**TM**	**+P**	**PT**	**TM**	**+P**	**PT**	**TM**
01/9/10	0	1	8	0	1	7	0	1	10	0	1	7	0	1	10

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15/9/10	0	1	12	0	1	12	0	1	13	0	1	9	0	1	13
29/9/10	0	1	11	0	1	11	0	1	13	0	1	10	0	1	12
13/10/10	0	1	10	1	1	9	0	1	11	0	1	8	0	1	10
27/10/10	0	1	9	0	1	8	0	1	10	0	1	9	0	1	8
10/11/10	0	1	7	0	1	7	0	1	10	0	1	6	0	1	7
24/11/10	0	1	5	0	1	5	0	1	7	0	1	4	0	1	5
Total	0	7	62	1	7	59	0	7	74	0	7	53	0	7	67
Grand Total	**A total of 315 female *Ae. aegypti* in 35 pool were collected in year 2010 ; 1 pool was found positive for dengue viral RNA**														

+P: Number of+ve pools; PT: Number of pools tested; TM: Total number of mosquitoes/pool

Table 4.18: Summary of the result of the detection of dengue viral RNA in mosquitoe pools collected from five sampling areas of Dhaka city during the year 2011

Date	Rampura			Dhanmondi R/A			Mirpur			Shegunbagicha			D.U.Campus		
	+P	**PT**	**TM**	**+P**	**PT**	**TM**	**+P**	**PT**	**TM**	**+P**	**PT**	**TM**	**+P**	**PT**	**TM**

25/8/11	0	1	8	0	1	10	0	1	8	0	1	10	0	1	9
9/9/11	0	1	11	0	1	11	0	1	11	0	1	10	0	1	9
26/9/11	0	1	9	0	1	11	1	1	12	0	1	11	0	1	12
11/10/11	0	1	10	1	1	12	0	1	10	0	1	12	0	1	12
26/10/11	0	1	11	0	1	9	0	1	13	0	1	13	0	1	11
10/11/11	0	1	9	0	1	8	0	1	10	0	1	12	0	1	9
25/11/11	0	1	8	0	1	7	0	1	9	0	1	8	0	1	7
9/12/11	0	1	2	0	1	2	0	1	3	0	1	2	0	1	1
Total	0	8	68	1	8	70	1	8	76	0	8	78	0	8	70
Grand Total	**A total of 362 female *Ae. aegypti* in 40 pools were collected in year 2011 ; 2 pools were found positive for dengue viral RNA**														

+P: Number of+ve pools; PT: Number of pools tested; TM: Total number of mosquitoes /pool

Table 4.19: Summary of the result of the detection of dengue viral RNA in mosquitoe pools collected from five sampling areas of Dhaka city during <u>the year 2012</u>

Date	**Rampura**			**Dhanmondi R/A**			**Mirpur**			**Shegunbagicha**			**D.U.Campus**		
	+P	**PT**	**TM**	**+P**	**PT**	**TM**	**+P**	**PT**	**TM**	**+P**	**PT**	**TM**	**+P**	**PT**	**TM**
2-7-12	0	1	2	0	1	2	0	1	3	0	1	1	0	1	2
9-7-12	0	1	1	0	1	1	0	1	3	0	1	1	0	1	2
17-712	0	1	2	0	1	2	0	1	2	0	1	1	0	1	2

24-712	0	1	3	0	1	3	0	1	3	0	1	2	0	1	3
1-8-12	0	1	3	0	1	3	0	1	4	0	1	2	0	1	3
8-8-12	0	1	4	0	1	4	0	1	4	0	1	3	0	1	4
16-8-12	0	1	4	0	1	4	0	1	5	0	1	3	0	1	4
23-8-12	0	1	5	0	1	5	0	1	5	0	1	4	0	1	5
31-8-12	0	1	5	0	1	5	0	1	5	0	1	4	0	1	5
7-9-12	0	1	6	0	1	6	0	1	6	0	1	6	0	1	6
15-9-12	0	1	6	0	1	7	0	1	7	0	1	6	0	1	7
22-9-12	0	1	9	0	1	9	0	1	10	0	1	11	0	1	9
30-9-12	0	1	10	1	1	10	0	1	11	0	1	10	1	1	13
7-10-12	1	1	12	0	1	11	1	1	12	1	1	12	0	1	11
15-10-12	1	1	10	1	1	12	0	1	10	0	1	9	0	1	11
22-10-12	0	1	9	0	1	9	0	1	9	0	1	9	0	1	10
30-10-12	0	1	7	0	1	7	0	1	6	0	1	5	0	1	7
7-11-12	0	1	6	0	1	6	0	1	5	0	1	5	0	1	5
15-11-12	0	1	4	0	1	4	0	1	3	0	1	4	0	1	4
22-11-12	0	1	3	0	1	3	0	1	3	0	1	2	0	1	4
30-11-12	0	1	2	0	1	2	0	1	3	0	1	0	0	1	2
7-12-12	0	1	1	0	1	1	0	0	1	0	0	0	0	1	1
14-12-12	0	1	1	0	1	1	0	0	0	0	0	0	0	1	1
21-12-12	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
29-12-12	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Total	2	24	116	1	23	117	2	21	119	0	21	100	1	24	122
Grand Total	**A total of 574 female *Ae. aegypti* in 113 pools were collected in year 2012 ; 6 pools were found positive for dengue viral RNA**														

+P: Number of+ve pools

PT: Number of pools tested

TM: Total number of mosquitoes /pool

4.2.4 Applying Nested RT-PCR Method for Detection of dengue viral RNA in field-caught mosquito specimens

Nested RT-PCR method was followed for simultaneous detection and typing of dengue virus in field caught mosquitoes. The correctly sized DNA product of 511 bp was obtained after first amplification with consensus primers D1 and D2. Each DNA product was then correctly typed by second round of amplification with the type specific primers. The expected sizes of second amplification products were 482 bp (dengue-1), 119 bp (dengue-2), 290 bp (dengue-3) and 389 bp

(dengue-4). Figure 4.21 represents gel analysis of DNA products of nested RT-PCR. Agarose gel analysis of all nine field caught dengue positive mosquito pools are shown. After first amplification reaction all positive samples showed single band of 511 bp on 2% agarose gel. First amplification could detect dengue viral RNA but could not be able to detect the specific serotype, for which second round amplification reaction with type specific primers were done.

M W 17 48 52 112 113 115 116 118 122 M

Fig 4.21: Gel-6: Analysis of DNA products of first round amplification reaction of nested RT-PCR; Lane M represent the100bp marke (lowest band shows 200bp);W- Water; Lanes 3-11, field caught mosquito pools with Dengue positive band at around 511 bp in pools 17, 48, 52, 112, 113,115, 116, 118 & 122.

4.2.5 Nested RT-PCR Method for Typing of dengue viral RNA in field-caught mosquito specimens

Representative photographs of agarose gel analysis of nested RT-PCR for detection and typing of dengue virus in 188 field caught mosquitoe pools are shown in figures 4.22 to 4.25. In each of these photographs lanes 1- 4 represent prototype of dengue type 2, 3, 4 & 1, respectively and lanes 6-15 represent 10 mosquito pools collected from the field in different sampling years. Prototype of DEN-2 band visible in 119bp, DEN-3 at 290 bp, DEN-4 at 389 bp and DEN-1 at 482 bp. In figure

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4.22 sample 17 shows one positive band of DEN-3 RNA at 290 bp. Figure 4.23 shows presence of DEN-3 RNA in sample 48 & 52. In figure 4.24 two samples (112 & 113) shows presence of DEN-3 RNA and one sample (115) shows presence of Den-2 RNA whereas in figure 4.25, sample 116 shows presence of DEN-3 RNA and sample 122 shows presence of Den-2 RNA.

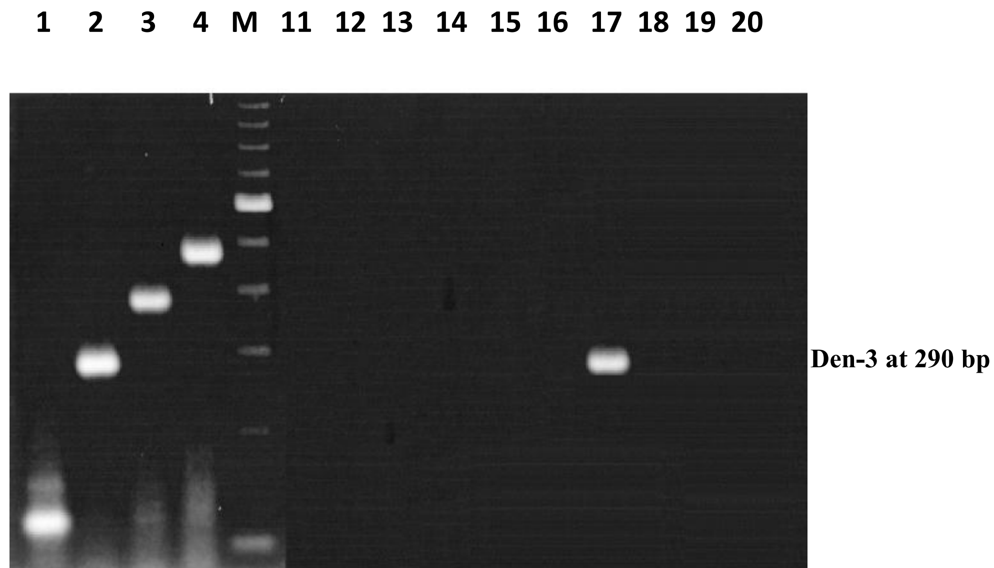
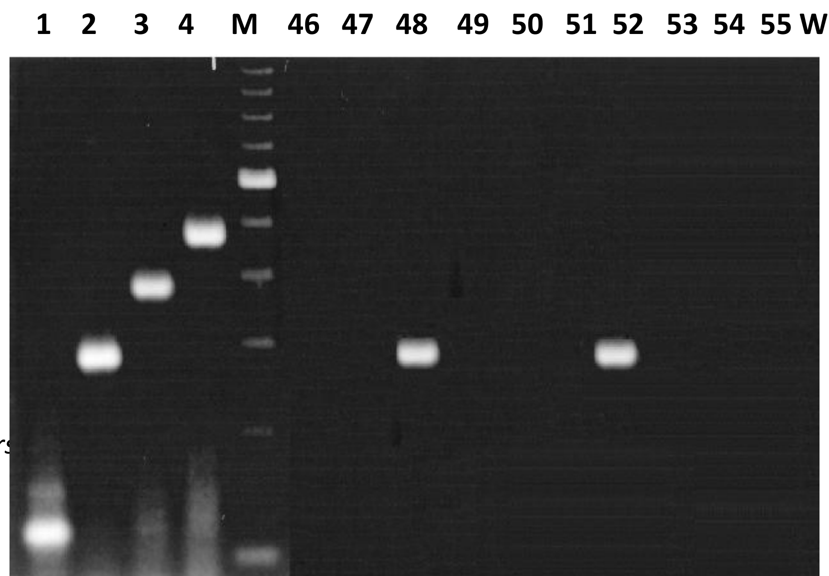


Fig 4.22: Gel-7: Molecular typing of dengue virus by nested RT-PCR. Lanes1-4 shows the positive controls for dengue-2, dengue-3, dengue-4 & dengue-1. Lane M ladder (lowest band shows 100bp); Lanes 6-15 represent field caught mosquito samples and lane 16 represent the negative control. One out of 10 mosquito samples shows 290 bp long den-3 DNA band



Den-3 at 290 bp

Fig 4.23: Gel-8: Molecular typing of dengue virus by nested RT-PCR. Lanes 1-4 shows the positive controls for dengue-2, dengue-3, dengue-4 & dengue-1. Lane M ladder with lowest band of 100bp, Lanes 6-15 represent field caught mosquito samples 46-55 and lane 16 represent the negative control. Two out of 10 mosquito samples shows 296 bp long den-3 DNA band

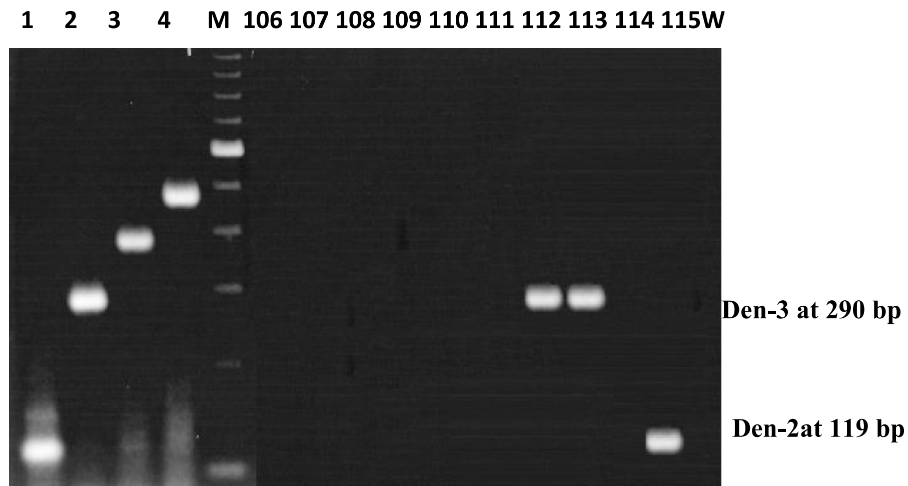


Fig 4.24: Gel-9: Molecular typing of dengue virus by nested RT-PCR. Lanes 1-4 shows the positive controls for dengue-2, dengue-3, dengue-4 & dengue-1. Lane M ladder with lowest band of 100bp, Lanes 6-15 represent field caught mosquito samples (106-115) and lane 16 represent the negative control. Two mosquito samples (112 and 113) shows 290 bp long den-3 DNA band and one mosquito sample (115) shows 119 bp long den-2 band in this gel.

1 2 3 4 M 116 117 118119120 121 122 123124 125W

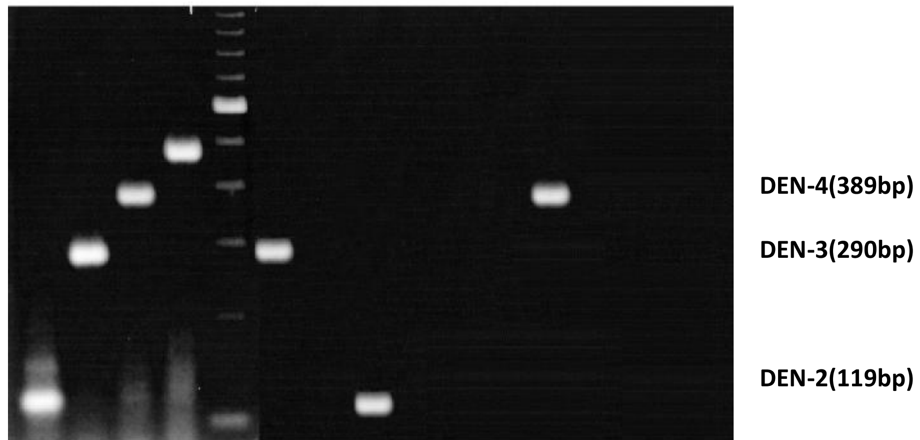


Fig 4.25, Gel-10: Molecular typing of dengue virus by nested RT-PCR. Lanes 1-4 shows the positive controls for dengue-2, dengue-3, dengue-4 & dengue-1 serotype. Lanes 6-15 represent field caught mosquito samples (116-125). Lane 5 represent ladder marker M with lowest band of 100bp and lane 16 represent the negative control. Sample 116 shows 296 bp long den-3 DNA band, Sample 118 shows 119 bp long den-2 band and Sample 122 shows 389 bp long den-4 band in this gel

4.2.6 Nested RT-PCR Method for Typing of dengue viral RNA in field-caught mosquito specimens

Detection and typing of dengue viral RNA in all nine positive mosquito pools was done by nested RT-PCR in one single experiment and picture of 2% Agarose gel electrophoresis result of this combined experiment is shown in figure 4.26. Samples 17, 48, 52, 112, 113 & 116 represent DEN-3 RNA, sample 122 represent Den-4 RNA and samples 115 & 118 represent DEN-2 RNA.

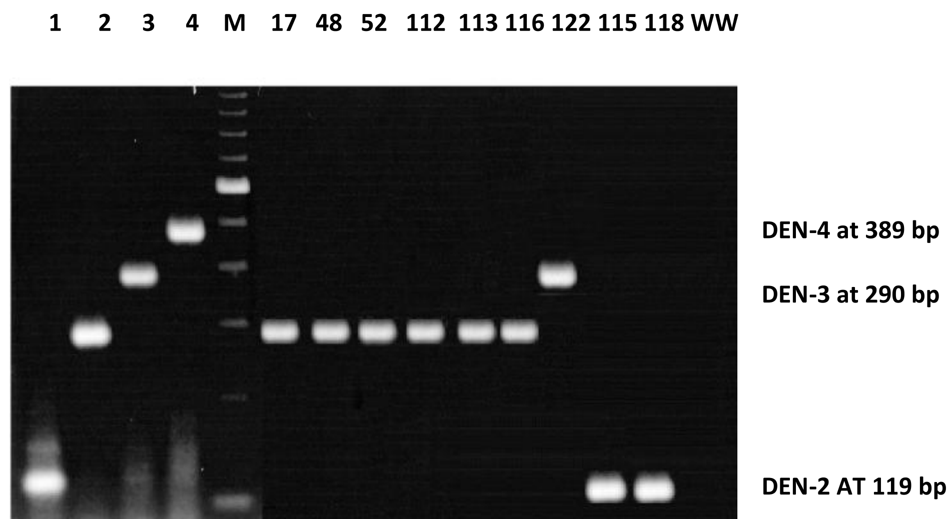


Fig 4.26, Gel-11: Nested RT-PCR analysis of Dengue virus positive field caught mosquito samples. Lanes 1-4 shows the positive controls for dengue-2, dengue-3, dengue-4 & dengue-1 serotype. Lanes 6-15 represent field caught mosquito samples. Lane 5 represent ladder marker M and lane 16 represent the negative control. Sample 17, 48, 52, 112, 113 & 116 shows 290 bp long den-3 DNA band, Sample 115 & 118 shows 119 bp long den-2 band and Sample 122 shows 389 bp long den-4 band in this gel

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4.3

4.3.1 Minimum Infection Rate of dengue positive *Ae.aegypti* mosquito pools in relation to dengue virus serotype detected

Minimum Infection Rate (MIR) of *Ae aegypti* caught from the five indoor stations was analysed. MIR ranged from 0.00-1.63 per 100 mosquitoes in tested stations with an average MIR of 0.72 for the combined stations. This means that the overall rate of dengue virus infection per 100 *Ae .aegypti* females during the study period was as high as 0.72 (Table-4.20). Dhanmondi residential area showed the highest (1.63) MIR and Shegunbagicha showed the lowest (0.00).

Table 4.20: Minimum infection rates (MIR) and serotype of *Ae. aegypti* collected from five prevalent areas of Dhaka city

Stations in Dhaka	No .of mosquitoes analyzed	No. of dengue positive mosquito	MIR	Serotypes found in different locations (no. of +ve mosq. pools)			Date of (+ve) pools collection
				Den-2	Den-3	Den-4	
Rampura	246	2	0. 81	0	2	0	7/10/12, 15/10/12-Den-3
Dhanmondi	246	4	1.63	0	3	1	(13/10/10, 11/10/11& 30/9/12) -Den3, 15/10/12-Den-4
Mirpur	269	2	0 .74	1	1	0	26/9/11-Den3,7/10/12-Den-2
Shegunbagicha	231	0	0. 0	0	0	0	
D.U Campus	259	1	0. 39	1	0	0	30/9/12-Den-2
All stations	1251	9	0. 72	2	6	1	

MIR = minimum infection rate per 100 females based on the assumption of a single infected female per infected pool (Gu et al., 2004).

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4.3.2 Dengue prevalent areas in Dhaka city on basis of RT-PCR positive dengue virus serotypes in field caught *Ae.aegypti* mosquito pools

Multiple dengue serotypes were found in two study area, Dhanmondi R/A & Mirpur (Fig-4.27). Number of Dengue positive mosquito pools were also found highest in Dhanmondi R/A. Both in Rampura and D.U Campus single serotype of Dengue virus had been detected. No Dengue positive mosquitoes had been found in Shegunbagicha in our study period. Dengue serotype 3 & 4 found in Dhanmondi R/A and Dengue 2 &3 found in Mirpur. In Rampura only Den-3 serotype and in Dhaka University Campus Den-2 serotype have been detected. Dhanmondi R/A was found to be the most prevalent area within the five study areas of Dhaka city for multiple dengue virus transmission (Figure 4.27).

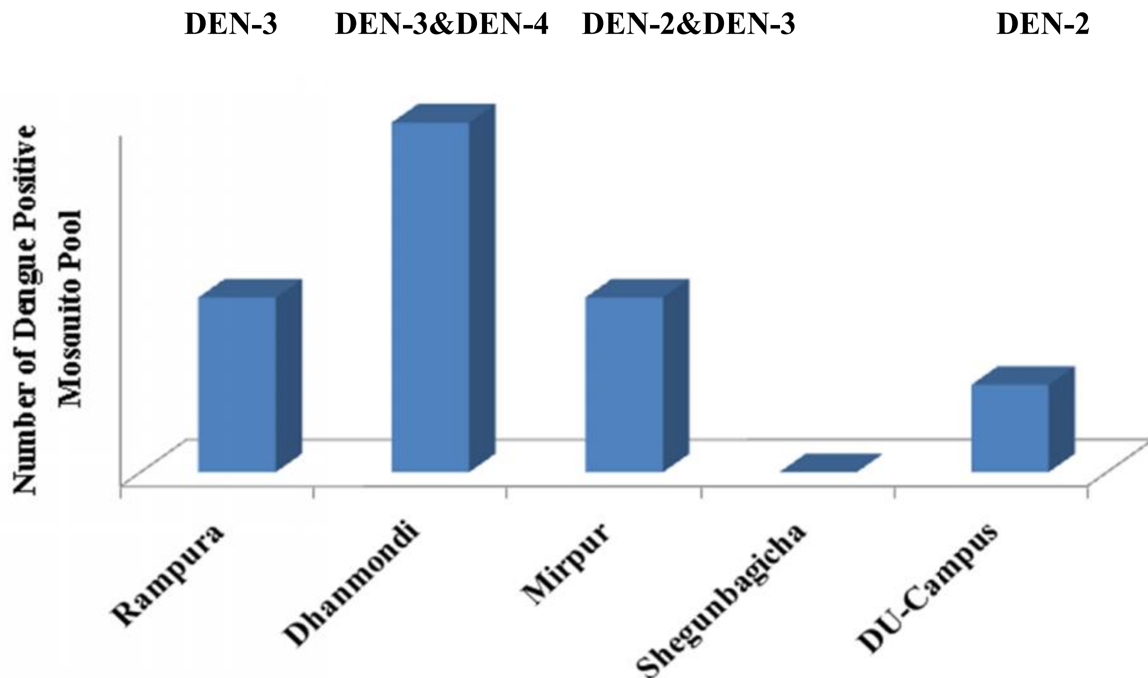

Fig 4.27: RT-PCR Based Detection of Positive Mosquito Pools in Dengue Prevalent Areas in Dhaka

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4.3.3 Detection of high risk dengue prevalent period on the basis of RT-PCR positive *Ae.aegypti* mosquito pools and dengue virus serotypes

All Dengue positive mosquito pools were found between the end of the September to mid October in three collection years as shown in figure 4.28. In year 2010 and 2011 dengue positivity was low and only Den-3 serotype was found in field collections, but in the year 2012 hight number of dengue positive mosquitoes were in circulation and a mixed infection with three serotypes DEN-3, DEN-2 & DEN-4 had been detected. In 2012, end of the September to the end of the October was the high risk period for multiple dengue virus transmission by mosquito vector.

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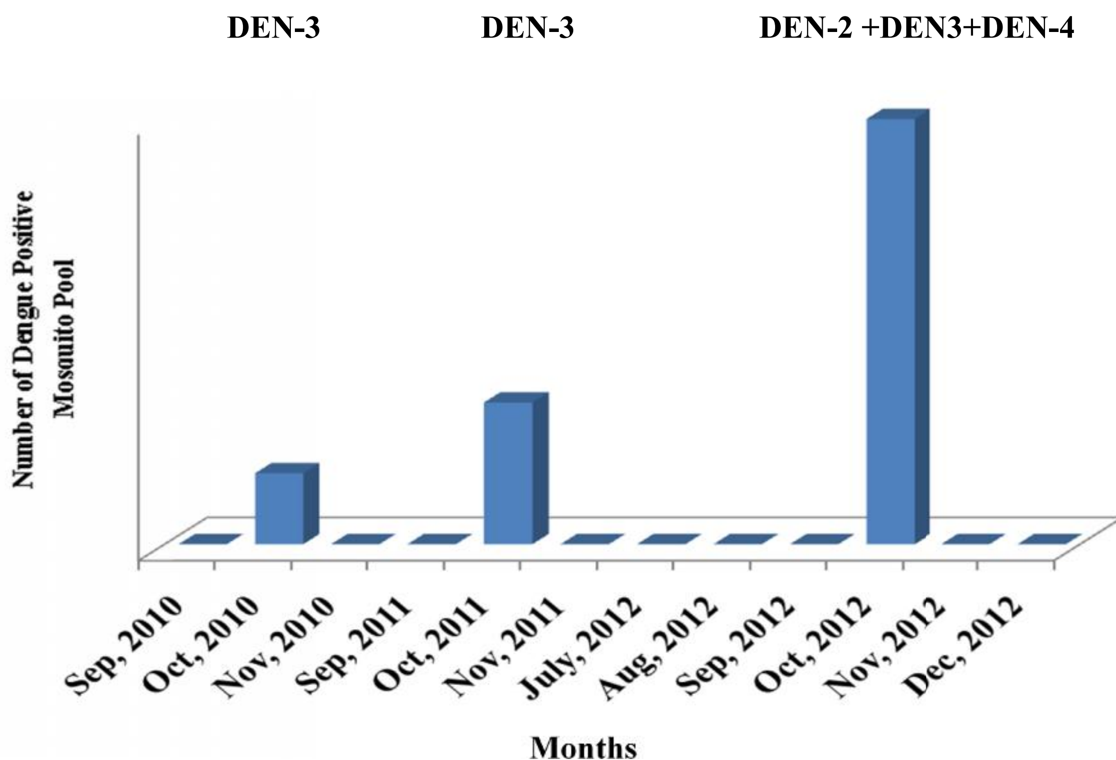

Fig 4.28: High risk period for multiple dengue virus transmission by positive *Ae.aegypti* mosquito

4.3.4 Detection of Predominant dengue virus serotype in field caught *Ae.aegypti* mosquito pools

During the study period of three calendar years, a total of 1, 251 *Ae aegypti* mosquitoes in 188 pools (ranging from 1-13 mosquitoes per pool) were caught and typing of dengue virus was done by semi–nested RT-PCR method. More than one viral serotypes (DEN-3, 2 & 4) were found to be circulating in vector mosquitoes in those five study areas. Majority of dengue virus-positive mosquitoes harbored dengue-3 virus and has been detected as the predominant dengue virus type in our study. Nine (9) mosquito pools out of 188 tested (4.8%) were found positive by nested RT-PCR. Of these six pools (66.66%) found positive for dengue type-3, two pools (22.22%) found positive for dengue type-2 and one pool (11.11%) found positive for dengue type-4. Dengue type - 1 was not found in any of the mosquito pools tested (Table 4.21 and Figure 4.29).

Table 4.21: Dengue virus Serotypes in field caught *A. aegypti* mosquito specimens

No of pools assayed	Total no. of mosquitoes	No. of positive pools	% of dengue virus serotypes (%) in positive pools			
			DEN1	DEN2	DEN3	DEN4
188	**1251**	**9(4.8%)**	0	2(22.22%)	6(66.66%)	1(11.11%)

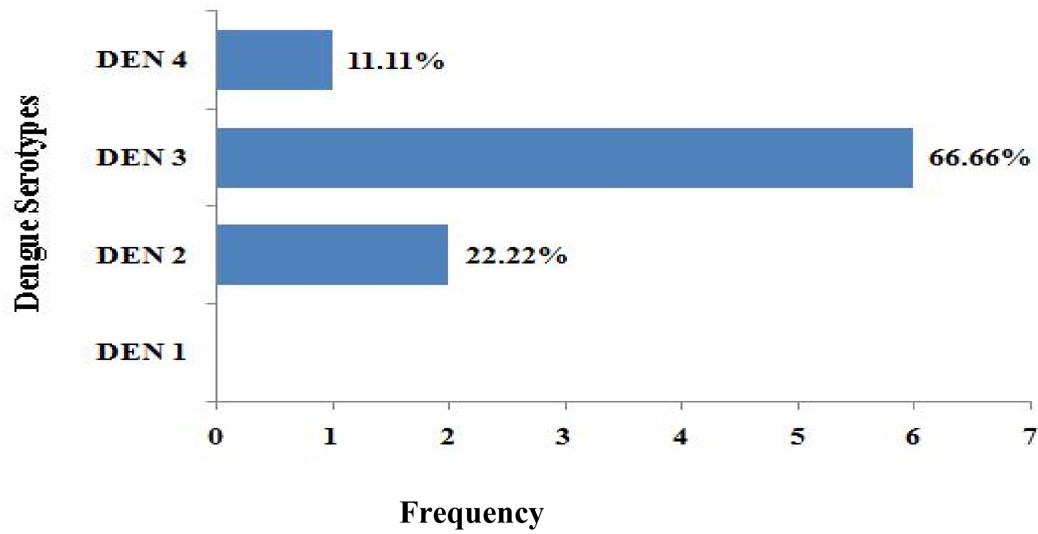

Fig 4.29: Percent o dengue virus serotypes in field caught *Ae. aegypti* mosquito specimens

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CHAPTER 5 *DISCUSSION*

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Binomics study of *Aedes aegypti* mosquito was done to understand breeding sites and seasonal prevalence of larvae and adult mosquitoes and to determine their probable relation to disease outbreak. Based on the findings of binomic studies mosquito samples were collected from the field for subsequent molecular studies. Indoor resting adults and larval surveys were carried out in five selected high-risk dengue prevalent areas in Dhaka city during July, 2009-June, 2010.

Binomic studies revealed that the breeding of *Aedes aegypti* takes place more in artificial container than in natural container. The empty flower pots, flower pot's saucers**,** flower-vases and glass jars with rooted plants which were kept in city dwellers houses or student hostels for recreation or kept in backyard carelessly has caused water-logging and created excellent places for *Ae.aegypti* to breed. Stagnated water in under-constructed area of the high rise buildings were the suspected breeding ground for mosquitoe since they prefer to live in fresh, stagnant water. In this survey cemented water reservoirs in under constructed residences were found to be good containers for *Aedes* breeding. This is an unique side of the problem since Dhaka is a growing city and many high rise buildings are seen under–construction in this city to cater the need of growing population.City dwellers carelessly thrown away bucket, jars, tin, pot, plastic bowls & bottles, jerry cans and discard appliances scatteredly on the back yards and streets. These containers were also found good breeding ground for *Aedes* larvae. The cut bamboo stumps were used to make the finch of garden or field in many places of surveyed area and many of these contained water inside and served as manmade natural place of larvae growth. Thrown away coconut shells and leaf axils or tree holes were the natural water container which were found good place for *Aedes* breeding. Man-made earthen containers like clay jars & pitchers, dumped tyres containing rain water were found good place for *Aedes* breeding. Water storage drums in residences also became ideal breeding ground of *Aedes* mosquitoes. Dhaka city's water supply was inadequate in many places including these five survey areas and people having shortage of water supply, made habit to store water in large drums. In our survey total 85.15 % of *Ae.aegypti* eggs, larvae and pupae were found in artificial container and 14.85% of *Ae. aegypti* were found in natural containers.

Nasiruddin *et al* (1952) claimed *Ae. aegypti* to be found in bamboo stumps and tree holes in Dhaka city. But Barraud *et al* (1934) had the opinion that it is unusual to find larvae of *Ae. aegypti* in tree holes or in bamboo stumps. Again, Basio *et al* (1971) found this species in both natural and artificial containers in the Philippins. Huang *et al* (1974) collected *Ae. aegypti* from artificial containers like tin can, water jar bucket, broken bottle and tyre from urban areas in Thailand. Another study in Dhaka city carried out by Bashar and his colleagues found that among the indoor containers, the highest relative frequency was in bucket, gallon, jar etc and the lowest was in AC drip pans. Density of larvae was highest in cemented tanks, holes, jars etc among the outdoor containers and lowest in leaf axils and money plant glass bulb. No larva was found in the bamboo stumps or coconut shells (Bashar *et al.,* 2006). Our findings supported the findings of Bashar *et al*., and Nasiruddin. Higher number of mosquito population in artificial container than in natural container indicated recent creation and availability of huge breeding ground for *Aedes aegypti*. Rapid urbanization, over population, city dwellers water storage habit and lacking of knowledge and practice about trash managenment in Dhaka city dwellers may have influenced higher mosquito densities by providing many more breeding sites. Findings of our study suggest that targeted awareness campaign and development of proper cleaning and sanitation habit among city dwellers can be an important component of dengues prevention strategy.

Usually *Aedes* lays egg from the month of May and mosquito population rises in the months of June-July after the rainfall. In Bangladesh usually rainy season starts from mid June. But monsoon was quite late in surveyed year 2009 and after a long hot & dry summer the monsoon came at the mid of July. The highest rain fall was recorded 333 mm/day on 28 th July, 2009 (Uddin, 2009). During August average rainfall was more than 400 mm. There was little rain (average 40 mm) during December, 2009-May, 2010. Our seasonal larvae occurrence survey showed that the post monsoon period (August -September 2009) was the most productive period for *Aedes* growth and transmission and winter has interrupted growth and biting activity.

The Breteau Index of 20 and more is considered as the risk level for dengue transmission. Our larva survey showed Breteau Index range of 20-40 during study period in five selected areas of Dhaka city which is well above the risk level for dengue virus transmission by the vector mosquitoes. Seasonal variation and areal variation of BI were also observed. Highest number of

positive containers was found in the month of September. From mid November 2009 to January 2010 the average BI was around 10, which then dropped to 0 (zero) during February-June, 2010, remained zero for next four 4 months (March-June, 2010) and BI again rose to 10 in the first week of July, 2010. Cold wave passed all over the country by the mid of the December, 09 to mid of the January, 2010 and no rainfall was seen until the end of June, 2010 in Dhaka city. The scattered rainfall was observed at the end of June, 2010. In this study we have also seen that winter and draught interrupted the mosquito's larval growth. In March, 2010 rainfall was very low and the population of *Aedes*, i.e, BI, HI & CI were the lowest. In the present study the highest larval population density was observed after the highest rainfall of more than 400 mm/month. Our observation supports the findings of Ahmed *et al.,* 2007. They found that the *Aedes* specieses were active in dry and wet seasons with peak during highest rainfall. The reduction of larvae population during the winter months was related to the low rainfall.

The Indoor resting adult mosquito population started rising from August with the peak mosquito population reached during September to November and is consistent with the findings of larvae survey. Adult population dropped from early December and few mosquitoes were found until the next monsoon came. From last week of December, 2009 to second week of January, 2010 the mean temperature of Dhaka city was around 10-12°C. which is below the required temperature (above 17°C) for successful hatching of *Ae. aegypti* (Christophers 1960, Campos and Macia, 1996, Micieli and Campos, 2003). The interruption of transmission occurred during winter but transmission may have occurred throughout the year in some areas, peaking in the monsoon season.

The peak season for hospitalization of dengue cases was during the month of November and December, 2009 which was again in consistent with the adult peak biting period. Therefore, it can be concluded that increased biting rate has probable relation to disease outbreak. Adult mosquito collection just after the monsoon has increased chance of getting dengue infected mosquitoes from nature. Though this bionomics study was done in small scale and in few selected areas of Dhaka city, it helped us to find the dengue prevalent season, dengue vector breeding sites and detect the peak season of vector population. These findings had increased the chance of getting more dengue infected mosquitoes from field. Findings of our binomic study also supported the findings of Ahmed & his team (Ahmed *et al.,* 2007).

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The binomics study revealed that all favorable environmental conditions conducive for growth transmission and maintainance of *Ae. aegypti* were present in Dhaka during study period. *Ae .aegypti* is a highly domesticated mosquito which can complete its entire life cycle within the confines of a single human dwelling. The female lays its eggs in small containers, such as flower vases, water storage jars and other containers holding water in houses. It also lay eggs in small amounts of peri domestic water which collects in tires, plastic containers and other debris associated with human settlement. When the embryo inside the egg of *Ae. aegypti* has developed to a certain stage, the egg becomes resistant to desiccation. It may then enter diapauses in which it can remain for about a year. When the eggs are flooded they hatch and the larvae commence their development immediately. This ability of *Ae. aegypti* to produce diapausing eggs enables the species to survive in areas with prolonged dry season while the rapid hatching of the eggs on flooding and the speedy development of the immature stages are adaptations to breeding in temporary collections of water. Adult *Ae.aegypti* emerging from breeding sites in indoors can complete their cycle without going outside. Swarming is not an essential component of mating. In domestic female blood feeding presents no problem because the female is strongly anthropophilic and feeds readily on human inhabitants of the dwelling. The blood-fed female rests in the house while maturing her ovaries and then deposits her eggs in domestic water containers. Dhaka city dwellers life style and uncontrolled population makes *Ae. aegypti* very much domestic and principal dengue transmission vector.

In Bangladesh, a comprehensive mosquito control program incorporates source reduction, public health education, and community participation against mosquito breeding. The *Aedes* control strategy has focused mainly on surveillance for and elimination of *Aedes* larval breeding habitats and emergency control of adults during outbreaks. Although this strategy has successfully reduced the *Aedes* mosquito population to a relatively low level, it has not prevented the emergence of progressively larger outbreaks in recent years. Outbreaks occur when a new dengue virus serotype is introduced into the human population and there are localized increases in the *Aedes* mosquito population.

Virologic surveillance based on the isolation and identification of dengue viruses infecting the human population provides an important means of early detection of any change in the prevalence of dengue virus serotype(s) (Gubler, 1989; Lam, 1993). The reappearance of serotype(s) that have not been widely circulating in human cases in the preceding years may signal an impending outbreak triggered by new emergent dengue serotype(s). The monitoring of the dengue virus type(s) infecting *Aedes* mosquitoes during inter- and intra-epidemic periods can complement the virologic surveillance for dengue outbreaks (Gubler and Rosen, 1974). Molecular techniques were used for dengue virus detection in artificially infected or field-caught *Aedes* mosquitoes and it was demonstrated that the NS3 gene-based RT-PCR assay coupled with cycle sequencing could successfully type and sequence dengue viruses in field caught *Aedes* mosquitoes (Chungue *et al.,* 1989; Lanciotti *et al.,* 1992). The use of broadly reactive consensus primrs for initial amplification in RT-PCR method ensures that all dengue virus isolates encountered in a diagonostic laboratory will be correctly identified. The nested PCR method is both more sensitive and easier for confirmation of the amplification product than any other method. The accuracy and speed of the RT-PCR assay make it an appealing method for the diagnosis and epidemiological surveillance of dengue. Virologic surveillance of dengue is traditionally performed by viral isolation and serotype identification and is considered as expensive, time-consuming and labor-intensive technique which often requires more than 7 days to obtain results. But RT-PCR assay can be completed within 30 h in laboratory, starting from RNA extraction to completing with agarose gel analysis. Therefore, RT-PCR technique constitute practical molecular diagnostic and epidemiologic tool for the virologic survelliance of dengue virus-infected *Aedes* mosquitoes to serve as an early warning system for dengue outbreaks.

Serum samples were collected from thirty hospitalized dengue patients and then intra thoracically inoculated into laboratory reared mosquitoes to produce lab infected mosquitoe population. Thirty serum sample and their corresponding lab infected mosquitoe pools were tested for the presence of dengue virus by two different methods: (a) indirect dengue Ag-capture ELISA to detect viral antigen and (b) RT-PCR to detect viral RNA. An in-house RT-PCR based molecular method was developed for detection and identification of dengue virus RNA through proper consultation of necessary reference articles.

Comparison of the result of ELISA and RT-PCR methods has revealed that the patient serum and inoculated mosquitoe had shown almost similar result by both method. Out of thirty samples tested from each groups nine shown dengue positive by RT-PCR. All nine serum samples which were found positive for dengue viral RNA by RT-PCR also showed positive dengue viral antigen by ELISA. But in inoculated mosquito group out of thirty tested, ten samples showed positive dengue antigen by ELISA, of which nine samples showed positive for dengue RNA by RT-PCR. One mosquito sample (sample no 25) was positive by ELISA but negative by RT-PCR. It may be indicated that viral phase had past of that patient serum before it was inoculated into the mosquito or their might be some error. No viral RNA could be detected in other 20 patient samples. All dengue positive samples showed positive band at 290 bp indicating the presence of Den-3 serotype. Therefore, it can be concluded that RT-PCR method that we developed and used in this study can detect and identify the specific type of dengue virus in mosquito specimens as well as in human serum. The RT-PCR method has the sensitivity of 90% (compare to ELISA) for detecting dengue virus RNA in mosquito samples.

The semi-nested RT-PCR method was applied for the detection of type specific dengue virus in field caught mosquitoes collected from five dengue prevalent areas of Dhaka city. The bionomics of *Ae .aegypti* of these five areas was studied previously to increase the chance of getting more dengue infected vector from the field. According to the findings of bionomics study we selected dengue prevalent season just after monsoon when *Aedes* population was in its peak. A total of 1,251 *Ae aegypti* in 188 pools (ranging from 1-13 mosquitoes per pool) were collected in three calendar years and samples were assayed by RT-PCR.

In our study the semi-nested RT-PCR detected and typed dengue viruses in a total of 9 pools of mosquitoes out of 188 tested (4.8%). Six pools (66.66%) were positive for dengue type-3, two pools (22.22%) were positive for dengue type-2 and one pool (11.11%) was positive for dengue type-4. The majority of dengue virus-positive mosquitoes harbored dengue-3 virus, which has been the predominant dengue virus type responsible for the local dengue epidemic since 1964. Multiple serotypes were found in two study areas e.g. Dhanmondi R/A and Mirpur. Dengue serotype 3 & 4 were found in Dhanmondi R/A and Dengue 2 & 3 were found in Mirpur area. Single serotype has been detected in Rampura area (Den-3) and in Dhaka University Campus (Den-2). No dengue virus detected from Shegunbagicha area. Dengue positive mosquito pools

were found during the months of September and October in all three survey years. During a dengue outbreak in Dhaka city in 1964, only one serotype, Den-3 was incriminated (Aziz et al, 1967). During the outbreak in 2000, circulation of all four serotypes of dengue viruses was observed in Dhaka city with DEN-3 being the predominant serotype (Pervin, M *et al*., 2002). A similar pattern of dengue serotypes was reported in 1998 from Chittagong (a city 200 km away from Dhaka) except that DEN-1 was not detected in (Emran *et al*., 2000). A PCR based molecular analysis of serum samples from the suspected dengue fever patients also indicated the presence of DEN-3, DEN-2 and DEN-4 during the outbreak of 2000 (Aziz *et al.,* 2002). Phylogenetic analysis of eight dengue virus strains isolated from clinically supected dengue patient's blood sample during outbreak of 2002 concluded that Den-3 was then circulating in Bangladesh which may entered from neighboring countries (Mohammed, *et al.,* 2006). Bangladesh was categorized into'B' category as an endemic country where DHF is an emergent disease and multiple virus serotypes co-circulating (WHO, 1999). These findings are in agreement with the findings of present study showing that Den-2, Den-3 & Den-4 infected mosquito vectors are circulating in Dhaka city where Den-3 is the predominant serotype.

As some strains of dengue viruses are more important for producing DHF, circulation of three serotypes of dengue viruses create a great threat of DHF epidemic to the Dhaka city dwellers. The predominant virus type and perhaps the most virulent serotype responsible for the current dengue fever is Den 3. Multiple virus serotypes are also co-circulating in some part of Dhaka city. Evidence of more than one circulating viral serotypes should be taken as a warning that outbreaks of DHF might occur in the future. However, we found that the overall rate of dengue virus infection per 100 *Ae .aegypti* females during the study period was as high as 0.72 which gives us an impression that a low grade infection and transmission is currently present in Dhaka city. During a dengue outbreak in 1960 in Singapoor, infection rate of 18.6 was reported for *Ae. aegypti* (Rudnick *et al.,* 1965). *Ae aegypti* caught at the five indoor stations had MIR (minimum infection rate) ranging from 0.0-1.63 per 100 mosquitoes with an MIR of 0.72 for the combined stations. Dhanmondi residential area showed the highest (1.63) MIR and Shegunbagicha area showed the lowest (0.00) MIR.

In the present study RT-PCR based molecular method was developed and tested for detection and typing of dengue virus in field caught *Aedes* mosquitoes. Use of this method as a tool for

vector surveillance can serve as an early warning monitoring system of dengue outbreak. Semi-nested RT-PCR is an affordable and reproducible molecular method with high sensitivity. Further study on molecular detection and serotype identification of dengue virus by RT-PCR in field caught mosquitoes with large scale samples is necessary before applying it as part of prevention strategy aiming to protect unprepared Bangladeshi population from DHF epidemic. RT-PCR-based surveillance data of dengue vector coupled with disease mapping data could serve as a useful epidemiological tool that might provide early warnings of dengue and dengue hemorrhagic fever (DHF) epidemics in Bangladesh.

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CHAPTER 6

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APPENDICES

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Annex-1: Materials required for rearing of *Ae. aegypti*

a. Aspirator
b. Mosquito rearing cages
c. Petri dishes
d. Paper cups
e. Beaker (Glass)
f. Glass Jars
g. Mosquito net & rubber bands for covering over paper cups
h. Thermometer
i. Hygrometer.
j. Spoon
k. Blotting paper
l. Plastic Bowls
m. Jalkanda-to prevent ants from getting into the mosquito cages
n. Dropper
o. Cotton
p. Sugar
q. Glass test tubes
r. Cerelac Baby food
s. Distilled water
t. Pigeons were used for feeding the adult female mosquitoes
u. Black container

Annex-2: a) Materials required for mosquito micro inoculation techniques

1. *Ae. aegypti* species of mosquitoes: Female adults of 1-5 days old.

2. Mosquito inoculating apparatus.

3. Other equipments and supplies:

a) Forceps

b) Scalpel or blades

c) Plastic cups with fine mosquito net and rubber band

d) Permanent over head projector marker

e) Glass line white paper

f) 1"x3" microscopic slide

g) Test tube with cotton stopper.

h) Beakers, Ice packs.

m) Compound Microscope.

b) Materials required for Incubation and preservation

i) Room heater and thermostat in winter ii) eppendrof tubes

Annex-3: Reagents and solutions required for mosquito inoculation:

a) Phosphate buffered saline diluents (PBS), Ph7.4

b) 5% Gelatin (Sigma Laboratories)

c) 5%heat inactivated (56°Cfor 30 minutes) calf serum (Life Tech)

d) Acetone

e) PBS p H 7.5

f) Acetone

g) Glycerol in PBS (Sigma Lab).

Annex-4: Composition of PBS (Phosphate Buffered Saline) 0.01 molar, p H -7.5

Na Cl	8.5 gm/ litre dist water
Na2HPo4	1.2gm/ litre dist water
NaH2p04	0.2 gm/ lit dist water
Distilled water	1 lit
Total	**1litre**

Annex-5: Composition of PBS Diluents pH 7.4 ((For patient serum dilutions)

PBS p H 7.5	95 ml
Gelatin	0.5 gm (Heat in small amount of PBS)
Inactivated Fetal calf serum	5 ml (56°C for 30 min)
Total	100 ml

Annex-6: Preparation of inoculums for mosquito inoculation (1:5 dilution):

PBS diluents 40 lμl

Patient serum 10μl

Annex-7: Preservation solution of mosquitoes head pools for ELISA

i) 500 μL solution of 20% acetone-extracted normal human serum in PBS, pH 7.4
ii) 0.05% Tween-20 and
iii) 0.02% sodium nitrite in PBS pH.4. The suspensions produced were stored at -70° C until testing.

Annex-8: Preservation solution of mosquitoes Thorax pools for RT-PCR :

i) Dissected thorax pool kept in 500μL PBS, pH 7.4 and were stored in -70°until testing

Annex-9:Materials for Identification of dengue virus by indirect dengue Ag-capture ELISA

I) ELISA plates (96-well U-bottom polystyrene plates, Titertek. Flow)

II) Anti-human goat globulin

III) Wash buffer; 0.01M PBS, pH 7. 4 , containing 0.05%Tween-20 (PBS-TW).

IV) 1% casein in PBS-TW

V) The polyclonal anti-flavivirus human IgG capture antibody

VI) Mouse anti-flavivirus monoclonal- detector antibody (4G2)

VII) Goat anti-mouse IgG conjugated to horse-radish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA.)

Dhaka University Institutional Repository

VIII) O-phenyline-diamine (Kirkegaard and Perry Laboratories, Gaithersburg ,MD , USA) diluted in 10 ml citrate phosphate buffer, Ph 5.0 and

IX) 3% hydrogen peroxide.

X) Stop Buffer

XI) Dynatech MR 600 reader to read the OD value.

Annex-10: a) Materials required for RNA extraction

I) Chemical Reagent TRIZOL LS (GIBCO BRL, USA)

II) Centrifuge machine

III) Isopropyl Alcohol

IV) RNA-se free water

b) Material required for c DNA Preparation

Extracted RNA 12µl

RT mixture 13µl containing 5× RT buffer

5X RT buffer

250 mM Tris -HCl-Ph 8.3

375Mm KCl

15 Mm MgCl2

1.25 mM of each d NTP's (Gibco BRL, Maryland, USA).

c) Materials required for the first round of PCR

Total	50µl volume
c DNA	12 µl
PCR mixture	38µl.

The PCR mixture contained 10 × PCR buffer

50 mM KCl

10 mM Tris-HCl-pH 9.0

1.5 mM $MgCl_2$ and

0.1% Triton X -100 (Promega, Madison, USA),

2.5 mM of each d NTP'S (Gibco BRL, Maryland, USA),

12 pmol D1 primer and

1 unit *Taq* DNA polymerase (Promega, Madison, USA).

The PCR reaction was carried out in a **Techne (Duxford Cambridge, UK) genecycler**

Annex-11: Materials required for Agarose gel Electrophoresis

Electrophoresis (Sigma, Missouri, USA) Tank

TBE (electrophoresis buffer)

Gel-loading buffer 6X

Ethidium bromide stained 2.0% agarose gel

100 DNA marker (100-2072bp) Gibco BRL , USA.

a)Composition of electrophoresis buffer:

Concentrated Stock solution (per liter) - 5X Tris -borate EDTA (TBE)

54g Tris base

27.5g Boric acid

20ml 0.5M EDTA (p H 8.0)

TBE was used at a working strength of 1x (i.e., a 1:5 dilution of the concentrated stock) for agarose gel electrophoresis.

b)Gel-loading buffer 6X

0.25% bromophenol blue

0.25% xylene cyanol FF

15% Ficoll (Type400; Pharmacia) in water, storage at room temperature

c)Ethidium bromide stock solution - 10mg/ ml.

In 2% agarose gel the amount of agarose in TBE was 2mg/ ml.