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DENGUE VECTOR DYNAMICS IN PAKISTAN

A Thesis submitted for the degree of PhD

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DEPARTMENT OF ANIMAL AND PLANT SCIENCES
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Dedicated to my family

Especially

To my Father and to the memories of my Mother

ABSTRACT

During the last few year dengue has become an emerging vector borne disease in Pakistan. *Aedes aegypti* once considered eradicated from almost whole of Pakistan was again collected from 13 different cities. This mosquito has not only started reinvading its previously occupied areas (Karachi, Peshawar and Lahore) but is also spreading to new areas where it was not present before (Attock, Haripur, Hasanabdal, Taxilla, Rawalpindi, Gujranwala, Sheikhupura, Faisalabad, Multan and Hyderabad). This mosquito is breeding in almost all types of artificial containers especially in tyres. Though this mosquito is mostly using outdoors containers for ovipositon but was also found breeding indoors in household. Eleven microsatellite markers were used to determine the genetic population structure of *Aedes aegypti* in Pakistan using mosquitoes collected from 13 different cities. There appears to be a single population of this mosquito in Pakistan with a pattern of isolation by distance rather than distinct sub-populations. The large scale of isolation by distance suggests long range passive dispersal which may be facilitated by the tyre trade in Pakistan. A decrease in genetic diversity from north to south suggests a recent spread of this mosquito from Karachi. A strong negative correlation between genetic distance and quality of road connections between cities suggests human-aided passive dispersal of *Ae. aegypti* in Pakistan. Dengue detection in mosquitoes collected from different areas of Pakistan with special reference to tyres shows that vertical transmission is not playing any significant role in the maintenance of dengue virus in mosquito of population. Absence of virus in larvae collected from tyres suggest that though tyres are playing important role in the spread of vector mosquito in Pakistan but its not playing any role in the spread of dengue virus in the study area. The long-range dispersal of *Aedes aegypti* in Pakistan could facilitate the strategy of introducing transgenic *Aedes aegypti* or an intracellular bacterium, like the endosymbiotic *Wolbachia,* for controlling the population of this dengue vector in Pakistan.

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Syed Basit Rasheed

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INTRODUCTION

"Dengue" is a Spanish word that means "fastidious or careful" and may refer to the gait of a person suffering from bone pain during dengue fever. Dengue may also have its origin in the Swahli phrase "Ke denga pepo" that means "a sudden cramp like seizure caused by an evil spirit" (Halstead 1980). It is an acute febrile disease caused by four closely related but antigenically distinct serotypes of Dengue virus belonging to genus *Flavivirus* of the family Flaviviridae. There are about 70 viruses in this family all of which are RNA viruses and are arboviruses (carried by insects) except for Hepatitis C virus (the cause of liver cirrhosis in humans) and the pestiviruses (viruses causing classical swine fever and bovine virus diarrhoea). Viruses belonging to this family are responsible for many diseases e.g. Yellow fever, West Nile virus, Congo cremian haemorrhagic fever, Hepatitis C, Japanese Encephalitis and Saint Louis Encephalitis and cause high morbidity and mortality in humans (Zanotto et al. 1996, Holmes and Twiddy 2003).

Dengue is a single stranded positive sense RNA virus with a genome size of approximately 11 kilo bases. It encodes a single open reading frame of 10,170 nucleotides corresponding to 3390 amino acid residues. The genome of dengue virus lacks a Poly-A tail at the 3′ end and encodes for three structural (core=C, membrane=M and envelope=E) and seven non structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins (Figure 1.1). The proteins are encoded in order 5`-C-pr(M)M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3`and the size of the virion is approximately 50nm in diameter. (Osatomi et al. 1988, Osatomi and Sumiyoshi 1990, Chevillon and Failloux 2003, Santos et al. 2008). Dengue virus has four closely related but antigenically different serotypes DEN-1, DEN-2, DEN-3 and DEN-4. The serotypes carry common epitopes on the envelope protein that result in extensive cross-reaction in serological tests. However, despite this antigenic similarity, they are different enough to elicit only temporary and partial protection, which lasts only for a few months and is not permanent (WHO 1999). As a consequence people living in dengue epidemic areas can be affected by two

serotypes during their lifetime if not four but only once by the same type. Hyperepidemicity (epidemics caused by more than one serotype of Dengue) occur in areas having multi serotypes of dengue virus (Gubler 1998, WHO 1999, Wang et al. 2000).

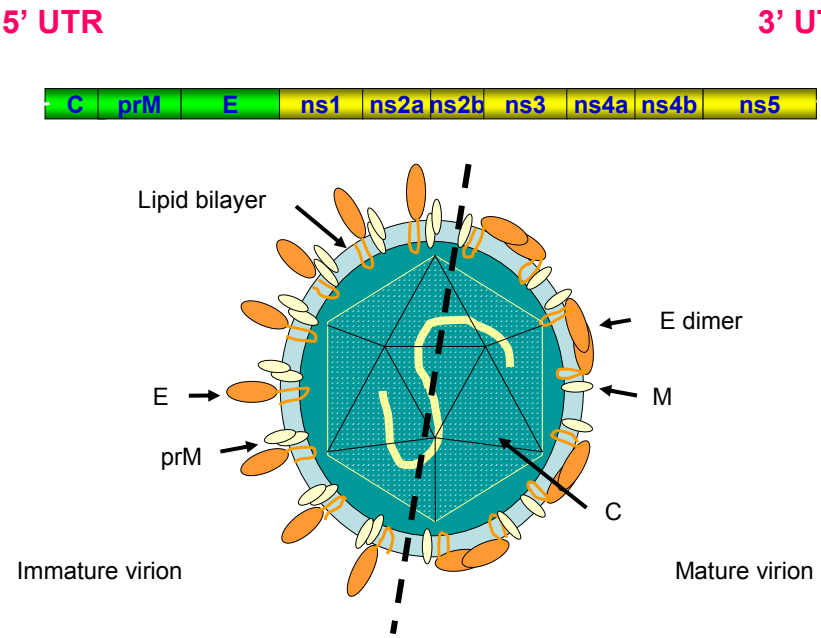

Figure 1.1: Dengue virus genome organisation and virion structure.

Dengue has become a major health problem in both the tropical and subtropical world and is currently considered to be the most important human arboviral disease. Humans, lower primates and mosquitoes are known natural hosts of Dengue. Female members of the genus *Aedes* are the arthropod vectors of Dengue in rural as well as urban area. *Aedes aegypti* is one of the most efficient mosquito vectors for arboviruses because it is highly anthropophillic, prefers habitats in close proximity to humans and often lives indoors (Arunachalam et al. 2008, Wang et al. 2000, Twiddy et al. 2002). *Ae. albopictus, Ae. polynesiensis,* and several species of the *Ae. scutellaris* complex are also considered to be responsible for Dengue outbreaks in different areas depending upon each species' particular geographical distribution; however, they are less efficient vectors than *Ae. Aegypti* (Gubler 1998, McBride and Ohmann 2000).

Each year about 50 million people are estimated to suffer from dengue virus with 55000 cases requiring hospitalisation, a large proportion of which are children.

At least 2.5% of these individuals die and the death rate could actually be up to twice as high as this. Approximately 2.5 billion i.e. about half of the world's population is said to be currently at risk of dengue (WHO 2008). The geographical distribution of dengue is similar to that of malaria. Like malaria it is distributed in tropical areas covering more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South and Southeast Asia and Western Pacific but unlike malaria it is found in urban areas. The distribution and incidence of the infection has increased over the past forty years (Gubler 1998).

1.1 HISTORICAL SITUATION

A Chinese medical encyclopaedia from the Chin dynasty (265-420 AD) reports the first recorded potential case of dengue fever. The Chinese knew that this disease is connected with flying insects associated with water and called dengue a "water poison". Though dengue was present earlier, Benjamin Rush reported the first recognized dengue case recorded in 1780 during the Philadelphia epidemic of dengue, (published in 1789). He also coined the term "Break bone fever" because of the symptoms of myalgia (muscular pain) and arthralgia (bone pain). Viral etiology and transmission of dengue by mosquitos was shown in the 20th century. In the 1780's the first epidemic of dengue like illness occured simultaneously in Asia, Africa and North America. People clinically having signs and symptoms similar to that of dengue occurred even earlier. The outbreaks of illness during the year 1635 in the French West Indies and that of Panama in 1699 could also have been dengue with similar clinical signs and symptoms. Thus, even before 18th century, when the first global epidemic of dengue like illness began, dengue or a very similar illness had a wide geographic distribution. There are no reliable reports that the epidemics of Batavia (Jakarta, Indonesia) and Cairo (Egypt) in 1779 were dengue but the Philadelphia epidemic of 1780 was dengue (Gubler 1998).

During the 18th and 19th centuries, epidemics of dengue like diseases were described globally in the tropics as well as in some temperate zones. The socio-economic impact of World War II created ideal conditions for increased transmission of mosquito borne diseases and resulted in increase spread of Dengue globally. In Manila and Philippines an epidemic began in the 1950's, which is

considered to be the first reported known epidemic of dengue in world, within 20 years the disease spread throughout South East Asia in an epidemic form and by 1975 denguehad become one of the leading causes of child death in many countries of south East Asia. Since then DHF has expanded geographically from Southeast Asian countries east to China and west to India, Sri Lanka, Maldives and Pakistan (Gubler 1998).

Between 1956 and 1989 more than 2.5 million cases of DHF were reported to the World Health Organization with 42,751 deaths (Kautner et al. 1997). After that Dengue spread so quickly that since the 1980's major epidemics had started occurring in the affected areas and had extended its boundaries to several Pacific islands (Niue, Palau, Yap, Cook Islands, Tahiti, New Caledonia and Vanuata) and through out the American continent where epidemic dengue was rare in the 1950's, 1960's and most of the 1970's. At the end of 1994 as many as 24 American countries, that had been free of the disease for between 35 and 130 years, reported laboratory confirmed cases of DHF. The situation became so bad in these countries that many countries' status regarding dengue disease had been changed from non-endemic (no endemic disease) or hypo-endemic (one serotype present) to hyper-endemic (multiple serotypes present). The reason for the wide spread of disease in this region is the re-emergence of the principal vector of dengue in these areas which was thought to have been eradicated from most of Central and South America. *Aedes aegypti* began to reinvade these countries after the discontinuation of the eradication programme in the early 1970's and by the 1990's it had nearly regained the geographic distribution it held before the eradication program was initiated (Rothman 2004). At the end of the 1990's dengue had become the most important mosquito born disease after malaria. In Africa only sporadic cases have been reported with increased epidemic of dengue fever but no major epidemic of DHF has as yet been reported (Gubler 1998).

1.2 DENGUE VECTORS

There are two transmission cycles of dengue; (i) the primitive sylvatic/enzootic transmission cycle involving canopy-dwelling *Aedes* mosquitoes and non-human primates reservoir host in the rain forests of Asia and Africa and (ii)

an epidemic transmission cycle found in humans as a reservoir or infective host and transmitted by *Ae. aegypti, Ae. albopictus, Ae. polynesiensis* and other members of the *Ae. scutellaris* group (Figure 1.2). *Aedes aegypti* plays a major role in urban transmission and other species act as a secondary vector of this disease although they are generally more susceptible to laboratory induced infections rather than natural infections (Wang et al. 2000).

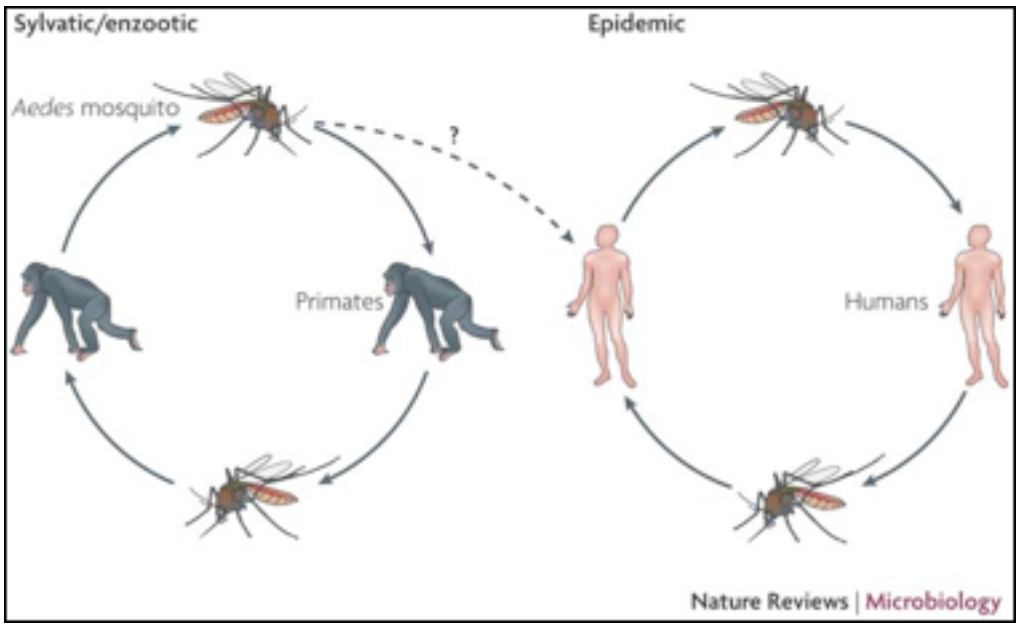

Figure 1.2: Sylvatic/enzootic and epidemic transmission cycles of dengue (*Whitehead et al. 2007*).

Aedes aegypti, the principal vector of Dengue was first identified in 1762 by Linnaeus and its role in transmission of dengue had been suggested by Bancroft in 1906 (Rosen 1993). It is a dark brown or blackish mosquito having white markings on its body. It has been shown to have two main forms. 1) *Aedes aegypti aegypti* the light coloured domestic form and *Aedes aegypti formosus,* a dark coloured forest form. *Ae. aegypti aegypti* is an urban and peri-urban mosquito species found around the globe in tropical and subtropical regions and is responsible for Dengue epidemics in humans. It is also responsible for the transmission of a number of other diseases including yellow fever, West Nile virus and Japanese Encephalitas. It is believed that *Ae. aegypti* has an African origin and was probably imported to the New World via the African slave trade (from the 15th to 19th centuries). Introduction

to Asia is believed to coincide with the development of the shipping industry (18th and 19th centuries) (MacDonald 1956). *Aedes aegypti formosus* is reputed to be the causal agent of sylvatic dengue cycles in West Africa so it is not considered to have an important role in the global dengue epidemic. The gene flow between the two sub species is thought to be restricted by spatial distribution. (Gubler 1998).

Generally *Aedes aegypti* distribution approximately corresponds to a winter isotherm of 10°C. Geographically it is distributed between latitiudes 35°N and 35°S. Although it has been reported from as far as 45°N, such invasions have occurred during warm weather, and the mosquito have not survived the cold season. Altitude is also a limiting factor for the distribution of *Aedes aegypti.* Its distribution is normally restricted by an altitude of 1000m but it has been reported at 2121m in India, at 2200m in Colombia, where the mean temperature is between 10°C and 19°C and at 2400m in Eritrea (WHO 1997). This tropical mosquito is found in or around homes and breed in artificial containers like old automobile tires, buckets used for collection of rainwater, flower vases and trash in general. They also breed in containers used for water storage and even in septic tanks thus breeding in large numbers near domesticated areas. Eggs of *Ae. aegypti* are highly resistant to desiccation, which increases their chance of survival and is a cause of failure in its control. They are an indoor resting mosquito, that feeds on humans during the daytime (Gubler 1998).

The biting activity of *Ae. aegypti* has two peaks, early morning for 2 to 3 hours after daybreak and in the afternoon for several hours before dark. The female mosquito can act both as a biological and mechanical vector and can infect several people in a single blood meal due to its nervous feeding behaviour. A slight movement can however disrupt the feeding process. The mosquito then returns to the same or a different person to continue feeding after some time therefore feeds on several people during a single blood meal. If this female mosquito takes a fresh viremic blood meal, the proboscis is infected with dengue virus, and can transmit the virus even if the mosquito does not take blood meal thus acting as mechanical vector. Several members of the same family may become ill with dengue fever within a 24 to 36 hours time frame suggesting that a single infective mosquito is

responsible for their infection. This behaviour of *Ae. aegypti* makes it an efficient epidemic vector of Dengue (Gubler 1998).

There are two ways by which a mosquito can get infected. The first is by sucking blood from a dengue-affected person in which the virus is transferred from human blood to the gut of the mosquito. The normal incubation period of the virus in the mosquito is 8 to 10 days but also depends upon ambient temperature. In the gut wall the virus replicates resulting in infection of the salivary glands making the mosquito able to transmit the virus for life (Gubler 1998). The second way of becoming infected is by vertical transmission in which the virus is transferred from a female mosquito to its offspring's during egg transfer in oviduct and egg development. It is also reported that the virus can be transferred from the male mosquito to its offspring through sperm during mating with an uninfected female (Lutomiah et al. 2007). Vertical transmission may be a strategy for dengue viruses for surviving adverse climatic conditions and is mainly observed in the summer months when dengue infection is low in the human population (Arunachalam 2008).

1.3 CLINICAL DIAGNOSIS

Humans are the main amplifying host for the virus in both the endemic and epidemic transmission cycles. The incubation period of the virus in humans is about 3 to 14 days depending upon the immune system of the infected person after which the signs and symptoms of disease can be observed. Dengue virus is not contagious without the mosquito but vertical trnsmission is possible i.e. it can be transmitted from infected mother to child at birth (Maroun et al. 2008). The response to the virus depends upon the immune system. There are three types of Dengue disease:

1. Classical Dengue Fever (CDF)
2. Dengue Haemorrhagic Fever (DHF)
3. Dengue Shock Syndrome (DSS)

A patient suffers from dengue for a short duration but it is a debilitating disease with high morbidity but low fatality/mortality rate. Dengue mostly affects

older children and adults but infants and young children frequently suffer from DHF that ultimately results in DSS. Symptoms of each type of dengue fever are as follows.

1.3.1 Classical Dengue Fever (CDF)

Older children and adults primarily suffer from classical dengue fever. The first sign and symptoms of dengue fever appear after 3 to 8 days of infection. Usually the fever lasts for 4-7 days and most people recover without any complications. In a few cases patients may take several months to recover. Dengue fever is also characterised by a "saddleback" type fever response because it usually starts suddenly with a rapidly climbing high fever and recurs periodically. Dengue resembles other diseases like malaria, influenza, measles, Colorado tick fever, scarlet fever, typhus, yellow fever and other haemorrhagic fevers. Good diagnosis is therefore very important in combating this disease. Signs and symptoms of CDF are:

- CDF starts with rapidly climbing high fever that may go up to 105°F.
- Retro orbital (behind the eye) pain.
- Severe joint and muscle pain.
- Nausea and vomiting.
- Maculopapular rash appears 3 to 4 days after emergence of fever. It is a characteristic red petechia that first affects lower limbs and chest and sometimes it appears on most of the body.

Some patients experience severe backache, sore throat or severe abdominal pain, which may be confused with appendicitis. Patients may also suffer from nausea, vomiting and thrombocytopenia with elevated serum hepatic enzymes. At this stage Dengue fever cannot be differentiated from other viral diseases and the need for supportive treatment is minimal as recovery is rapid. This condition may last up to 6 days. The rash then fades away and the patient start recovering. Dengue fever is generally self limiting and rarely fatal (Gubler 1998).

1.3.2 Dengue Haemorrhagic Fever (DHF)

The incubation period of DHF is thought to be similar to that of dengue fever. Patients of DHF are mostly children under the age of 15, although adults may also suffer. DHF also starts with a suddenly climbing fever that usually last for 2 to 7 days along with other non-specific symptoms. During the acute phase it is difficult to distinguish the signs and symptoms of DHF from dengue fever and other tropical diseases. Diseases like measles, rubella, influenza, typhoid, leptospirosis, malaria, other viral haemorrhagic diseases and any other non-specific viral syndromes should be included in differential diagnosis during the acute phase of the illness. Upper respiratory infections are frequently caused in children due to concurrent infections with other viruses and bacteria. There is no particular or specific sign or symptom for DHF during the acute stage however when the fever fades away, accurate clinical diagnosis in many cases is possible by the appearance of characteristic manifestations of plasma leakage (Gubler 1998).

In DHF the critical stage is the time of defervescence. Signs of haemorrhagic manifestation or circulatory failure may occur from a day before to a day after the temperature falls to normal or below. Common hemorrhagic manifestations include marked damage to blood and lymph vessels due to increased vascular permeability and abnormal haemostasis that can lead to hypovolaemia and hypotension and in severe cases result in hypovolaemic shock complicated by severe internal bleeding. Bleeding starts from nose, gums or under the skin; causing purplish bruises and from the GI tract. The patient also has severe abdominal pain, passage of blood through the vagina and vomiting of blood. Patients suffering from DHF have cold clammy skin of legs and hands alone or in combination. Lethargy and drowsiness are more marked than CDF. The tourniquet test which shows capillary fragility in patients, may be diagnostically helpful to the physician. As a result of hemorrhagic manifestation scattered petechiae can be observed on the extremities, trunk and other body parts (Gubler 1998).

1.3.3 Dengue Shock Syndrome (DSS)

DSS is the most severe form of dengue disease. Some patients experience shock from blood loss during DHF due to failure of early diagnosis and proper management. Symptoms of shock are circulatory failure including narrow pulse pressure or frank shock, fluid leaking outside blood vessels and massive bleeding. The liver may be palpable and tender with liver enzymes mildly elevated but jaundice is rare. Petechia is more common on the face in patients with severe dengue shock syndrome (DSS). Four characteristic features of shock are intense, sustained abdominal pain, restlessness or lethargy, persistent vomiting and a sudden change from fever to hypothermia with sweating and prostration. On the appearance of these signs and symptoms the patient should be admitted to hospital immediately to prevent shock. Volume replacement helps the patient to recover rapidly but during the period of excessive capillary permeability may result in recurrence of the shock. Early detection and treatment can reduce the fatality rate to as low as 0.2% contrary to fatality rate of as high as 12% to 44% in the case of shock (Gubler 1998).

1.4 LABORATORY FINDINGS

In DHF thrombocytopenia and haematoncentration are reported in almost all patients. The platelet count decreases to below 100,000 per mm^3 often before or simultaneously with a change in the haematocrit of usually between the third and eighth day of illness. Plasma leakage is an indication of a rise in the haematocrit level, it is more pronounced in shock cases but also present in non-shock cases. An increase of the haematocrit value by 20% or more in haematoconcentration is considered to be the definitive evidence of increased vascular permeability and plasma leakage. The drop in platelet count and the rapid rise in haematocrit are unique to DHF and it occurs before defervescence and before the occurrence of shock (Gubler 1998).

In DHF the white blood cells count varies at the onset of illness, ranging from leucopenia to mild leukocytosis but it drops due to reduction of neutrophills near the end of the febrile illness. In most patients a coagulation assay or

fibrinolytic factors assay show a reduction in prothrombin, fibrinogen, factor VIII, factor XII and antithrombin II. Reduction in α-antiplasmin (α-plasmin inhibitor) is also observed in some cases. Prothrombin factors that are vitamin-K dependent, such as factor V, VII, IX and X also decrease in severe cases of DHF with marked liver dysfunction. Partial thromboplastin time and prothrombin time are prolonged in about one-half and one-third of DHF patients respectively. In severe cases the thrombin time is prolonged. There is impairment in platelet function and serum complement levels, particularly that of C3, are reduced (Gubler 1998).

Loss of albumin results in hypoproteinaemia. Hyponatraemia and elevated levels of serum aspartate aminotrasferase are also observed. Metabolic acidosis may frequently be found in prolonged shock and blood urea nitrogen is elevated at the terminal stage of shock. Chest X-rays of DHF patients reveals pleural effusion, mostly on the right side, the severity of which can be correlated with the severity of disease. Bilateral pleural effusion is a common finding in shock (Gubler 1998).

DHF generally occurs in patients previously infected by different serotypes of Dengue virus. This is due to antibody dependent enhancement (ADE); a phenomenon in which the heterotypic antibodies from a previous dengue serotype infection result in promotion of viral replication within the mononuclear leucocytes during a secondary infection of a different serotype. It was originally suggested by S.B. Halstead in the 1970s that DHF is more likely to occur in patients who are previously exposed to dengue infection and secondary infection leads to a phenomenon explained by ADE. An immunological phenomenon, known as original antigenic sin, the immune system fails to respond to a stronger secondary infection and instead of eliminating the virus, the virus start replicating in the leukocytes thus resulting in a far more serious form of secondary infection. This process is known as super infection. It is also postulated that like other animal viruses, dengue virus also varies and changes genetically as a result of selection pressure due to replication in humans and mosquitoes resulting in some virus strains that have greater epidemic potential. These genetic changes result in increased virus replication, virulence and epidemic potential (Wang et al. 2000, Lobigs et al. 2003, Yang et al. 2001, Halstead 2008)

1.5 LABORATORY DIAGNOSIS

In epidemics early and accurate diagnosis of dengue is very important for the control of the disease. Surveillance of vector populations and the detection of dengue virus in field caught mosquitoes is also an important aspect of epidemiological studies of dengue. Surveillance of mosquitoes infected with dengue virus can help in monitoring of the infection rates within vector populations carrying a specific serotype and provides an early warning of a possible outbreak of dengue in that area (Sameul and Tyagi 2006, Gurukumar et al. 2009). Blood samples should be taken immediately from patients suspected of suffering from dengue disease. Dengue can be confirmed either by serological tests or by using modern molecular techniques. Five basic serological tests; Haemagglutination inhibition (HI), Complement fixation (CF), Neutralization test (NT), Immunoglobin M (IgM) capture enzyme-link immunosorbent assay (ELISA) monoclonal antibody capture (MAC-ELISA) and indirect IgG ELISA are used for the diagnosis of dengue. High cross reactivity in these tests acts as a limiting factor requiring a comprehensive pool of antigens, including all four serotypes, another flavivirus (Yellow fever virus, Japanese encephalitis virus or St. Louis encephalitis virus) and in some areas other viruses like Oropouche, Mayaro or Chikungunya virus that cause similar clinical symptoms to those of flaviviruses. Dengue antibodies are detected around the fifth day of disease onset, which result in the failure of rapid diagnosis. These serological tests are based on the principle of a rapid four fold or greater rise in titer of specific antibodies between acute and convalescent phase serum samples (Gulber 1998, De Paula et al. 2004).

1.5.1 The Hemagglutination Inhibition (HI) Test

HI has been the most frequently used test for many years, because of its high degree of sensitivity and ease of performance. Dengue specific HI antibodies persist for long periods (up to 48 years or more) making it ideal for seroepidemiological studies and to differentiate between primary and secondary infection. The HI test is recommended by the World Health Organization (WHO) to discriminate between primary and secondary dengue virus infections. HI antibodies can be detected on day 5 or 6 of illness. Antibody levels remain high for 2 to 3 months in some patients

but begin to decrease by 30 to 40 days and its level reaches below 1,280 in most patients. The major disadvantages of the HI test are its lack of specificity, the need of paired samples and its inability to identify the infecting virus serotype (Gubler 1998, De Paula et al. 2004, Matheus et al. 2005, Sa-Ngasang et al. 2005)

1.5.2 Complement Fixation (CF) Test

The CF test is fairly difficult to execute as it requires highly trained personnel and is rarely used for routine dengue diagnostic serologic testing in humans. The principle of the CF test is that complement is consumed during antigen-antibody reactions. CF antibodies are generally detected later than HI antibodies and usually persist for shorter periods, making it of limited value for seroepidemiological studies. They are very specific in the primary infections, contributing the determination of the infecting serotype, as demonstrated by the monotypic responses observed in primary infections (Gubler 1998, De Paula et al. 2004).

1.5.3 Neutralizing Test (NT)

NT is the most sensitive and specific serological test for dengue diagnosis in humans. Serum dilution plaque reduction NT protocol is used to quantify the titre of neutralising antibody for a virus. Generally neutralizing-antibody titres rise at about the same time or slightly more slowly than HI and ELISA antibody titers but more quickly than CF antibody titres. Since neutralizing antibodies can last for a long time (for at least 48 years) NT can be used for seroepidemiological studies. Due to the sensitivity of neutralizing antibodies it can be detected in some patients with past dengue infection even in the absence of detectable HI antibodies. As a relatively monotypic response is observed in patient serum during the convalescent phase, the NT can be used for serotype identification in primary infection due to high specificity. In the case of secondary and tertiary infection determination of the infecting virus serotype by NT is not reliable. NT is not used routinely for dengue diagnosis in laboratories because it is expensive, time consuming and technically difficult (Gubler 1998).

1.5.4 Immuno-Florescence Assay (IFA)

IFA is a simple and reliable method for detection of multiple viruses in patients suffering from multiple serotypes. In mosquitoes monoclonal antibody capture indirect IFA is developed for dengue detection. IFA with serotype specific monoclonal antibodies produced in tissue culture or mouse ascitic fluids and an anti mouse IgG florescence isothynate conjugate is the method of choice for dengue virus identification (Sameul and Tayegi 2006).

1.5.5 Enzyme-Linked Immunosorbent Assays (ELISAs)

In the past few years ELISA has become the most useful and widely used serological test for dengue diagnosis both in humans and mosquitoes due to its high sensitivity, simple procedure and requirement of very little sophisticated equipment. Serotype specific monoclonal antibodies (MABs) capture ELISA and antigen capture ELISA are used for the identification of dengue in mosquitoes. ELISA can detect acute phase IgM, convalescent phase IgG antibodies as well as antigens. ELISA can also be used to differentiate between primary and secondary dengue infections. In primary infection IgM antibody titres are significantly higher than in secondary infection and IgG antibodies are absent. In secondary infection patients have high antibody titres of IgG, IgM production is much lower and transitory and some patients even have secondary infection with no IgM antibodies detected (De Paula et al. 2004, Sa-Ngasang et al. 2006, Velathanthiri et al. 2006).

In Monoclonal antibody capture ELISA (MAC-ELISA) there is no need for convalescent samples as anti-dengue IgM antibodies appears within five days of disease onset. Production of IgM varies considerably among patients. In some patients IgM can be detected by the 2^{nd} to 4^{th} day after disease appearance, while others do not have detectable IgM until the 8^{th} day after disease onset. HI test is more sensitive than MAC-ELISA in paired samples collected during the acute phase of the disease. In monotypic response the specificity of MAC-ELISA is similar to that of HI in primary infections as well as secondary infections. ELISA cannot identify different virus serotypes, due to IgM monotypic response to different virus serotypes. MAC-ELISA acts as a valuable tool for surveillance of

dengue and DHF/DSS. It has the advantage of fast detection of the propagation of transmission (De Paula et al. 2004).

IgG ELISA can be compared with HI test as it can be used for the differentiation of primary and secondary infections of dengue. The test is simple and easy to perform and a great number of samples can be analysed in a short time. Like HI, IgG ELISA is highly sensitive and might be useful in seroepidemiological studies. The major disadvantages of ELISA are it's cross reactivity with other flaviviruses (De Paula 2004) and inability to detect the infection during the early phase of disease (Gurukumar et al. 2009).

1.5.6 Viral Isolation

Dengue virus can be isolated by four methods; intracerebral inoculation of newborn baby mice, inoculation on mammalian cell cultures, intrathoracic inoculation of adult mosquitoes and inoculation of mosquito cell cultures. Isolation of virus in cell cultures or in infant mouse brains remains the gold standard for diagnosis of dengue virus but it is expensive, time consuming, less sensitive and the viruses frequently require multiple passages before inducing cytophatic effects (syncytium formation, presence of multinucleated giant cells) in the infected cells that makes it impractical in most situations (Gubler 1998, Gurukumar et al. 2009).

The most sensitive but least used method for dengue virus isolation is intrathorax inoculation of adult mosquitoes. *Aedes aegypti, Ae.albopictus, Toxorhynchities amboinensis,* and *T. splendens* are used in this method and both sexes of these species are susceptible. In a short period of four to five days dengue virus replicates to high titires depending on the incubation temperature. Detection is made through indirect immunoflorescent assay (IFA) usually done on the brain or salivary gland tissue of infected mosquitoes. The disadvantages of this method are the long time the procedure takes, the need for insectaries for mosquito breeding and experimentaion and the precautions that need to be taken to avoid the release of infected mosquitoes (De Paula et al. 2004).

A modern technique for dengue virus isolation is mosquito cell culture. C6/36 clones of *Aedes albopictus* cells are now widely used for this process. Dengue antigen can be detected by IFA. This technique is less sensitive than intrathoracic inoculation of adult mosquitoes but easy handling of many samples at a time makes it the standard technique for dengue virus isolation. *Aedes albopictus* cell culture has a higher sensitivity than mammalian cell culture, the virus can be grown and maintained at room temperature and virus culture in *Ae. albopictus* cells can be maintained for up to 14 days without changing the medium. The degenerative changes in the cells, associated with the multiplication of dengue virus is difficult to detect in mosquito cell cultures (De Paula et al. 2004).

1.5.7 Molecular Detection

In recent years several new techniques have been developed for dengue virus diagnosis. These methods have proven very useful because of their ability to detect dengue virus readily during the acute phase and occasionally during the convalescent phase.

1.5.7.1 Nucleic Acid Hybridization

In nucleic acid hybridization RNA extracted from infected cell culture supernatants, pools of infected *Aedes* or tissue obtained in autopsies are identified either by hybridizing with biotinylated probes or ^{32}P labelled probes. Generally radioactive labelled probes are used for direct viral identification in clinical samples because this method is more sensitive than biotinylated probes. RNA-RNA hybridization is a sensitive technique and requires fresh samples. This technique has more often been used as a research tool rather than a diagnostic method due to difficulties in working with RNA and the requirement of highly skilled technicians to obtain reproducible results. (Gubler 1998, De Paula et al. 2004)

1.5.7.2 Polymerase Chain Reaction (PCR)

PCR is the most reliable and rapid technique of dengue virus diagnosis. According to the World Health Organization (WHO) PCR is a powerful diagnostic

tool for dengue diagnosis. PCR is reliable when serum samples are taken at the febrile stage (Sa-Ngasang et al. 2005). The different types of PCR used for detection of dengue virus are as follows.

a) Simple PCR

This PCR is performed in two steps. In the first step RNA is extracted from acute phase patient sera or supernatants of virus infected cell cultures. This RNA is reverse transcribed into cDNA by a cyclic reaction in a thermocycler. This cDNA is then subjected to PCR and the product is then sequenced for the detection of Dengue virus. A separate reverse transcriptase step followed by PCR has the advantage that various RNA virus PCRs can be carried out by using the DNA produced as a result of reverse transcriptase activity in the lab (Lindergen et al. 2005).

b) Reverse Transcriptase PCR (RT-PCR)

RT-PCR has revolutionized the laboratory diagnosis of several RNA viruses in the past few years. Linciotti et al. developed RT-PCR in 1992 and it was modified to single tube format by Harris et al. in 1998. This is a rapid, sensitive and simple method used for genome diagnosis in human clinical samples, biopsies and autopsy tissues and mosquitoes. Using whole blood instead of serum or plasma for RNA extraction results in a higher detection rate in RT-PCR thus making the sample handling easy. This method not only identifies the virus but also its serotype by amplifying different regions of the genome. RT-PCR along with IgM and IgG ELISA increases the accuracy and sensitivity of lab diagnosis of dengue virus infection especially in secondary infection. RT-PCR and the virus isolation system using C6/36 cell cultures have similar sensitivity but in RT-PCR poor handling, storage, the presence of an antibody or any other virus usually do not influence the outcome of PCR as they do with virus isolation. RT-PCR is however highly sensitive to amplicon contamination and without proper quality control false positive results may occur (Gubler 1998, De Paula 2004, Sa-Ngasang et al. 2005, Chonticha et al. 2007, Gurukumar et al. 2009).

c) Real time RT-PCR (rtRT-PCR)

Real time RT-PCR is a recent technique used for diagnosis of dengue in humans only. Real time RT-PCR is a simpler, rapid, quantitative, highly specific and easily standardized one step process utilizing a single pair of primers. It has an advantage over traditional PCR based assays in that large numbers of samples can be handled in less than 4 hours. Use of SYBR green dye makes this technique less expensive than RT-PCR. High sensitivity of rtRT-PCR could be useful in early warning of a possible outbreak of the dengue disease. This technique can also quantify virus load in blood samples making it a useful tool for investigating the role for viremia in pathogenesis. (Bai et al. 2008, Santos et al. 2008, Gurukumar et al. 2009).

1.6 PREVENTION AND TREATMENT OF DENGUE

There is no proper treatment or drugs at present for Dengue disease. Drugs such as steroids, antivirals and carbazochrome are not effective against this disease. Non-steroid drugs like asprin should be avoided as it increases the risk of a more severe stage of dengue i.e. dengue Haemorrhagic fever (Gibbons and Vaugn 2009). The only treatment is supportive treatment in which the patient is encouraged to keep oral intake of fluids to prevent dehydration. If the patient is unable to maintain oral intake, supplementation with intravenous fluids may be necessary to prevent dehydration and significant haemoconcentration. A platelet transfusion is rarely indicated if the platelet count significantly drops or if there is significant bleeding but transfusion is only recommended on platelet count falling below 20,000 without bleeding or approximately 50,000 with bleeding (WHO, 1997).

Safe and effective vaccine development for the prevention of dengue is still not a reality though it has been a priority for WHO for decades (Swaminath and Khanna 2003). The major problem in development of a vaccine is the possible immunological response that the vaccine might trigger, which would lead to antibody dependent enhancement and will make the situation further worse exposure to a different serotype. Thus a monovalent vaccine, which induces protection against one-dengue serotypes, could actually increase the risk of developing potentially fatal DHF

and DSS (Kautner et al. 1997, Swaminath and Khanna 2003). Hence a successful dengue vaccine should be tetravalent which can simultaneously induce high levels of long lasting immunity to all four serotypes. The current strategy for producing a tetravalent dengue vaccine is based on producing vaccine formulation by combining all four monovalent vaccine viruses.

At present scientists are working on six different virus based vaccines, four are chimeric recombinant vaccine viruses developed using infectious clone technology and two are traditional tissue based live attenuated vaccines. Mahidol University, Thailand and US Armed Forces at Walter Reed Army Institute of Research (WRAIR) separately have developed the most advanced live attenuated tetravalent vaccine by combining attenuated virus of all four types. The vaccine developed by Mahidol University has successfully completed the phase 2 clinical trials, which showed that it is safe and immunogenic trials and is ready for phase 3 clinical trials conducted in 2012 in Thailand. On the contrary the vaccine developed by US armed forces is in phase 2 trials. Despite the safety features of live attenuated tetravalent dengue vaccine the questions of reversion of the vaccine virus to the virulent form of dengue virus is still there (Gibbons and Vaugn 2009). However the efficiency of both of these vaccines is limited by unbalanced immune response due to viral interference, which tends to skew the immune response predominantly towards one serotype (Swaminath and Khanna 2003). Despite of the efforts researchers have not achieved any success in making dengue vaccine till now (Wiwanitkit 2009).

The risk involved in creating a tetravalent vaccine has shifted the focus of scientists towards the creation of a single recombinant vector system that may permit the creation of a single vaccine vector capable of tetravalent protection. Recombinant vaccine vectors (derived from *Vaccinia* virus) carrying different flavivirus genes have been constructed and trialed on animal models but they appear to be associated with an inconsistent antigen specific immune response. Along with immunological issue, the unavailability of a suitable animal model to evaluate candidate dengue vaccine are the major obstacles in development of dengue vaccine (Swaminath and Khanna 2003).

1.7 Practical Implications of Population Genetics

A dengue vaccine is still under development and there is no proper treatment for this disease. Vector control by using insecticides is less effective due to insecticide resistance (Huber et al. 2002). The only effective dengue prevention technique is to control the vector of this disease (Ravel et al. 2001). To develop new strategies for vector control, knowledge of population genetics can act as a valuable tool. In the absence of an effective vaccine, specific treatment or curative drug, prevention and control strategy of dengue transmission has solely shifted towards the control of vector population. Conventional control methods are based on the use of insecticides, removal of mosquito breeding sites and minimising vector human interaction (Kaunter et al. 1997). The control of adult mosquitoes alone could not help in vector control unless and until the breeding habitats have been destroyed. Vector-human interaction could be minimized by destroying the breeding habitats near the residential areas and by covering the whole body to prevent mosquito bites. The only successful strategy at the moment to control the spread of disease is vector control and minimising the vector human interaction (Gulber 1998). In the Americas the strategy of vector eradication was successful but as soon as the control programme was discontinued or given lower priority dengue re-emerged. Continuous efforts will be required to prevent the vector population from reoccurring. Due to extended use of insecticides in some areas, insecticide resistance is also posing a great problem in controlling the vector population (Kaunter et al. 1997).

The failure of vector control strategies with standard mosquito control programmes due to insecticide resistance and ecological factors have shifted the prospects of WHO towards adopting a new strategy of incorporating population and evolutionary genetic information about the vector into the design of effective control measures. The physiological, ecological and behavioural heterogeneity that exists between different vector populations acts as a logical basis for designing this strategy. This variation between different populations gives each population a separate entity, which could be used in formulating strategies that could yield long-term success. The presence of cryptic species complex in many major vectors has heightened the use of such an approach during the recent decades (Collins 2000). Various studies have confirmed that members of these complexes founds in similar geographical area have

different disease transmission rates, dispersal ability, insecticide resistance, habitat preference and biting behaviour, this information about variation in biological characteristics could be incorporated into vector control programmes: some examples are described below.

The variations discussed above are not only present in subspecies/sibling species but geographical variations have been observed within several species of mosquito including *Aedes aegypti*. Intra-specific variation has been observed for insecticide resistance all over the world in vector mosquitoes (Hemingway and Ranson 2000). This is well illustrated for two vector species in mosquitoes, *Anopheles culicifacies* and *Aedes aegypti*. Insecticide resistance for malathion has been investigated for different species of the *Anopheles culicifacies* species complex. In Srilanka malathion resistance has been reported for *Anopheles culicifacies* species B only but in the neighbouring country of India resistance was observed in two speices B and C (Hemingway and Ranson 2000). When geographical strains of *Aedes aegypti* from Asia (Bangkok and India), Trinidad and Virtudes were compared for insecticide resistance, Asian strains were found susceptible for DDT, malathion and deltamethrine whereas the African strain was resistant to all three classes of insecticides (Mourya et al. 1993). The genetic basis of variation in the behavioural characteristics in *Anopheles arabianses* and *Anopheles gambie* has been evaluated in Nigeria. It was observed that mosquitoes carrying certain inversion karyotypes are more frequent outdoors then indoors due to variation in tolerance to humidity. This change in behaviour results in non uniform exposure of the mosquito population to residual insecticide spray in houses thus explaining the mediocrity of the results of malaria control strategies in African Savana based on house spraying against endophilic vectors (Coluzzi et al. 1979). Intra-specific differences in vectorial capacity can act as a major factor in governing the regional differences in epidemiology of many vector borne disease e.g. significant variation in susceptibility of arboviruses has been observed in several species of mosquitoes belonging to different parts of the world including *Aedes aegypti* (Huber et al. 1999). It is becoming evident that several barrier systems which are under genetic control prevents the infection of various tissues in mosquitoes, give rise to intra-specific variation in vector competence to different extents in these mosquitoes. Such differences are attributed to the absence of dengue outbreaks in Western Africa where yellow fever transmission occurs at a high rate (Failloux et al.

2002). These observations provide evidence that while designing an efficient control program different vector populations should be considered separately with respect to their vectorial capacity and insecticide resistance.

The molecular/genetic markers used in estimating the level of genetic variability in a population depends upon the assumption that they are selectively neutral. Processes such as genetic hitchhiking may result in the linkage of some alleles of putatively neutral markers to genes that are actually under selection. Such alleles at closely linked loci may then be used as indicator of selective processes like the development of resistance to insecticides. In western Kenya the spread of permethrin resistance to a large study area of permethrin impregnated bed nets was determined by using this approach of building in the markers for insecticide resistance genes (Collins et al. 2000). It has also been proposed that genome wide scans should be conducted to look for markers associated by hitchhiking with loci under selection including those responsible for traits of potential relevance to transmission like host attraction, resting behaviour, activity cycles and susceptibility to infection. These markers could then be used in screening of that vector activity in some other area. Therefore, simple genetic assays could provide basic information needed for more efficient vector control without lengthy ecological and behavioural studies. For example, significantly reduced variation at an indicator locus in early evening outdoor biting vector rather than late night indoor biters had provided an early indication of behavioural selection towards the avoidance of a late night control strategy such as insecticide-impregnated bed nets (Collins et al. 2000).

In addition, scientists have also turned their focus to genetically modifying the vectors to alter their ability to transmit pathogens (James 2005). *Aedes aegypti* has been genetically modified to express abundantly antimicrobial immune effector molecules in the haemolymph and attempts are under way to make *Anopheles gambiae* incompetent to malaria. A strain of *Anopheles stephensi* has already been engineered so that it is unable to transmit malaria in mice (Beaty 2000, Morel et al. 2002). Another strain of *Aedes aegypti* has been developed with tetracycline-repressible dominant lethality in both males and females causing death at larval pupal boundary in he absnce of tetracycline only (Phuc et al. 2007). These genetically modified vectors could then be released in natural populations to decrease the vectorial

capacity. However in situations where population bottlenecks occur this could result in changes of allele frequencies and some alleles may disappear totally which could result in the loss of the refractory genes. In such situations the need for the reintroduction of these transgenic mosquito increases and a good understanding of the extent of population structuring, as well as the temporal scale of fluctuations in gene frequency become essential (Collins et al. 2000).

All these facts show that knowledge of the population genetic structure and gene flow between mosquito populations is useful in maintaining effective control strategies. Such information not only gives an insight into vector genetic diversity and vector competence for the pathogen but would also allow discrimination between resistance developement and the reinvasion process after insecticide treatments (Ravel et al. 2001). The amount of genetic exchange between groups not only provides an estimate of gene flow within the mosquito population but also indicates the type of mosquito movement or dispersal. This knowledge of gene flow along with their ability to harbour and transmit a disease is necessary for the development of effective control. In addition, using the population genetic structure information the potential threat of invading populations can be assessed by genetic delineation of vector populations according to geographic regions (Failloux et al. 2002). A knowledge of population genetics could also be used to determine the best period to initiate insecticide treatment and also in making a model that can predict the rate of insecticide resistance development and to study or develop conditions that allow transgenic mosquitoes to spread efficiently to replace the wild type vector thus blocking pathogen transmission (Paupy et al. 2004).

1.8 Genetic Variation in *Ae. aegypti*

Aedes aegypti is systematically a complex species exhibiting extensive behavioural and morphological variation. The polytypic nature of *Aedes aegypti* was formally recognized by Mattingly in 1957 who proposed two subspecies of *Aedes aegypti* on the basis of morphological and geographical distribution. These two subspecies could be easily distinguished on the basis of differences in scale colouration on the first abdominal tergite. The darkly and usually sylvan form *Aedes aegypti formosus* is confined to south of the Sahara Desert and tropical african forest

and breeds in forest habitats and is predominantly zoophilic (preferring to bite non-human animals). The pale to brownish black domestic form *Aedes aegypti aegypti* is found on the costal plains of Africa and outside Africa in the tropics and subtropics and breeds specifically in human generated containers and is strongly anthropophilic (preferring to bite humans) (Tabachnick and Powell 1979, Failloux et al. 2002). Later the sylvan form was also reported from domestic and urban habitats in Africa, South East Asia and from islands in the Indian Ocean (Failloux et al. 2002). Along with colour variation differences in oviposition site preference, host preference behaviour, house entering behaviour, and resistance to desiccation, dengue infection rates (Failloux et al. 2002) and biochemical differences also confirm the polytypic nature of this species (Tabachnick and Powell 1979).

The lighter scale pattern of *Aedes aegypti aegypti* predominates in urban and domestic populations of Africa and all non-African populations show considerable variation in scale colouration within populations making the original distinction between subspecies by Mattingly somewhat doubtful (McClelland 1974). Some authors have suggested a third intermediate peridomestic form that displays wide colour variation and was thought to be a hybrid between domestic and sylvan populations (Trpis and Hausermann 1975). Genetic studies conducted later using isoenzyme loci provided support to Mattingly classification suggesting that at least in East Africa the sylvan and domestic form of *Aedes aegypti* clearly represent two separate sympatric gene pools (Tabchnick and Powell 1979, Fallioux et al. 2002).

Tabachnick and Powell in 1979 proposed the sylvan form as the ancestor of *Aedes aegypti*. The *Stegomyia* subgenus has a total of 34 African species and only one species is found to occur outside the Ethiopian region which has domesticated itself and is preferring to breed in mans drinking water. The possibility exist that evolution of traits allowing this mosquito to breed in man made habitats have facilitated its spread through out the tropical world. According to this model of the evolution of *Aedes aegypit* the expansion of Sahara Desert towards North Africa converted them to arid areas, leading to intense selection pressure on behavioural traits for adapting to man made containers. Eventually the newly evolving domesticated form along the North cost of Africa was isolated by geographical barrier in the form of the Sahara Desert from the ancestral form in south of Sahara (Tabachnick and Powell 1979) and

progressively differentiated into *Aedes aegypti aegypti*. The spread of this mosquito to the urban areas of Africa and rest of the world is quiet recent and human trading activity has played an important role in this spread. Development of the shipping industry and the African slave trade has played an important role in the spread of the mosquito outside Africa where it has become the predominant *Aedes (Stegomyia)* species. The geographical distribution of this mosquito expanded to the Pacific Islands during the 2nd World War along with the movement of troops (Failloux et al. 2002). With the passage of time these introductions have led to the evolution of genetically different geographical strains of this mosquito in different parts of the world.

A study using variation at 11 isoenzyme loci suggested the presence of 8 genetically different geographical groups (Wallis et al. 1983). These variants include East Africa aegypti, East Africa formosus, West Africa, Asia, South-Eastern United states, South Western United States with Mexico, South and Central America with Trinidad and the Caribbean groups. This study was not only in agreement with previous studies regarding the genetic differentiation between East African aegypti and East African formosus. Some interesting facts were revealed in this investigation that East African sylvan group was genetically different from West African *Aegypti* and also that the domestic type *Aedes aegypti aegypti* is totally absent in West Africa. This finding goes against the popular belief of most of the entomologists and epidemiologists that the New World got its domestic form from West Africa. Asia and South Eastern United states groups are more closely related to sylvan forms whereas the New World populations show more genetic affinity to domestic form of East Africa (Wallis et al. 1983). The population of *Aedes aegypti* of Asia is clearly different from all the other geographical strains, which were also reflected in vectorial capacity. The asian strain is unable to transmit yellow fever virus in contrast to populations that occur outside Asia (Aitken et al. 1977). Historical evidence of the absence of yellow fever epidemic in Asia also supports the fact that the Asian strain is not an efficient vector of yellow fever. The genetic isolation of Asian *aegypti* from other domestic groups indicates its probable spread from East Africa to Asia and the low heterozygoisty at isoenzyme loci is attributed to its recent introduction to Asia (Tabachnick and Powell 1979, Wallis et al. 1983).

In another study using isoenzyme markers three major clusters of *Aedes aegypti* were identified. One of the group is formed by the *Aedes aegypti formosus* from West Africa and some islands in the Indian ocean and the other two clusters are formed by *Aedes aegypti aegypti*, one cluster that of Southeast Asia and South America and second group from the South Pacific Islands. This clustering was based on sampling from Africa, Southeast Asian and some islands of Inidan Ocean, South Pacific Islands and South America. This study also showed that the populations of sylvan form have evolved in greater isolation from one another than the populations of domestic aegypti form because of role of human in spreading the domestic aegypti mosquito and shaping its recent evolution (Failloux et al. 2002). The use of ND4 (NADH dehydrogenase subunit 4) sequence in *Aedes aegypti* identified two different mitochondrial lineages in South America [i.e in Mexico (Gorrochotegui-Escalanta et al. 2000), Peru (Costa-da-Silva et al. 2005) and Brazil (Scarpassa et al. 2008)] and Asia [i.e in Thailand (Bosio et al. 2005) and Southeast Asia (Hlaing et al. 2009)] separately and also in a much wider study using samples from Americas (Brazil, Peru, USA, Guatemala, Venezuela), Asia (Tahiti, Cambodia, Singapore) and Africa (Guinea, Senegal, Uganda), which probably reflects the existence of historic, distinct matriarchal lineage within the species that probably arose through prolonged, historical separation between the populations (Gorrochotegui-Escalante et al. 2000). Further more the presence of these lineages in different regions support the hypothesis that they may have evolved before the exporting of *Aedes aegypti* from its presumable ancestral source in North Africa. This hypothesis still needs to be confirmed by testing samples from other parts of the world especially from Africa (Bosio et al. 2005).

In a more recent study using mosquitoes collected from 13 countries on five continents and then clustering them by Bayesian method using microsatellite loci also support the existence of two divergent groups in *Aedes aegypti*, furthermore high genetic difference between pan tropical non-African populations and African populations correspond with previous descriptions of subspecies *Aedes ae. aegypti* and *Aedes ae. formosus* based on geography and genetics. The overall lack of mixing between the two subspecies further supports the distinction between them. The within population genetic diversity observed in this study also support the previous hypothesis that a founding non-African pantropical populations split allotropically and spread across the globe from ancestral African *Ae. aegypti*. This study also makes it

clear that genetically cohesive *Ae. ae. formosus* is much more ecologically variable than had been suggested traditionally as shown by the clustering of all urban populations in West Africa in *Ae. ae. formosus*, suggesting independent invasions of human habitats by *Ae. ae. formosus* across the Africa. This evidence also supports the view that sympatric or near sympatric breeding of both species in West Africa is not a strict analogue of the situation in coastal east Africa, where the two species are very different from the genetic perspective (Brown et al. 2011).

Some mosquito populations with similar behavioural, morphological and ecological characteristics are genetically similar irrespective of geographical distance e.g. domestic *Aedes aegypti* of East Africa are genetically more related to New World populations rather then sylvan population that are geographically very near. In contrast, West African formosus shows greater genetic affinity to the East African Sylvan population then to all other domestic populations that occur throughout the world (Tachnick and Powell 1979). In the absence of such variation in characteristics mosquito populations remain genetically uniform over 100 kilometres (Wallis et al. 1984, Apostol et al. 1996) although it varies from 90 to 250 kilometres but are likely to differ at distance more than 250 km because of change in the allele frequency and types of alleles (Gorrochotegui-Escalante et al. 2000). However exceptions may occur with high genetic differentiation within a few kilometres as observed in Brazil (Ayres et al. 2003). The intensive use of insecticides might establish a cycle of extinction and recolonization of *Aedes aegypti* in Brazil, as a consequence of genetic drift, increase the genetic difference between populations resulting in highly structured population as compared with other parts of the world (Ayres et al. 2003). Such observations show that geographical distances could not be considered as a good indicator of genetic difference between populations. Distances compatible with species migration distance can contribute a lot towards reducing the variation by gene flow between populations. Along with the rate and mode of gene flow other parameters like the spatial distribution of population, the size of the population and the process of extinction and recolonisation can significantly affect the distribution of genetic variability (Huber et al. 2002).

The availability of oviposition sites also contributes a lot towards shaping the population strucuture of mosquitoes at a local level (Apostol et al. 1994). *Aedes*

aegypti dispersal is to some extent driven by oviposition behaviour and this behaviour varies with availability of ovipositon sites (Edman et al. 1998). Therefore, the number and proximity of breeding sites and dense human population contribute to genetic differentiation by limiting mosquito dispersal (Huber et al. 2002). This was observed in Ho Chi Minh City, Vietnam where population differentiation was found in both the city centre and the outskirts. Two studies using different genetic markers (Huber et al. 2002, Tien et al. 1999) showed that availability of oviposition sites could act as an important factor in shaping the genetic structure of *Aedes aegypti* in Ho Chi Minh City, Vietnam. Populations in the city centre show less differentiation because of abundant larval ovipositon sites and dense human populations for adults to feed upon. In contrast, the water storage activity in the outskirts as a consequence of a lack of piped water tends to limit mosquito dispersal due to non-availability of water storage container acting as larval breeding sites and low human population size to feed upon. This results in an increase of genetic differentiation indicating the lack of gene flow to reduce the genetic differentiation at the corresponding distances (Huber et al. 2002).

Human activities and environmental factors play an important role in shaping mosquito population differentiation (Paupy et al. 2004). In the outskirts of Phnom Penh city human activities like the storage of water, subjected the mosquito populations to important extinction/recolonization phenomenon, which along with genetic drift increase the genetic differentiation among mosquito populations in the outskirts of Phnom Penh city (Paupy et al. 2004). Dispersal is inversely correlated with the availability of oviposition sites. In the dry season, temporary breeding sites/containers dry out, as a result population density of mosquito decreases. This drying out of oviposition disperses the female further to find suitable sites to lay eggs, decreasing the level of differentiation among the mosquito populations. This phenomenon was suggested to be responsible for the increase in differentiation among mosquitoes collected during the rainy season in the city of Rio de Janerio compared to higher genetic homogeneity in the dry season (Costa Riberio et al. 2006).

Dispersal of *Aedes aegypti* either occurs naturally through adult flight, although they have a short flight ranges of about 10-800 metres (Huber et al. 2004),

or passively though the transport of eggs, larvae and adults. Long distance movement although not as common probably arises by the movement of desiccated eggs, larvae or both moved in containers like discarded bottles, cans, appliances, tires and cargo containers along trade routes (Gorrochotegui-Escalante et al. 2000, Endersby et al. 2009). This human mediated dispersal of mosquito through goods favours population admixture over large spatial distances (Paupy et al. 2008). This phenomenon was detected by using microsatellite and AFLP markers that showed the unintentional movement of *Aedes aegypti* from Guaymas to Hermosillo in Mexico, by high truck traffic between the two towns (Ravel et al. 2001). Lower genetic differentiation between samples from Vietnam and Cambodia that are separated by hundreds of kilometres was attributed to genetic exchange by accidental transportation of eggs larvae and adult mosquitoes through human transportation (Huber et al. 2004). The weaker relationship of genetic distance and geographical distance in mosquitoes from Australia (Endersby et al. 2009) and North Eastern region of Argentina (Duenas et al. 2009) was also attributed to passive movement of aquatic stages of *Aedes aegypti* in containers moved by humans. Similarly the recolonization of *Aedes aegypti* in Uruguay was though to have taken place via passive movement of eggs and larvae from Argentina and Brazil (Soliani et al. 2010). In Southern Thailand genetic similarity was found among distant locations connected by major roads suggesting human transportation routes have resulted in passive long distance migration of *Aedes aegypti* (Hlaing et al. 2010). According to international sanitary regulations mosquito control in harbours and airports is obligatory in endemic areas as well as for the transport carrier from infected countries (OMS, 1973).

Estimating mosquito movement could give information on disease spread (Huber et al. 2000). Genetic differentiation of *Aedes aegypti* was observed in Chiang Mai, Thailand with characteristic high values of infection rates, that were not similar all over the geographical area under study (Mousson et al. 2002). In Ho Chi Minh City the *Aedes aegypti* population in the city centre were genetically differentiated and their infection rates to dengue virus 2 differed from those of the populations in the commuter belt (Tien et al. 1999). Multiple introductions of the vector have an immense epidemiological impact on dengue, because gene flow may assist the spread of transmission related genes thus enhancing vector competence.

This phenomenon was thought to be responsible for the first outbreak of dengue in Brazil in the city of Boa Vista, the gateway city of Brazil to countries of northern South America. It was also observed that *Aedes aegypti* from this city had the highest susceptibility to DENV2 as compared to collections from other places of Brazil. Similarly multiple introductions were thought to be responsible for the large number of DHF cases in Manaus, Brazil (Scarpassa et al. 2008).

Although, all studies conducted on *Aedes aegypti* in different geographical regions reported significant difference among the populations, the overall genetic differentiation was reported to be low. The direct relationship between genetic distance and evolving time suggests that the divergence between *Aedes aegypti* populations is fairly recent (Fraga et al. 2003). These studies also showed that the efficiency of a vector population to transmit a disease depends upon the geographical origin of the population (Fallioux et al. 2002) e.g. the sylvan formosus form show low infection rates to dengue virus as compared to *Aedes ae. aegypti*. Further, the vectorial efficiency of mosquitoes from Southeast Asia and French Guinea were transferring dengue more efficiently than Pacific Islands. Although infection rates found in lab grown stains do not exactly represent the efficiency with which natural mosquito populations transmit the disease, they are thought to give a good estimate of the transmission rate of dengue viruses in terms of the salivary gland infection (Gubler and Rosen 1976).

The spread of non-malarial mosquito borne tropical diseases like dengue has shifted the attention of research workers to investigate the vector dynamics of dengue in affected areas. A lot of work has been carried out in the last two decades to understand the biology, ecology and population structure of *Aedes aegypti*. Keeping in view all this, a study was designed to investigate the population structure of this mosquito in Pakistan using microsatellite markers.

1.9 Microsatellite As Markers For The Study Of Population Genetic

Microsatellites are simple-sequence repeats (SSRs) of one to six nucleotide motifs present in the non-coding region of DNA in a wide variety of eukaryotes of varying complexity and genome size. They have become the marker of choice in

many studies because of their high level of polymorphism, co-dominant inheritance, ease and reliability of scoring alleles and short length which make them useful for studies of DNA from hair or faeces collected in the field and from fossils (Luikart and England 1999, Meglecz et al. 2006, Lovin et al. 2009). At first microsatellites were regarded as sequences of no particular interest but with the rise of PCR they were realized to be the most powerful Mendelian marker ever found whose molecular structure and mutation rate information is relatively easy to retrieve. The allele size of a microsatellite at a locus may be known with an accuracy of one base pair and they are characterized by their number of repeats at the locus analyzed (Jarne and Lagoda 1996). The mechanism underlying microsatellite allelic variation in populations seems to be related to their mutation rate, which varies between 10^{-4} and 5.10^{-6} (Huber et al. 1999). Slipped-Strand Mispairing generates new length alleles during DNA replication in mitosis and meiosis causing the number of microsatellite repeats to vary at a locus. This results in a continuously different repeat number among individuals within species (Fragerberg et al. 2001).

Microsatellite genome wide abundance varies in different taxa of organisms. In some organisms, especially insects, a large proportion of microsatellites group into sequenced families based on similarity in flanking regions which along with a relatively low abundance of microsatellites make it difficult to isolate usable microsatellites from the genome (Meglecz et al. 2007). The PCR reaction analysis of microsatellite is based on the assumption that the flanking sequence of each microsatellite locus is unique and mutates much more slowly than the microsatellite repeat motif. The use of primers developed for one species in other related taxa, is potentially valuable by saving time and effort in searching for microsatellite markers. Potential problems in using microsatellite marker are the occurrence of null alleles. Null alleles occur due to mutation in the primer attachment sites of flanking region resulting in mistyping of heterozygote individuals as homozygotes affecting population genetic structure analysis (Bruford and Wayne 1993). Null alleles may be detected in the population by testing against frequencies that are expected under the HW equilibrium provided that occurance of heterozygote deficiencies has no other reason like mating system (Jarne and Lagoda 1996).

Microsatellites have been successfully applied to parentage and relatedness testing in many species including humans, proving especially valuable where non-invasive sampling methods are necessary (Bruford and Wayne 1993). Gene flow and genetic distances between populations can be estimated through analysis of allelic richness and calculation of heterozygosity (Xiao-Gu et al. 2006). These markers have been successfully used for population genetic studies of social insects like ants and in insect vectors of medical importance like mosquitoes and *Glossina* (Tse-tse fly) (Ravel et al. 2001). Microsatellites are abundant in the genomes of Anopheline mosquitoes but not so common in *Aedes aegypti* (Fragerberg et al. 2001). Along with *Aedes aegypti* microsatellites have been successfully isolated in mosquito species of medical importance like *Anopheles gambiae* (Zheng et al. 1993), *An. maculatus* (Rongnoparut et al. 1996), and *An. stephensi* (Verardi et al. 2002).

Isoenzymes studies have been reported for more than 40 years aimed at studying the population structure of *Aedes aegypti*. More recently microsatellite markers have been used to study genetic variation or to detect gene flow (Costa-Ribeiro et al. 2006). Microsatellites have been proven to be a promising molecular tool for the study of genetic variability. They can be helpful for analyses of information on distance, direction and rate of dispersal of genes of *Aedes aegypti* populations and this knowledge can be used to develop vector control strategies (Huber et al. 1999). In *Aedes aegypti* microsatellite loci are not abundant or are defiant to common methods of identification. These methods include examining expressed gene coding sequences, construction and screening of microsatellite enriched genomic library and oligonucleotide based screening of selected cosmid genomic clones. The combined effect of these studies were still disappointing resulting in only 40 useful microsatellite marker loci several of which have low polymorphism. The close association with repetitive elements as opposed to the microsatellite frequency in the *Aedes aegypti* genome was suggested to be one of the causes for the low yield of microsatellites (Lovin et al. 2009).

Initially the expense and time required to obtain microsatellite markers from plasmid libraries were a major hindrance to their development. The development of new techniques, increasing the proportion of microsatellite sequences in a genomic

library by hybridizing oligonucleotide probes bound to magnetic particles in solution with complementary repeated sequences, has proved more efficient along with decreasing the risk of analyzing false positive results. This new technique of enriching a conventional plasmid library in association with PCR screening of positive clones has enabled the isolation of 4 microsatellite loci from a library of 120 recombinant clones, which previously proved unsuccessful from a conventional library (Huber et al. 1999). This was the first report of developing usable microsatellite loci in *Aedes aegypti* but no information about the polymorphism of these two loci or their use on populations of *Aedes aegypti* is available. In the same year three more microsatellite markers were identified by screening the sequences obtained from the Gene Bank. These three loci were tested for allele diversity and observed heterozygosity by studying six populations from different areas of Asia, the Carribean and Africa. Although there were only 50 individuals the number of allele observed were 3, 5 and 6 respectively for these loci, seeming sufficient enough to allow population studies (Barbazan et al. 1999). Both these two sets of primers designed for microsatellite loci have never been applied to genetic studies until now.

In another study microsatellite repeat sequences were searched for within the available data banks of *Aedes aegypti* and five microsatellite loci were observed including the two already reported by Barbazan et al. (1999). Two of the loci were present in the coding region but due to the report of significant polymorphism in the coding region despite the assumed selective pressure on such sequences, these were also included in the analysis. Primers were designed for both new and old loci and were used in two different population genetics studies (Ravel et al. 2001, Ravel et al. 2002). In one of the studies only three of these loci, one new and two old isolated by Barbazan et al. (1999) were used along with AFLP to analyse genetic variability and the reinvasion of *Aedes aegypti* in Mexico. Collections were made from two districts each of two cities, Guaymas and Hermosillo, separated by 120 km but connected by train and a main road. In this study low polymorphism was observed in mosquitoes from Mexico as compared to Cameroon (Barbazan et al. 1999) and the Ivory Coast (Ravel et al. 2002). These three microsatellites were not able to distinguish between mosquitoes collected from Hermosillo and Guaymas but analysing the data on the basis of districts within cities showed genetic differentiation between the mosquitoes collected from the two districts of Hermosillo. Heterozygote observed for one of the

loci were higher in the northern district of Hermosillo as compare to the other three districts. This was attributed to a greater mixing of mosquito populations in that district. Overall the study suggested that the southern district of Hermosillo vector populations might be genetically closer to those of Guaymas or be introduced from the city probably as a result of high truck traffic between the two cities (Ravel et al. 2001).

The same set of all five marker isolated by Ravel et al. (2001) were used for geographical and temporal analysis of genetic variability in *Aedes aegypti* from the Ivory Coast. Two sets of samples were collected from four different areas i.e. Kabolo, Bouake, Abidjan Plateau and Abidjan Port Bouel at a distance of one year apart. High polymorphism was observed at two loci as compared to *Aedes aegypti* collected from Cameroon and Mexico. Although these markers were less polymorphic but they revealed the existence of genetically distinct populations of *Aedes aegypti* along the north south transect of the Ivory Coast with no isolation by distance. Furthermore, the population of Kabolo was found to be genetically distinct from the other three populations. Genetic differentiation at the microgeographic scale was observed in Bouaka where dramatic changes in the structuring of vector populations had been observed over time resulting into two genetically distinct groups of mosquitoes. No possible explanation was found for this variability. Insecticide resistance had been excluded because of the absence of intense insecticide treatment in the area during this period and no information was available regarding the breeding of the different groups (Ravel et al. 2002). This was the first study to assess the ability of microsatellite loci to discriminate between populations on a microgeographic scale. This could be of great importance in studies on the molecular taxonomy and the epidemiological importance of *Aedes aegypti* (Ravel et al. 2002).

The non-availability of the genome sequence of *Aedes aegypti* was a major hindrance in isolating microsatellite loci. To overcome this difficulty Huber et al. (2001) constructed a $(CAA)_n$ enriched partial genome library of 5000 recombinant clones of *Aedes aegypti*. Out of these 172 positive clones were detected and sequenced. Fifty seven of these clones were found to contain one or more microsatellite sequences among which only four were finally selected and appropriate primers were designed for them. Four more loci selected from other sequence databases were also assessed in this study. Six of these loci (two from the recombinant

clone library and four from other sequence databases) give satisfactory amplification results with polymorphism range from 5 to 14 alleles at each locus and follow Mendelian inheritance (Huber et al. 2001). These loci were later on used in different studies to investigate the genetic differentiation and gene flow among mosquitoes in a number of geographical areas.

Huber et al. (2002) used the previously isolated six-microsatellite loci not only to define the pattern of genetic differentiation of *Aedes aegypti* population in Ho Chi Minh City, Vietnam but also to evaluate the potential of microsatellite markers for the study of the population genetics of *Aedes aegypti*. Mosquitoes were collected from the city centre and outskirts and checked for variation using microsatellite loci. Mosquito populations from the city centre were less differentiated as compared to the outskirts where substantial differentiation was observed among *Aedes aegypti* populations suggesting that that the population is panmictic in central Ho Chi Minh City due to the availability of larval breeding sites. Larger genetic differences due to the process of genetic drift in the outskirts indicated that the gene flow was not high enough due to water storage practices and lack of piped water supply to decrease the genetic differentiation at the corresponding distances. No significant correlation was found between genetic and geographic distances either in the city centre or in the outskirts. This study showed that human activities associated with urbanization, type, density and the location of breeding sites as well as the human density in the urban areas could play an important role in shaping the genetic structure of *Aedes aegypti* population. Further more, this study also proves that microsatellites could act as an important tool in investigating population genetic structure of *Aedes aegypti*.

Estimating the population genetic structure and gene flow of *Aedes aegypti* could provide an insight to dengue epidemiology. The role of vector in the changing pattern of dengue in Southeast Asia had been assessed by evaluating the genetic differentiation of *Aedes aegypti* collected from Ho Chi Minh City (Vietnam), Phnom Penh (Cambodia) and Chiang Mai (Thailand) using microsatellite markers isolated by Huber et al. (2001). This study showed that the mosquito population from Phnom Penh and Ho Chi Minh City were genetically less differentiated than from either of them and Chiang Mai. Phnom Penh and Ho Chi Minh City are 250 Km apart from each other but passive dispersal through human transportation in the form of direct

flight and much flow of surface traffic probably result in gene flow and decrease in genetic differentiation. In contrast this phenomenon is absent between either of the two cities and Chiang Mai making it genetically different from the mosquitoes of these two cities. This passive dispersal could favour the movement of dengue infected eggs and larvae thus spreading the disease and potentially resistance to insecticide treatment (Huber et al. 2004). This showed the importance of microsatellite markers in retrieving information regarding gene flow and movement of mosquitoes. This information could in turn be used in planning effective and efficient mosquito control to stop the spread of vector borne diseases like dengue.

Paupy et al. (2004) compared the amount of variation and pattern of gene flow detected by AFLP with isoenzyme and microsatellite markers. The Fst value was three to four folds higher in the case of AFLP markers but still these three different markers reveal the same population structure having significant variation among populations of six sampled sites in Phnom Penh (Combodia). The amount of variation and pattern of gene flow at local levels using microsatellite and isoenzyme markers was analysed and compared by Costa-Riberio et al. (2006). Mosquitoes were collected from five districts of Rio de Janerio. Samples were divided into city centre and the outskirts on the basis of the density of inhabitants. Five microsatellites isolated by Huber et al. (2001) and six isoenzyme were used for genetic analysis. Microsatellite markers were more polymorphic as compared to isoenzymes therefore they were able to differentiate within the city centre whereas isoenzyme markers revealed genetic differentiation among the samples from the outskirts spread on a larger geographical scale. This study demonstrated that microsatellites markers are better for studying variation within a small geographic scale like a city than isoenzyme analysis.

The availability of the *Aedes aegypti* genome sequence and the development of techniques to enrich genomic DNA for microsatellite motifs have improved the isolation of new microsatellite loci. Chambers et al. (2007) isolated and identified 11 new microsatellite sequences from multiple genomic libraries for *Aedes aegypti*. Six single copy simple microsatellites from 3 plasmid libraries enriched for $(GA)_n$, $(AAT)_n$ and $(TAGA)_n$ motifs and five single-copy microsatellites from cosmid libraries of *Ae. aegypti* were identified. In the beginning 30 unique sequence clones containing a microsatellite were selected from the plasmid library developed from genomic DNA

enriched for the repeat motifs. However PCR primers for unique sequences flanking the microsatellite were prepared for only 12 of these sequences because of the difficulty in designing quality primers for the remaining sequences. On testing these sequences for amplification four of the primers pairs amplified multiple PCR products indicating that they likely recognize repetitive sequences. Seven of the remaining eight microsatellite were single locus markers and six have three or more than three alleles. One has only two alleles, which makes it less important in genetic studies. One of the six loci has a perfect dinucleotide repeat, 2 have imperfect dinucleotide repeats, 3 have perfect trinucleotide repeats and 1 has a perfect tetranucleotide repeat. The *Aedes aegypti* cosmid library was also screened for repeat motifs and primers were designed for nine microsatellite sequences out of which only five were single locus, polymorphic and have a trinucleotide repeat motif. Overall 11 microsatellite loci were isolated, six from the developed plasmid library and 5 from the available cosmid library (Chambers et al. 2007)

The availability of a partial *Aedes aegypti* genome in 2005 provided an opportunity to uncover 13 more polymorphic microsatellite loci. The partial genome was screened for six dinucleotide repeats; $(AT)_n$, $(CT)_n$, $(GT)_n$, $(AC)_n$, $(AG)_n$, and $(GC)_n$. Thirty three microsatellites were selected on the basis of having at least eight uninterrupted repeats and not having a string of single nucleotides next to the repeat. Primers were designed for only 17 loci, which were examined for variability by using mosquitoes collected from Thailand and Kenya. Amplification of two loci was poor and inconsistent at different annealing temperatures and two loci failed to amplify consistently in laboratory reared mosquitoes so were excluded from the analysis. The number of alleles observed for these loci raged from 2 to 10. Thus the addition of these 13 highly polymorphic loci to *Aedes aegypti* would greatly facilitate a wide variety of studies of this important vector (Slotman et al. 2007).

The success of vector control method depends on the population structure of the focal species along with its population size and the rate of movement of individuals among populations. The release of transgenic mosquitoes could target those populations that show a high degree of isolation from neighbouring populations since this would allow the modified individuals to become established with ultimate dispersal to other regions (Endersby et al. 2009). Microsatellite markers were used to

assess the population structure of *Aedes aegypti* in Australia and its mixing with neighbouring population of Vietnam and Thailand in order to evaluate the success of releasing modified individuals containing *Wolbachia*. Six microsatellite markers were used in this study, two from the Slotman et al. (2007) primers, three from Chambers et al. (2007) and one was designed for this study by screening the *Aedes aegypti* genome. Analysis showed that populations from these three regions were genetically significantly different from each other and all these populations were isolated by distance from each other. Isolation by distance was also observed within mosquitoes collected from Australia, although this correlation was weak. These analyses suggest that very limited dispersal occurs within the regions and the weak correlation within Australia is because of the role of human in passive dispersal of this mosquito throughout the region. Lower allelic richness in Australia also showed that *Aedes aegypti* might have gone through a bottleneck during colonization. The study concluded that a large number of modified individuals would be required to be released in the Australian region to establish them in natural populations. Alternatively the number and size of the target population should be reduced through insecticide treatments especially in the dry season to remove the desiccated eggs before establishing new variants. These two strategies could help in establishing the population of modified vector mosquitoes and could help in control of this mosquito in that area (Endersby et al. 2009).

In another study Slotman et al. (2007) set of markers were used in estimating the hierarchical population structure of mosquitoes collected on large spatial scales of more than 2000km in Southeast Asia. Mosquitoes were collected from mainland Southeast Asia (Myanmar, Thailand and Cambodia) and also from Sri Lanka and Nigeria. They showed that low but significant genetic structuring was observed at all spatial scale even within 500 meters. It was also revealed that spatial distance was not the only factor shaping large scale population structure but genetic heterogeneity and genetic uniformity between distant locations connected by major roads suggested the role of human transportation in long distance passive dispersal of *Aedes aegypti* in the region. The relative strength of this passive dispersal could greatly affect the vector control in those areas. The restricted movement on small geographical area can make localized control efforts and transgenic insect technology effective for disease control

but prevention of the establishment of insecticide resistance genes or spreading genes of interest in using transgenic technology could be challenging (Hlaing et al. 2010).

It is also important to understand the process of colonization and adaptation of disease-vectoring arthropods to human habitats. *Aedes aegypti* probably originated as a wild, zoophilic species in Sub-Saharn Africa, where it can still be found in sylvan habitats. Some populations within Africa and almost all populations outside Africa have domesticated themselves to human environments and become anthropophilic. It was not clear whether all domestic populations are genetically related and represent a single domestication event or whether the association with human habitats has evolved multiple times independently. This hypothesis was tested by using 12 microsatellite loci, including eight previously reported markers by Slotman et al. (2007) and four new markers developed by screening the *Aedes aegypti* genome library for 8-20 uninterrupted trinucleotide repeats without having sequences in either of the flanking region. 24 worldwide populations were screened for genetic variability at these 12 loci. Two distinct genetic clusters, one including both domestic and sylvan populations of *Aedes aegypti* within Africa and the other including all domestic populations outside Africa were indentified. This showed that human association of *Aedes aegypti* occurred independently in Africa than than in domestic populations across the rest of the world. It also suggested African *aegypti* is the ancestral form (Brown et al. 2011).

In this study the population genetic structure of *Aedes aegytpi* in Pakistan was investigated by genotyping mosquitoes collected from Pakistan using thirteen microsatellite loci isolated by Slotman et al. (2007). The Slotman microsatellites were used because they are highly polymorphic and consist of perfect dinucleotide repeats whose alleles could easily be recognized while genotyping.

1.10 Vertical Transmission of Dengue Virus in *Aedes aegypti*

Dengue displays a seasonal pattern related to temperature and rainfall in most endemic countries. The question of how dengue overwinters or survives during dry or cold season has always puzzled investigators. One possibility could be the survival of infected mosquitoes throughout and the subsequent re-introduction of the virus in the next season. *Aedes* mosquitoes remain infected for life and the

longest life span recorded to date is 174 days, although the normal survival rate is only 1-2 weaks (Kyle and Harris, 2008). Another possibility is venereal and vertical transmission i.e. transfer of virus from male to female during copulation and transfer of virus from the infected mosquito to its progeny respectively (Hutamai et al. 2007). Vertical and venereal modes of transmission may not only play an important role in the persistence of this arbovirus in nature (Rosen 1987) but also are important in the persistence of dengue during dry periods and when the invertebrate host is not available during cold periods (Cecillo et al. 2009).

Transovarially infected mosquitoes can transmit the virus orally and are fully capable of initiating Human-Mosquito-Human transmission cycles and once a mosquito is infected with virus, the virus could be transferred transovarially up to the 5^{th} generation (Rohani et al. 2008) although some studies have suggested that this is possible up to the 7^{th} generation (Mouraya et al. 2002). The natural history of dengue virus also suggests that biologically these viruses are highly adapted to their mosquito host and prior to their adaptation to humans and lower primates dengue was most likely a mosquito virus (Joshi et al. 2002). In the past few decades modern means of transportation has increased the movement of people and commodities between regions of the world leading to increased movement of both mosquito and dengue, resulting in a dramatic increase of the global prevalence of dengue. The expanding geographic distribution of the mosquito vector also results in the spread of dengue. The recent geographic spread of *Aedes albopictus* has been accredited to the used tyre trade so the virus as well was transported in desiccated eggs to other regions of the world as the virus could replicate in the diapausing eggs (Guo et al. 2007). So if a mosquito gets an opportunity to feed on a viremic host prior to summer and lays eggs in these micro niches and if these eggs survive through the inter epidemic period, the emerging adult mosquitoes may initiate the interspecific transmission cycle (Joshi et al. 2002). This could be a possible reason for the occurrence of a dengue epidemic when a vector is introduced into previously dengue free area.

Transovarial transmission of dengue had been observed experimentally in *Aedes* species during early 1980s (Khin and Than 1983). The ecology and mechanism of involvement of vertical transmission of dengue virus in disease outbreaks remain

unknown due to inadequacy of data (Lee and Rohani 2005) but different studies had been conducted to explain its occurrence in nature. The possibility that not only the oral secretions, but alimentary canal and genital organs of dengue infected mosquitoes might contain the virus capable of infecting the other mosquitoes was investigated by conducting a series of experiments on *Ae. aegypti*, which gave the first evidence of transfer of dengue virus from mosquito to mosquito by copulation. This transfer takes place either directly or after some time when the virus matures in the males. These experiments suggested that transmission of dengue virus by copulation is possible, though with great difficulty, so it occurs in nature rarely and is regarded as an unimportant and improbable means of virus persistence in mosquitoes. This was the first report of mosquito-to-mosquito venereal transmission of dengue (Simons et al. 1931). In a similar study male *Aedes albopictus* were experimentally infected with dengue virus types 1,2,3 or 4. These infected male mosquitoes had transmitted their infection to females and such transmission was enhanced when the female had taken a blood meal 2 to 7 days prior to mating. Dengue can also be transmitted from an infected male to its progeny as infected progeny were found among those derived from eggs laid greater than or equal to 3 days after mating but not among those individuals derived from eggs laid prior to that time. It was suggested that probably the virus was not transmitted directly to the ova; rather the virus first underwent prior replication in the female genital tract. No infection was found among males mated with experimentally infected females supporting the hypothesis that male mosquitoes acquire their natural infection vertically rather than sexually from infected females (Rosen 1987). Previous studies (Watts et al. 1973, Aitken et al. 1979, Tesh and Cornet 1981; cited in Roen 1987) suggested that in nature arboviruses transmits transovarially in mosquito but the study carried out by Rosen suggested that this is not the only mechanism true for dengue virus. Dengue infection can also be transferred to the next generation when a fully developed egg enclosed in chorion is fertilized at the time of oviposition, enabling the virus to continue infecting the progeny following a single maternal blood meal. The entry of virus into the egg enclosed in thick chorion takes place via micropyle, a specialized apparatus present at the anterior end of the egg, which allows the spermatozoa to cross the thick impervious chorion and reach the ovum at the time of fertilization. This phenomenon explains the vertical transmission of virus after the first blood meal when the virus has not infected the surrounding

ovarian tissue, once it infects the ovary then the transmission may be transovarial (Rosen 1987).

Transovarial transmission of all four serotypes of dengue had been observed experimentally in *Aedes albopictus* in the early 1980s but at that time its occurrence remained doubtful and it also remained a mystery, how the virus persisted during the interepidemic period (Khin and Than 1983). At that time direct fluorescent Antibody test (DFAT), indirect fluorescence using type specific dengue monoclonal antibody test and complement fixation test were used on field-collected larvae to investigate the occurrence of vertical transmission in nature. In one of the studies mosquitoes were grouped in pools and checked for dengue antigen by DFAT and then typed by complement fixation test for detection of dengue 2 virus in larvae collected from the field in Rangoon Burma. Though the minimum infection rate was low (1:2,067 and 1:3865), it still showed that transovarial transmission of dengue virus occurs in nature (Khin and Than 1983). The same tests for detection of dengue virus failed to detect natural transovarial transmission of dengue virus in *Aedes aegypti* and *Aedes albopictus* in Thailand (Watts et al. 1985). Type specific monoclonal antibodies were used for detection of vertical transmission of dengue 4 virus in Trinidad, which further confirmed the occurrence of this phenomenon in nature (Hull et al. 1984).

Fauran et al. (1990) carried out a study on vertical transmission of dengue virus in the South Pacific after the dengue outbreak. Larvae and pupae of *Ae. aegypti* were collected from Noumea and in Wallis Island from the areas with dengue cases. The immature stages of mosquitoes were divided into 15 pools of which 3 pools appeared to be infected with Dengue virus type 1 having a minimum infection rate of 1:12. In this study they confirmed vertical transmission of dengue in nature and also suggested that vertical transmission ensures the survival of dengue virus during the interepidemic period. Vertical transmission of Dengue virus type 3 by *Aedes aegypti* in nature was also reported from Jalore town in India (Joshi et al. 1996). Larvae were collected and reared to adulthood in the laboratory and tested for the presence of dengue antigen by indirect fluorescent antibody test (IFAT). Mosquitoes were also obtained from an area without any reported cases of dengue fever and were inoculated with Dengue 3 virus intrathoracically. The F1 progeny of lab induced infected

mosquitoes were also tested for the presence of dengue virus antigen showing that 3.6% of the wild caught females were positive for dengue virus whereas in experimental studies 88% of the artificially infected mosquitoes F1 progeny were positive for dengue virus. In both cases none of the males were found to be infected with dengue virus. This was the first report of transovarial transmission of dengue virus 3 in nature as well as to the F1 progeny of experimentally infected female mosquitoes in the Indian subcontinent (Joshi et al. 1996). This high MIR of Den 3 in field collected larvae could be possibly due to the fact that these mosquitoes originated from eggs, which remained, desiccated for long time. Dengue virus may not undergo latency in desiccated eggs so the virus keeps multiplying in the embryo at a slow rate due to a slow rate of metabolism (Mouraya et al. 2001).

Thenmozhi et al. (2000) studied natural vertical transmission of dengue virus in *Ae. aegypti* in Southern India. Immature stages of *Aedes aegypti* were collected from different breeding habitats, reared to adulthood, separated by sex and pooled for further analysis. The resting and landing collection method was also used to catch wild males. Samples were divided into 213 pools of wild males, 190 pools of reared males and 204 pools of reared females, each pool consisting of 20 mosquitoes and were tested for infection by Mab-ELISA. Pools found to be positive were further tested by inoculation into *Toxorhynchites splendens* and tested with IFA (Toxo-IFA) to confirm dengue presence. Eight pools of reared males and 4 pools each of wild males and reared females were found positive for flavivirus antigen. Of these none of the female pools were positive for dengue where as 3 and 1 pools of wild caught males and reared males respectively were positive for dengue. Further analysis revealed the presence of dengue type 2 and 3 in these pools. Isolates were made in relatively cool months though the temperature was not sufficiently low to show overwintering suggesting that vertical transmission is maintained by endemicity of the virus during a period when dengue is not normally apparent.

It is possible to predict a dengue outbreak six weeks in advance of the occurrence of the 1st human case (Chow et al. 1995). However the interval between transovarial dengue detection and the first human case ranged from 7 to 41 days but it is still difficult to predict the time interval between detection of the virus from emerging adults and the appearance of clinical cases due to the presence of viremic

asymptomatic or mildly symptomatic cases. The ecology and mechanism of involvement of vertical transmission of dengue virus in disease outbreaks remain unknown due to inadequacy of data. Early detection of dengue virus in the mosquito prior to its introduction to the human population could help a lot in predicting an impending dengue outbreak (Lee and Rohani 2005). Serological diagnosis methods like serum dilution-plaque reduction neutralization assay and complement fixation test of hemagglutination inhibition assay could not diagnose the virus specifically due to cross reactivity of antibodies to flaviviruses particularly between different serotypes of dengue. Secondly paired samples are needed for these assays, which could delay diagnosis and rarely give clear-cut results. Virus isolation using mosquito or cell culture, though is sensitive but it is also times consuming and is not always successful because of small amounts of viable virus in the inoculate, virus antibodies complexes and inappropriate handling of samples. To overcome these problems Polymerase Chain reaction (PCR) has been developed which can be performed rapidly, is sufficiently sensitive, specific and is clinically and epidemiologically useful (Linciotti et al. 1992).

For the first time Kow et al. (2001) used type-specific reverse transcriptase Polymerase Chain Reaction (RT-PCR) to detect dengue virus in wild caught males of *Ae. aegypti* and *Ae. albopictus*. This study was conducted in the dengue sensitive area of Singapore. Adult males were collected weekly for one year from indoor using a back packed battery-operated aspirator and from outdoors by using human bait collection. Heads of the male mosquitoes were excised and homogenised, RNA was extracted and subjected to single step RT-PCR followed by semi-nested PCR using an upstream consensus primer and four type specific primers within the NS3 gene of dengue virus. The diagnostic fragments were of sizes 169, 362, 265, and 426bp for DEN-1, DEN-2, DEM-3 and DEN-4 serotypes respectively. The virus infection rate in Singapore was 1.33% and 2.15% for *Ae. aegypti* and *Ae. albopictus* adult males respectively. DEN-1 was the dominant serotype in *Ae. aegypti* and DEN-4 for *Ae. albopictus*. This study showed the potential in both species for males being infected in the natural environment, which may play an important role in the maintenance of virus even in the absence of infectious host.

Mouraya et al. 2001 studied horizontal and vertical transmission of DEN-2 in highly and low susceptible strains of *Ae. aegypti.* Oral feeding and individual rearing was used to establish two lines of isofemales highly and lowly susceptible to dengue 2. These were then allowed to feed on Bovine albumin phosphate saline pH 7.2 (BAPS) through membrane feeding. The presence of dengue virus in the probed BAPS was determined either by using ELISA or intrathoracic inoculation of mosquitoes or both. Progenies were obtained from these two female lines and checked for vertical transmission. It was found that when the eggs were allowed to develop after two months the rate of vertical transmission of virus was very high. A possible explanation of this was that at room temperature, the virus multiplied and increased more rapidly increasing its number in the quiescent embryos. The progeny of these female lines were allowed to probe on BAPS through a membrane feeder and the presence of virus indicates their capability of transmitting the virus horizontally. The higher transmission rate in mosquitoes obtained from eggs desiccated for longer times and then the horizontal transmission of virus by them is of potentially epidemiological significance since it shows the potential for the persistence of virus during the dry and winter season.

Joshi et al. (2002) carried out a laboratory experiment on the persistence of DEN-3 virus through transovarial transmission passage in successive generations of *Ae. aegypti*. In this study female mosquitoes were intrathoracically infected with DEN-3 virus and were reared through many generations. Virus was isolated from the seventh generation showing that it could persist in successive generations through vertical transmission. Vertical transmission initially increased in the F1 and F2 generation but then it was found to become stable. The larval duration of transovarially infected batches increased as compared to the control mosquitoes. The fecundity and fertility of vertically infected mosquitoes was also different from that of control mosquitoes. This was the first study on persistence of dengue virus in mosquitoes by vertical transmission in successive generations.

Gunther et al. (2007) analyzed vertical transmission of dengue in *Ae. aegypti* in two dengue endemic localities of Oxaca state, Mexico. Immature stages of *Ae. aegypti* were collected, reared in the laboratory and vertical transmission was investigated using a semi-nested RT-PCR either in larvae or newly emerged

mosquitoes. Dengue was not detected in larvae but in adults 4 out of 43 pools were positive when amplified for the viral genome. Serotypes detected were DEN-2, DEN-3 and DEN-4. It was concluded that vertical transmission is there in both localities of Mexico helping the virus to survive unfavourable conditions in quiescent embryos. Hutamai et al. (2007) collected immature stages of *Ae. aegypti* and *Ae. albopictus* from different localities of two provinces in northern Thailand. On rearing to adults they were tested for the presence of virus but none of the samples were found positive suggesting that transovarial transmission may not play a significant role in the epidemiology of dengue infection in Thailand perhaps due to its very low occurrence in vector populations. In another study conducted by Thenmozhi et al. (2007) the occurrence of vertical transmission of dengue virus was examined in larvae and wild caught *Ae. albopictus* males in Kerala state of Southern India. Dengue antigen was detected in males and females emerged from field-collected larvae. Dengue serotype 2 was isolated from males collected in the field suggesting that vertical transmission plays an important role in maintenance of dengue virus in *Ae. albopictus* during the dry season.

The role of different species in the transmission and retention of dengue virus in semi-arid areas of Rajasthan, India was investigated by Angel and Joshi (2008). The larvae of *Ae. aegypti, Ae. albopictus, Ae. vittatus* were collected from various breeding habitats of different rural and urban areas of Jodhpur, Jaipur and Kota. Larvae were reared to adulthood in the laboratory and checked for the presence of dengue virus by IFA. In Jodhpur the mosquito infectivity rate was 15.7% in *Ae. albopictus* followed by 12.6% in *Ae.aegypti*, in Jaipur 20% by *Ae. vittatus* followed by 18.7% by *Ae. albopictus* and 13.3% by *Ae. aegypti* and in the town of Kota maximum dengue virus infection rate was shown by *Ae. albopictus* i.e. 14.2%. Vertical transmission rates were very high in this study showing that it can be an important factor in virus persistence and transmission but the samples were not confirmed by a much more reliable test like RT-PCR. In Chenai, India a study was undertaken by Arunachalam et al. (2008) to comprehend the natural vertical transmission dynamics in *Ae. aegypti* and to assess its epidemiological importance. In this study resting and landing collections were used to collect *Ae. aegypti* males. The collected samples were tested for dengue virus infection by ELISA and if found positive by ELISA the sample was further examined by insect bioassay, Toxo-IFA using type speicific Mabs. 15 out

of 509 pools were positive for the virus having the highest minimum infection rate in June i.e 2.8%. DEN-2 and DEN-3 were identified in these mosquitoes. The presence of dengue virus in wild caught males of *Ae. aegypti* shows that they are infected by vertical transmission. The occurrence of vertical transmission in summer months suggest that dengue virus adopts a novel strategy of surviving adverse climatic conditions when dengue infections in humans were low. In another study larvae collected from the field in India were also detected with dengue antigen using monoclonal antibodies and this vertical transmission was thought to be the reason for the maintenance of this virus during summer months in Dehli and Jaipur (Bina et al. 2008).

Zeilder et al. (2008) also carried out a study on the detection of dengue virus in larval forms of *Aedes aegypti* and the association of the vector with rainfall and incidence of dengue in a city of Northern Brazil. They installed two oviposition traps per home and removed them after every week. 44 pools of at least 30 larvae each were formed and tested by RT-PCR and hemi-nested PCR. Despite the high incidence of dengue in that area none of the pools were found positive for the presence of dengue virus showing that vertical transmission was present in a very low frequency and therefore virus persistence may not depend upon such transmission. Given this context, the current study had tried to investigate the presence of dengue virus in adult mosquitoes and vertical transmission of dengue in field-collected larvae from dengue-affected areas in Pakistan. Moureau et al. (2007) reported degenerative primers, which can amplify the N terminal end of NS5 gene in 51 flavivirus species and 3 tentative speices. Due to the high sensitivity of real time PCR, using SYBR green along with Moureau et al. primers were used for the detection of dengue virus in adults and larvae collected from Pakistan.

1.11 AIMS

The objective of the thesis was to gain a better understanding of the Dengue situation in Pakistan using a wide range of medical entomological techniques. In addition to surveys I focussed on the population genetic structure of the dengue vector mosquito; *Aedes aegypti* larvae collected from different types of container and

examined the prevalence of dengue virus and flavivirus like sequences in the *Aedes aegypti* genome. The main aims of the study are:

1. To investigate the spatial distribution and to identify the typical breeding containers of dengue vector in Pakistan (Chapter 3).
2. To investigate the genetic structure of populations of *Aedes aegypti* in Pakistan in order to determine its origin, mixing and spread (Chapter 4).
3. To detect dengue virus in mosquitoes and larvae collected from different types of containers and in particular tyres (Chapter 5).
4. To detect flavivirus like sequences in the *Aedes aegypti* genome and discuss their evolutionary implications (Chapter 6)

1.12 Thesis Layout

In chapter 2 I present a review of dengue as an emerging vector borne disease in Pakistan. I give an over view regarding the occurrence of the primary vector of dengue *Aedes aegypti* in Pakistan along with serological findings regarding dengue exposure in Pakistan in the 1960's. I also describe and discuss the first epidemic of dengue in the study area during 1994 along with dengue epidemics occurring from 2006 onwards with a particular focus on the most recent epidemic of 2011. In addition I also discuss the routes of spread of dengue in Pakistan and consider different control methods. Chapter 3 describes an entomological survey of dengue vectors in Pakistan. Two surveys were conducted in Pakistan to determine the spatial distribution of *Aedes aegypti* across a wide area of Pakistan. Mosquitoes collected in these surveys were then used for investigating population genetic structure (Chapter 4) and detecting the virus (Chapter 5). I discuss different types of containers that *Aedes aegypti* use for oviposition. At the end of the chapter different control strategies are discussed which could be adopted in the study area to decrease the mosquito vector population. Chapter 4 describes a substantial part of the thesis that was focussed on the population genetic structure of the dengue vector (*Aedes aegypti)* in Pakistan. Microsatellite markers were used to genotype the larvae collected from different areas of Pakistan to examine the population structure and therefore the spread of the mosquito. Then I examine whether this pattern correlates with landscape only or also with other factors such as passive dispersal. A number of different statistical methods were employed to

carry out this analysis. Currently there is no control strategy in Pakistan to control this mosquito either before or during a dengue epidemic. The results of the study may help to design an effective control program in Pakistan. Chapter 5 describes the approaches taken to detect dengue virus in field-collected mosquitoes. First I used the cDNA from mosquito ribosomal RNA to check that the RNA extraction method and cDNA formation was working properly. The virus detection was carried out by using real time PCR, using SYBR green and Moureau et al. (2007) primers. The aim was to then compare viral incidence with the pattern of spread of the vector mosquito. In chapter 6 I examined flavivirus-like sequences within the mosquito genomes. I give details of the detection of flavivirus-like sequences in mosquito DNA. These sequences were then compared by using neighbour-joining polygenetic trees using the P-distance algorithm. This enabled me to examine the genetic relatedness between them as a percentage identity. Finally in chapter 7 I present a general discussion of the results of the thesis and describe future studies that need to be carried out, building on this work in order to gain a good understanding of the Dengue vector situation in Pakistan. From this I describe possible routes to the better management of this important emerging disease.

Chapter 2

A REVIEW OF DENGUE AS AN EMERGING DISEASE IN PAKISTAN

Dengue is an acute febrile disease caused by a single stranded RNA virus with four closely related but antigencially different serotypes DEN-1, DEN-2, DEN-3 and DEN-4 (Gubler 1998). It has become a major health problem in both the tropics and subtropics and is currently considered to be the most important human arboviral disease (Arunachalam et al. 2008). According to WHO approximately 2.5 billion which is about half of the world's population is said to be currently at risk of dengue. Every year about 50 million people are estimated to suffer from dengue virus with approximately 55,000 cases requiring hospitalization, a large proportion of which are children (WHO 1999). At least 2.5% of these individuals die but the death rate may be twice as high as this. (WHO 2008). Mosquitoes of the genus *Aedes* are the vectors of Dengue in both rural and urban areas. *Aedes aegypti* is the most important mosquito vector of arboviruses because it is both highly anthropophillic and prefers habitats in close proximity to humans (Wang et al. 2000, Twiddy et al. 2002). Here we review the Dengue situation in Pakistan where it is both an emerging disease and an increasing health problem.

Pakistan, a country characterised by its geographical and climatic diversity is located in the north west of South Asia. It is situated between the latitudes 23.45º and 36.75º North and between longitudes 61º and 75.5º East. Administratively it consists of four provinces; Punjab, Sind, Khyber Pukhtoonkhwa (KPK), Balouchistan, and the independent state of Azad Jammu and Kashmir. Due to its subtropical location and climatic suitability for vectors Pakistan is faced with problems associated with many vector born diseases including Malaria, Leishmaniasis, Crimean-Congo Haemorrhagic Fever, Dengue Haemorrhagic Fever, West Nile Virus, Japanese Encephalitis and Scrub Typhus (Sugamata et al. 1987, Country Report 2003). Malaria and some other vector born diseases have been reported for more than half a century in Pakistan but Dengue Fever is quiet recent. It has extended its range during the last few years resulting in morbidity in the thousands and is causing a number of deaths due to

complications of Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS).

2.1 Dengue Vectors

Reports of Dengue vectors in the region predate the creation of Pakistan. In 1934 Barraud collected *Aedes aegypti* the primary vector of Dengue from Peshawar, Dera Ismail Khan, Lahore, Larkana and Karachi. In 1949 Qutubuddin reported *Ae. aegypti* from Kohat-Hangu valley of Northern Province of Pakistan. The distribution of *Aedes aegypti* decreased significantly after 1950 as a by-product of a malaria vector eradication program (Suleman et al. 1996). Its return was in two episodes in Pakistan, one in the south in the 1980s and then in the north in 1993. In the southern metropolis of Karachi *Ae.aegypti* was again reported by Kamimura et al. (1983) in a survey conducted in 1983 to determine the species of mosquito acting as vectors of different diseases. However at that time the distribution of *Ae. aegypti* was limited to the port city of Karachi and they failed to collect it from the neighbouring District of Thatta.

The second re-emergence of *Ae. aegypti* occurred in Landi Kotal a border town of Khyber Agency located 40 km from Peshawar and 10 km from the Afghan border. In 1993 there were reports of an abundance of an unusual diurnal human biting mosquito in this area. A survey was conducted by a team of entomologists from Peshawar University lead by Dr. Suleman. On surveying the area *Ae. aegypti* were collected both in adult and immature forms. This was the first time that the spread of this mosquito was associated with tyre trade in Pakistan. The mosquito was breeding in tyres stored in warehouses (Suleman et al. 1996). In 2000 all 5 districts of Karachi were again surveyed for the presence of *Ae aegypti* and it was collected from all of them indicating an increase in its distribution. (Tariq and Zafar, 2000). In another survey of Karachi District in 2010 the presence of the vector mosquito was again confirmed from all 18 towns of Karachi (Tariq et al. 2010). It is clear therefore that from eradication in the 50s *Ae. aegypti* has recently re-established itself in Pakistan associated with the tyre trade.

2.2 Dengue Disease

Dengue virus may have been endemic in Pakistan although no outbreak was reported untill the 1994 epidemic. However according to seroepidemiological studies conducted in the 1960s and 1980s a substantial proportions of the apparently health population in Rawalpindi and Peshawar (Burney 1966, Burney and Munir 1966), parts of Punjab province (Hayes and Burney 1981, Hayes et al. 1982) and Karachi (Sugamata et al. 1986) were tested positive for neutralization and haemagglutination inhibition antibodies for dengue virus. In another study conducted between 1983 and 1985 on the prevalence of three of the flaviviruses (West Nile Virus, Japanese Enciphalitis and Dengue) in the city of Karachi, 65% of the total population aged between 6 and 65 showed positive results for Haemagglutination Inhibition (HI) antibodies against one or more of the three flaviviruses. The age group between 6-20 years showed an increase in the HI seroantibody positivity rate for Dengue type 2 between the months of July and October (Sugamata et al. 1987). The first outbreak of Dengue in Pakistan started in August 1994 and continued through November 1994 in Karachi leading to morbidity in the thousands. Epidemiological data for this epidemic was not recorded but the number of patients seen by physicians was in the thousands. There is no proper estimate of the number of fatal cases but at least two deaths occurred in different hospitals of Karachi (Chan et al. 1995).

A few independent small level studies had been conducted to confirm this outbreak as dengue. In most of the investigations the presence of Dengue type 1 and Dengue type 2 were confirmed by detecting Immunoglobins M (IgM) using an enzyme linked immunosorbent assay. These investigations were made on adults as well as children (Chan et al. 1995, Qureshi et al. 1997, Akram et al. 1998, Ansari et al. 2001). In one of the studies Chan et al. (1995) used semi nested PCR to detect the dengue virus in sera of patients and also isolated Dengue type 1 and type 2 from these serums. This was the only study in which molecular technique was used and the virus was isolated from the serum of infected patients. As such we have only a limited understanding of the extent and nature of this first epidemic.

Dengue cases continued to occur in 1995 but this time in a different city. A contractor building a powerhouse in Hub 60 km west of Karachi reported pyrexia of

unknown origin in most of its employees (Country Report Pakistan 2003). IgM and IgG antibodies for all four serotypes of dengue virus were determined by using ELISA. Again IgM antibodies for serotype 1 and 2 were used in this investigation suggesting that the disease persisted in the southern area of Pakistan for two consecutive years. In this outbreak patients were not only local but also expatriates hired by the company, which suggests that the disease was transmitting locally. No entomological work was carried out alongside the seroepidemiological studies but the employees were complaining of mosquito bites in the daytime. When all the possible habitats of mosquitoes in the construction sites of the power plants were destroyed the disease did not occur again in that area (Paul et al. 1998).

One reason for the 1994 and 1995 outbreaks may have been the introduction of another serotype of Dengue virus to the population. Sugama et al. (1987) reported only serotype 2 in 1987 but in the 1990s epidemics serotype 1 was also detected in the patients. The presence of multiple serotypes in a population results in an increased risk of antibody dependent enhancement (ADE); a phenomenon in which the heterotypic antibodies from a previous dengue serotype infection results in promotion of viral replication within the mononuclear leucocytes during secondary infection of different serotype (Cummings et al. 2005). It was originally suggested by S.B. Halstead in the 1970s that DHF is more likely to occur in patients who are previously exposed to dengue infection. The 1994 and 1995 outbreaks of dengue results in illness of thousands of people but no serious steps were taken to prevent future epidemics. In particular no rules were implemented to quarantine people coming from different dengue endemic areas and the tyre trade was not monitored.

After a break of 10 years another serotype of Dengue virus was reported in Pakistan in 2005. During the intervening period there was no major outbreak though cases were still present. According to the National Institute of Health Islamabad, in 2005 about 395 cases of dengue were confirmed in their laboratory all of which were from Karachi. Initially a local hospital of Karachi investigated some cases symptoms of which indicated signs of Dengue Fever by conducting ELISA and RT PCR. A total of 106 patients were included in the study of which 9 died and 97 recovered. 42 of the suspected cases were positive for anti-dengue virus IgM

antibody. Of the 9 patients 6 patients had both dengue IgM and IgG according to ELISA while 3 only had dengue IgM. RT PCR confirmed 3 patients dengue status but in this investigation a step further was taken and the PCR product was sequenced to determine the serotype and the phylogenetic relationships of the virus. The analysis showed it to be serotype 3, that had a significant similarity with an Indian strain of dengue serotype 3 isolated from Delhi (Jamil et al. 2007).

From 2006 the disease extended its range from the south to the northern parts of Pakistan causing epidemics where thousands of people were affected with hundreds of deaths over the last 5 years (Figure 2.2). In 2006, 5800 suspected cases of dengue were reported across Pakistan with 3000 confirmed cases and 52 deaths (Tang et al. 2008). The epidemic started in May and continued till November after which it started to decline. The maximum number of cases was reported from August to October, when the country receives heavy rains due to the Monsoon. Karachi was the most affected city with more than 4500 cases, 1500 of which were confirmed by IgM and IgG antibodies and 50 people succumbed to death (Khan et al. 2007). Dengue cases were also reported from the northern provinces of Pakistan. About 800 cases were reported from the Punjab and 31 cases with one death were reported from the North-Western Province of Khyber Pukhtoonkhwa. In Punjab, Lahore and Rawalpindi were the most effected cities with more than 400 cases in each city. The capital city of Islamabad was also hit by this epidemic resulting in 480 cases and one death. This was the first epidemic of dengue in Pakistan infecting such a large number of people over a large proportion of the country. There was no strategy present at that time for either the control of dengue or the treatment of patients. Cases continued to occur till November and after that the number of affected patients decreased because of the colder weather not favoring the mosquito.

In 2007 again dengue hit the southern and northern part of Pakistan, this time the intensity was lower than the previous year. 3342 patients suspected with dengue were reported in different hospitals with 1208 confirmed by ELISA and 22 deaths. Dengue cases were reported throughout the year with the maximum cases reported in the month of November followed by October. Karachi had the maximum number of cases, 2900 suspected and 950 confirmed with all 22 deaths

reported this year followed by Lahore with 258 cases. Cases were reported from the interior of the Punjab also but they were only one or two cases in each city, which were therefore likely to be imported cases. Attock located in the north of Punjab was new to the disease in epidemic form but it was restricted to two villages Basal and Thatta Khalil. According to hospital records in the months of August and September about 80% of these two villages population suffered from disease having symptoms like Dengue. Later Dengue was confirmed in the patients by using ELISA.

In 2008 3280 suspected cases with 30 deaths were reported from all over Pakistan. The balance has shifted towards the north with 1450 confirmed cases and 20 deaths reported from Punjab. Karachi reported 1470 cases out of which only 585 were confirmed by ELISA with 6 deaths in total. 290 cases were reported from Rawalpindi/Islamabad with 11 deaths and 70 cases with 30 confirmed dengue cases and 4 deaths were reported from Khyber Pukhtoonkhwa (KPK) province. In 2008 Dengue affected the capital city of Punjab more severely than Karachi. Lahore witnessed 1358 confirmed dengue fever cases with 9 deaths. No cases were reported from January to March and then only eight cases were brought forward from April to August. The number of patients continued to rise from September reaching a maximum number in November and then there was a sudden decrease in number of patients from 995 in November to only 39 in December thus the disease continued to occur till the end of the year. The number of patients suffering from dengue decreased to half in 2009 as compared to previous years with only 1650 patients with 950 confirmed by ELISA and 16 deaths throughout Pakistan. Only 112 cases with 2 deaths were reported from January to August. In September when the monsoon rains started, providing good breeding conditions to vector mosquitoes, the number of cases rose to more than 1500 with about 800 confirmed cases. In total 14 people died during this period out of which 7 were from Haripur, a district of Khyber Pukhtoonkhwa province and 7 from Karachi.

In 2010 a number of areas were affected by the worst flood of the decade, which not only resulted directly in the loss of lives and property but also increased the breeding opportunities of the vector mosquitoes. As a consequence according to the press releases of health departments the number of dengue cases reported in 2010 were

the highest recorded. In total more than 9000 cases were reported across Pakistan. Approximately 5000 cases were reported from Sindh with 35 deaths 16 of which were from Karachi. More than 4000 cases were reported from Punjab with only 3 deaths. In the last week of October 1500 cases were reported followed by 1400 in the first week in November. Like previous years Karachi and Lahore remained the most affected areas with Karachi having the maximum number of patients suffering from dengue with 16 deaths in total.

This year (2011) has proven to be even worse in regard to dengue in Pakistan. Dengue has been found in almost every city of the three affected provinces of Pakistan. In 2011, according to the health departments approximately 22,562 confirmed cases of dengue with 363 deaths were reported in Pakistan, unofficial reports claim more than 35,000 confirmed dengue cases and approximately 420 deaths in the country. The epidemic started in August and continued till November (Figure 2.1). Punjab province was the most affected province of Pakistan. More than 21,300 cases and 337 deaths were reported from Punjab alone. Dengue cases were reported from almost every city of Punjab (Table 2.2). Lahore the capital city of Punjab topped the list with 17493 cases and 290 deaths. Other cities of Punjab having death reports due to dengue included Rawalpindi, Chakwal, Faisalabad, Dera Ghazi khan and Chichawatni. This year dengue mostly affected the areas of Punjab and especially Lahore but still cases were reported from Sindh and KPK provinces. Sindh witnessed 952 cases with 18 deaths, 755 cases among these were reported from Karachi alone with 15 deaths. In Sindh cases were reported from Larkana, Dadu, Ghotki, Hyderabad, Jamshoro, Khairpur, Nowshehro feroz, Nawab shah, Sanghar, Skkhar, Tando Muhammad Khan, Tando Allah yar. Rajan Pur and Umer Kot. Compared to the Punjab very few cases were reported from KPK. Dengue cases in KPK were reported from Peshawar, Nowshehra, Haripur, Mansehra, Bannu, Mardan, Battagram, Laki marwat, Khyber agency, Mingora and Abbottabad. Only 296 confirmed cases were reported from KPK out of which 92 patients had a travel history to Punjab. These patients probably got the infection in Punjab and then they travelled to KPK carrying the virus inside their body and later on showed signs and symptoms in KPK. The disease reduced at the end of November due to cool weather in which it was difficult for mosquitoes to survive.

2.3 Routes of Spread of Dengue In Pakistan

Dengue was not common until 2006 in Pakistan. It was mostly restricted to the southern part of Pakistan and especially to the port city of Karachi. Indeed no cases were reported from other parts of Pakistan until 2005. The spread of dengue to other parts of Pakistan could be explained either by the movement of people carrying the virus from dengue affected area to non-dengue areas or by vertical transmission of dengue in mosquitoes. One interesting example is the two villages Basal and Thatta Khalil in the District Attock located at the border of Punjab and Khyber Pukthoonkhwa provinces. These two villages are located in a small valley like location in Attock. The nearest village to them is about 5 kilometers away. In 2007 there were reports of pyrexia of unknown origin with symptoms consistent with dengue fever affecting about 80% of the total population of these villages. The area was visited and people were interviewed in which they confirm mosquitoes having day biting activities, indicating *Aedes*. According to locals the disease started when a couple from Karachi, who were suffereing from a fever consistent with dengue, came to the village to attend the death ceremony of one of their relatives (*Personal communication*). The vector mosquito was already present in the area and it is likely that the couple acted as a reservoir for dengue thus spreading the dengue in the local population. The disease did not spread to other areas of the district because these two villages were somewhat isolated from other areas of the city because of the hilly nature of the area. A similar case was also found in Rawalpindi when a dengue infected Medical student travelled from Lahore and suffered from dengue in Rawalpindi.

In 2011 most of the patients in KPK arrived from the Punjab before becoming symptomatic. This movement of infected people is likely to be the main cause of such a rapid spread of Dengue in Pakistan in less than a decade. The movement of dengue infected people combined with the presence of the vector in the dengue free areas could probably be the cause of dengue cases in almost every city of the three affected provinces of Pakistan. Another hypothesis is that dengue is imported into Pakistan and then distributed within Pakistan through tyre trading. Tyres from dengue endemic countries may carry desiccated eggs, which hatch during the rainy season. This hypothesis needs to be tested by collecting and testing

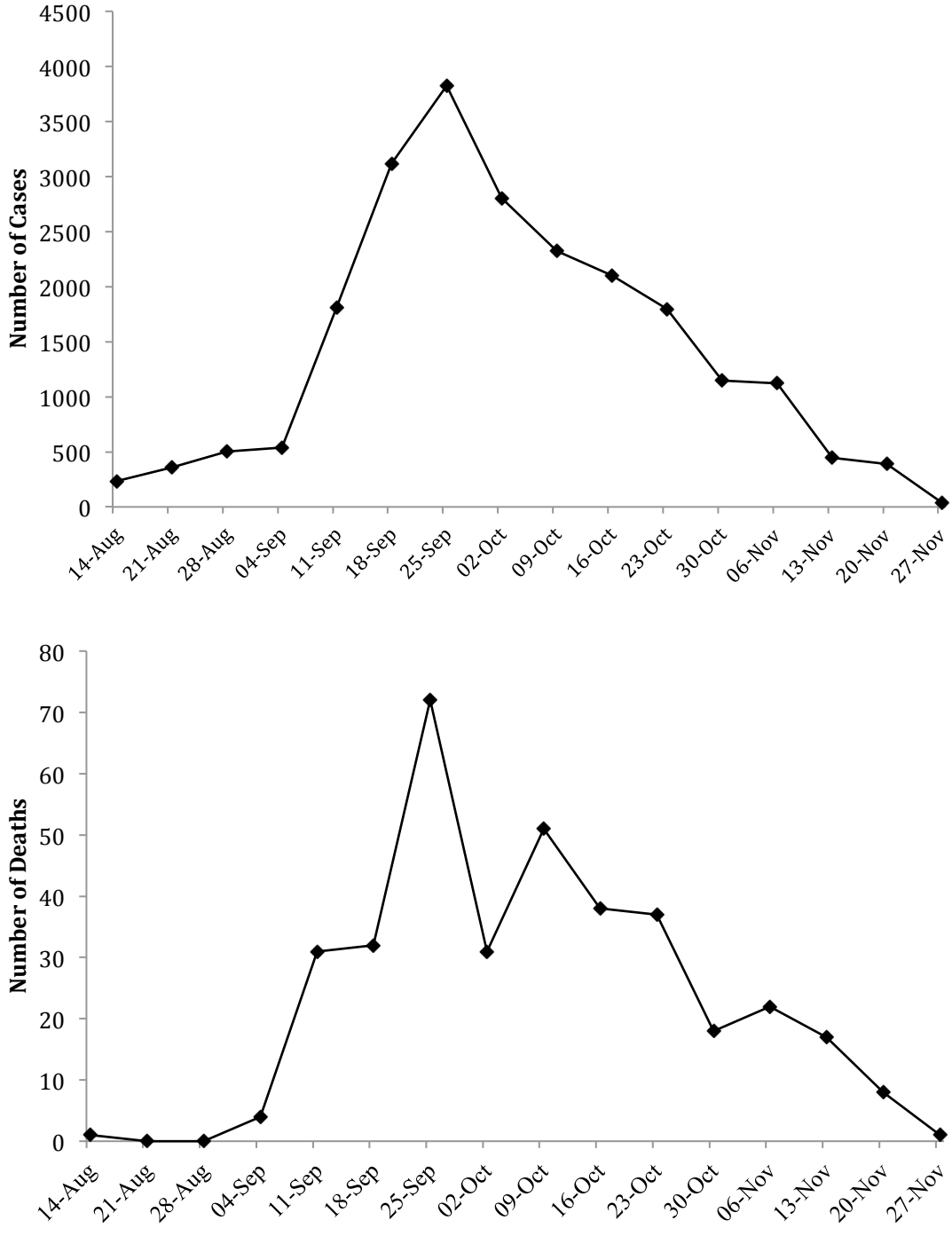

Figure 2.1: The number of weekly Dengue cases and deaths due to Dengue from 9th August till 28th November 2011

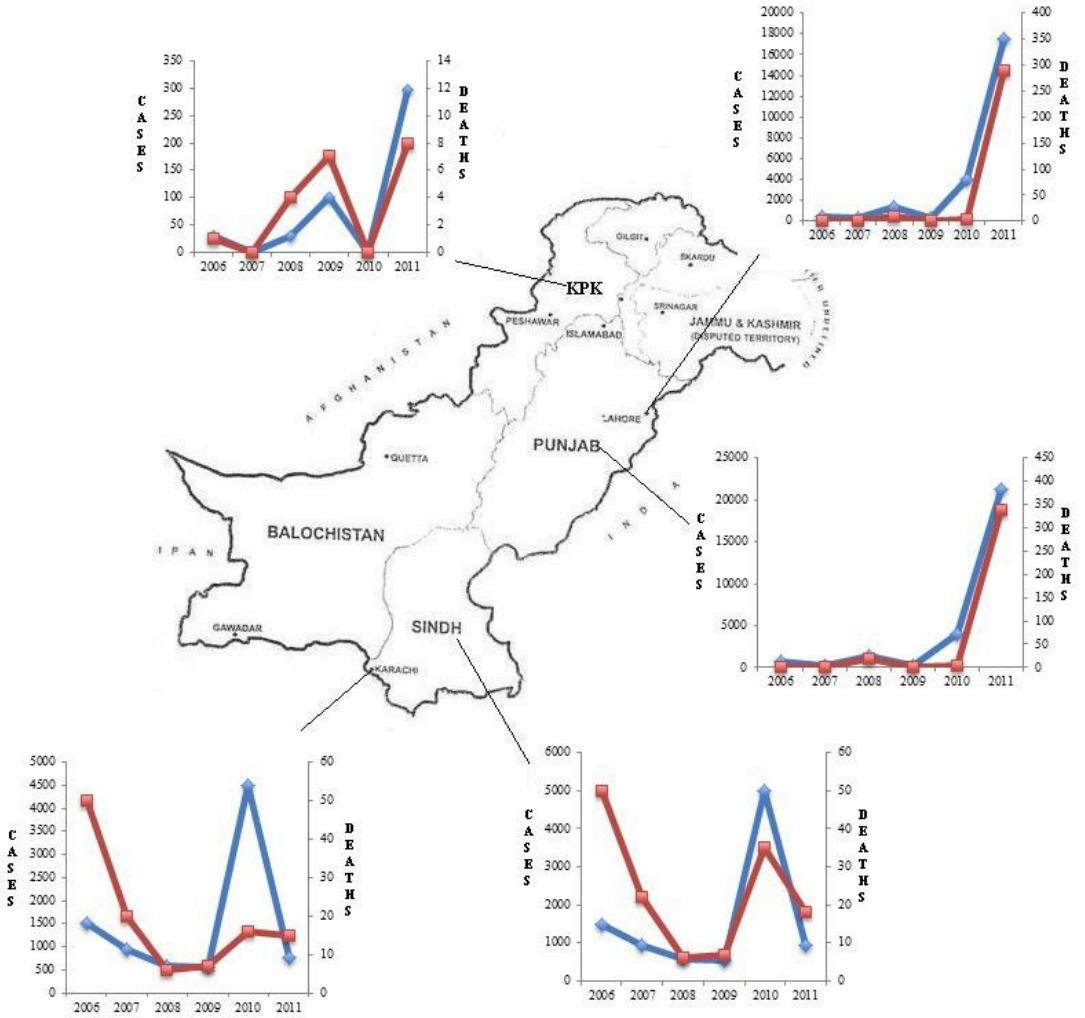

Figure 2.2: Number of dengue cases (blue lines) along with number of deaths due to dengue (red lines) in the three provinces and two mainly affected cities (Karachi and Lahore) of Pakistan from 2006 to 2011.

Table 2.1: Number of dengue cases* reported from different cities of Punjab during the 2011 dengue epidemic in Pakistan.

City	No. of Cases	City	No. of Cases
Lahore	17493	Dera Ghazi Khan	38
Faisalabad	1149	Narrowal	36
Multan	538	Jhang	36
Bahawalpur	305	Sahiwal	33
Sargodha	232	Muzaffar Garh	30
Rawalpindin	181	Bhakkar	24
Sheikhupura	156	Chakwal	23
Okara	118	Nankana Sb	23
Rahim Yar Khan	109	Toba Tek Singh	20
Gujranwala	107	Miawali	16
Taxilla	90	Gujrat	15
Vehari	80	Hafizabad	14
Jehlum	71	Mandi Bahawadin	11
Pakpatan	69	Chiniot	11
Sialkot	58	Lodhran	8
Layyah	55	Attock	7
Khanewal	52	Rajanpur	7
Bahawalnagar	51	Khushab	5
Kasur	43	**Total**	**21314**

*Data downloaded from http://dengue.punjab.gov.pk/?q=system/files/Summary_13_Oct.pdf and then updated by health department reports.

for Dengue of a very large sample of adult as well as immature stages of mosquitoes from tyres and a large number of cities of Pakistan.

2.4 Control of Dengue

Most of the disease control effort has been made to treat the dengue infected people as soon as possible and therefore to reduce the number of deaths However no or very little effort has been made to stop or reduce the number of infected cases through vector breeding control. In all the cities only fumigation is carried out to kill adult mosquitoes. Better control may be achieved through three main routes: (1) **Chemical Control:** A combination of larvicide and fumigation having adult knock down effects has the potential to reduce the mosquito population. Chemical control is quick and efficient but its disadvantages include resistance to insecticides in mosquitoes (Kaunter et al. 1997). (2) **Biological Control:** Small fish like *Gambusia affinis* and Tilapia could be released in water tanks, which could help a lot in reducing mosquitoes by eating the larvae that are produced in the tanks. Biological control is slow but could prove to be a good method to control the mosquito population growth. (3) **Education.** No strategy will be successful until and unless there is the involvement of local people. Using electronic and print media to tell people how to eradicate the mosquito breeding points, how to protect daily use utensils like drums, buckets, water tanks, plant pots and other small containers from becoming mosquito factories can help a lot in controlling the vector and thus controlling the disease.

2.5 Summary

As described in this review, Dengue has become a major health problem in Pakistan. Once relatively rare it has moved from localised infrequent epidemics in a few cities to a widespread problem with larger numbers of cases. There is a critical need for large-scale entomological surveys in addition to epidemiological ones and an urgent need for a vector control programme. Without these, now that is established, Dengue is likely to become a much bigger health problem in the coming years.

Chapter 3

AN ENTOMOLOGICAL SURVEY OF THE DENGUE VECTOR IN PAKISTAN

3.1 Introduction

Pakistan as a sub-tropical country supports a variety of disease vectors including mosquitoes, sandflies, ticks, mites, fleas and bed bugs. In turn these lead to problems associated with many vector born diseases including Malaria, Leishmaniasis, Crimean-Congo Haemorrhagic Fever, Dengue Haemorrhagic Fever, West Nile Virus, Japanese Encephalitis and Scrub Typhus (Sugama et al. 1987, Country Report 2003). The major mosquito born diseases in Pakistan are malaria and increasingly dengue. Dengue is an acute febrile disease caused by a single stranded RNA virus with four closely related but antigencially different serotypes that has become a major health problem in both the tropics and subtropics (Gubler 1998). Indeed it is currently considered by some authors to be the most important human arboviral disease (Arunachalam et al. 2008). Dengue epidemics are relatively recent to Pakistan with the first epidemic reported in 1994 (Chan et al. 1995) and second after a gap of 12 years in 2006 (Tang et al. 2008). After the reappearance of dengue in 2006 epidemics are occurring every year in Pakistan. In the absence of effective vaccination and intervention, an understanding of the vector status in Pakistan is critical to the prediction and control of Dengue epidemics.

There has been a considerable amount of work on the distribution and ecology of malaria mosquitoes i.e. *Anopheles* in Pakistan (Aslamkhan and Salman 1969, Reisen 1978 and Reisen et al. 1982). However there is much less information on Dengue vectors including the most important one *Aedes aegypti.* It was first reported from Peshawar, Dera Ismail Khan, Lahore, Larkana and Karachi by Barraud in 1934 (Barraud 1934) and Qutubuddin collected it from Kohat-Hangu valley in 1949 (Qutubuddin 1960). After 1950 *Aedes aegypti* was coincidently eradicated with the malaria mosquito (Suleman et al. 1996) and was not reported

again until the early 1980's from Karachi (Kamimura et al. 1985). In 1996 *Aedes aegypti* was collected in the north of Pakistan from Landi Kotal near Peshawar and was again reported in 2010 from Karachi only (Tariq and Zafar 2000, Tariq et al. 2010). No information is available regarding the distribution and ecology of this mosquito in Pakistan outside of these few reports. The recurrence of dengue epidemics every year after 2006 however has increased the need to examine the spread, distribution and ecology of this mosquito in Pakistan.

Given the lack of information on the distribution of *Aedes aegypti* in Pakistan we carried out a nationwide entomological survey over two years. The aim was to firstly to gain an insight into the distribution and some idea of the prevalence of the mosquito in different cities in Pakistan. Secondly we will gain some information on its breeding habitats. Finally the mosquitoes we collect will be used in the population genetics study (chapter 4) and for our dengue detection study (chapters 5 and 6). This is important baseline data for our understanding and potentially suggesting a strategy for the control of Dengue in Pakistan.

3.2 Materials and Methods

Sampling was carried out for two consecutive years. In both these surveys collection sites were selected not only on the basis of occurance of dengue cases in the past few years but also areas with no dengue cases were searched for the presence of *Aedes aegypti* larvae. A total of 25 cities were selected for entomological survey. Some of these cities like Karachi, Lahore, Rawalpindi, Sheikhupura, Faisalabad and Multan were having cases of dengue while other cities were close to these affected areas. The number of sites selected depends upon the number of town within cities, which vary from 2 in Sheikhpura and Gujranwala to 18 in Karachi. Minimum of two towns were selected from each city. Each selected area was visited and searched for the presence of larvae inside the houses as well as around the houses. Along with different domestic containers inside houses, large containers like tanks and drums were thoroughly checked for larvae. Small discarded containers on the roofs and outside houses were also checked for larvae. Tyre shops near the houses as well as large tyre markets were checked if these tyres

or other containers present in the market were positive for *Aedes aegypti* larvae or not.

The first survey was conducted from April to July 2009 while the second collection was conducted from April to October 2010. In the first survey sampling was carried out in 11 different cities of Pakistan. This survey was conducted exclusively to collect the primary vector of dengue i.e. *Aedes aegypti* with a focus on understanding its population genetic structure in Pakistan. Geographical coordinates and the types of container from where the larvae were collected were recorded for each collection site. All larvae were preserved in a separate labeled twist cap 1.5ml tube containing 70% ethanol.

A second field survey was conducted to collect larvae from dengue-affected areas with the main aim of investigating the status of dengue virus in mosquito larvae and adults. Samples were also used to add data in order to improve the analysis of the population genetic structure. Since the focus of this survey was to examine dengue virus within mosquitoes, live larvae and freshly killed adults were transferred from collection containers into separate labeled twist cap 1.5ml tubes and then kept in a 3 liter liquid nitrogen container having a temperature of -196°C for preservation. The samples were then transferred to a bigger liquid nitrogen container of 35 liter at the Department of Zoology, University of Peshawar until transferred to the research lab in England. The mosquitoes were transported back to the UK using Dry ice (solid CO_2) having a temperature of -80°C and then stored at -80°C until analysis.

In both these surveys collection sites were selected not only on the basis of occurance of dengue cases in the past few years but randomnly selected areas were also searched for the presence of *Aedes aegypti* larvae. Each selected area was visited and searched for the presence of larvae inside the houses as well as around the houses. Along with different domestic containers inside houses, large containers like tanks and drums were thoroughly checked for larvae. Small discarded containers on the roofs and outside houses were also checked for larvae. Tyre shops near the houses as well as large tyre markets were checked if these tyres or other containers present in the market were positive for *Aedes aegypti* larvae or not.

Figure 3.1: Map showing cities in Pakistan from where *Aedes aegypti* was collected (black dots) and from where *Aedes aegypti* was not collected (open circles). Cities from where *Aedes aegypti* was collected is having number of sites (patches) in the brackets from where this mosquito was collected.

Larvae from tyres and small containers were collected by using a suction bottle while those from other containers with a large amount of water such as tanks and drums were collected by using a 500ml plastic cup. Adults were collected by using a battery operated suction sweeper (aspirator) with a 6-volt fan for suction and a mosquito net for capturing adults. Both adults and larvae were identified to species level by using the mosquito identification key of Barraud (1934) while pupae were reared to adults and then identified. *Aedes aegypti* larvae were identified on the basis of the shape of the siphon to distinguish genera and the presence of a thorn like structure on the thorax and branched scales at the base of siphon for species. Adults were identified by the pattern of lines on the thorax of the mosquito, in the case of *Ae. aegypti* there are two characteristic crescent like lines on the thorax.

3.3 Results

In this study two surveys were conducted to firstly confirm the presence and distribution of the primary vector of dengue *Aedes aegypti*. *Aedes aegypti* was collected from 13 cities including all dengue-affected areas of Pakistan during the two surveys (Figure 3.1). All three stages i.e. adults, larvae and pupae were collected in both surveys from each city.

The first survey was partly conducted to provide the material for the population genetic structure of this mosquito in Pakistan (Chapter 4). I therefore wanted to sample widely across Pakistan and in total collections were carried out from 11 cities. These larvae were collected from three different places in Peshawar, Lahore, Karachi and Faisalabad, two each in Attock, Rawalpindi and Haripur, and one each in Taxilla, Sheikhupura, Gujranwala and Multan. A total of 898 specimens most of which were larvae and some adults were collected from 57 different containers. Hasanabdal, Jehlum, Kasur, Okara, Sahiwal, Khanewal, Bahawalpur and Quetta were also surveyed but were found to be negative for the the presence of *Ae. aegypti.* Table 3.1 shows the number of specimens collected for population genetic work during both surveys.

The second survey was conducted partly to investigate the prevalence of dengue virus in field collected *Ae. ageypti* larvae. Sampling was carried out from 9 different cities of the country. Four of these cities i.e. Attock, Rawlapindi, Lahore and Karachi were surveyed both for vertical transmission investigation and adding samples to improve the population genetics structure data. Collection was carried out from Hyderabad, Hasanabdal, Taxilla, Sheikhupura and Multan to improve the population genetics structure data only. In a previous survey no larvae were collected from Hyderabad and Hasanabdal but this time larval and adults collection was made from these two cities thus increasing the number of cities to 13 (Figure 3.1). More than 5000 specimens, most of which were larvae were collected from approximately 250 containers during the six months period of the fieldwork. The number of larvae per container varied from 2 to 30. Sukkar, Sadiqabad, Kashmore and Bahwalpur were checked for the presence of *Ae. aegypti* but I failed to collect any specimens. The maximum numbers of larvae were collected from Karachi followed by Lahore whereas he lowest numbers were collected from Haripur as shown in table 3.2.

In the second survey the number of larvae collected from Lahore were very low before the rainy season due to larvicidal spray by the health department of Lahore. However after the monsoon season in September, a sudden increase in the vector population was observed. This sudden increase in the population size of *Ae. aegypti* was observed in almost every city from where collections were carried out. In Karachi no larvae were found in water tanks, which had small fish or naiads of dragon flies (Order Odonata: Class insecta). This suggests that there is a potential for effective biological control as these fish and naiads are carnivorous and feed on the larvae of mosquitoes. Guppy fish and Tilapia were found to be an effective tool in controlling the mosquito populations in some areas of Karachi (Tariq et al. 2011)

The majority of the larvae i.e. 64.4% in both surveys were collected from rain filled tyres (Figure 3.2). These tyre shops vary from a small tyre puncture mending shop (35%) to large shops and warehouses in tyre markets (65%) (Figure 3.3). Tyres exposed to rainfall and placed in shady places were acting as preferred breeding places. The number of mosquitoes varied from two larvae to hundreds of larvae per tyre. In tyres larvae were present both in clear water as well as dirty water.

Table 3.1: Number of mosquito larvae collected for Population Genetics work during both surveys.

City	Total Number	Number Used For Population Genetics
Peshawar	133	76
Attock	161	80
Hasanabdal	16	16
Haripur	146	68
Taxilla	50	41
Rawalpindi	90	89
Gujranwala	43	20
Sheikhupura	36	30
Lahore	113	99
Faisalabad	71	37
Multan	41	27
Hyderabad	16	16
Karachi	368	251
	1284	850

Most of the tyres harboring larvae had dead leaves in the water. The dirty water ranged from turbid water to old and rank water. 35.6% larvae were collected from containers such as buckets, plastic bottles, drums, matka, pitchers, plant pots, water tanks (under ground, over ground and seepage tanks), drinking water coolers and water tubs (Figure 3.3). Among non tyre containers (Figure 3.4) the most larvae were collected from pitchers (31.3%) followed by water drums (28%) and water tanks (23%) whereas the minimum number of larvae were collected from water coolers (1.13%). The larvae were collected from muddy water in tanks and drums as well as portable water in pitchers, matka (earthen big containers) and water coolers whereas in Karachi near the coastal area larvae were also collected from buckets having brackish water. Chi-square test was performed to see if there is any association between the occurance of larvae in tyres and domestic containers in north and south

The mosquito was collected both from outside as well as inside houses (Figure 3.5) however the majority were collected from outside houses (71.7%). Most of the indoor collection was from containers, which were not regularly emptied and cleaned such as big water tanks, pitchers, matka and drums. The surveys were conducted along with officials from the health department so the people were advised by the officials to clean the containers regularly. This practice makes the chance of collecting larvae from the same indoor container very rare. In the case of outdoor containers no care was given to them especially in big tyre markets making it possible to collect mosquitoes from these containers regularly. Each site was visited at least three times during both these surveys and mosquitoes were collected from all sites in every visit to outdoor sites. of Pakistan (Table 3.3), which showed a highly significant association (χ^2 = 675.42, df= 1, $P < 0.0001$) between the occurance of larvae in tyres in the north of Pakistan and in domestic containers in south of Pakistan.

Some other species of mosquito were also found in the containers in which *Ae. aegypti* were breeding. These were *Anopheles stephensi, Anopheles culicifacies, Culex pipiens quinqufasciatus, Aedes albpictus, Aedes unilineatus* and *Aedes pseudotaeniatus. Anophles stephensi* was found in Attock and Karachi, *Anopheles*

Table 3.2: Number of mosquitoes (larvae and adults) collected during second survey used for detection of dengue.

City	Larvae	Adults			Total
		Male	Female	Total	
Attock	517	8	70	78	595
Haripur	417	35	85	120	537
Rawalpindi	623	36	107	142	765
Lahore	733	12	45	57	790
Karachi	1658	90	364	454	2112
	3948	181	671	851	4799

Table 3.3: A 2 x 2 table for chi square test to check for the association of occurance of breeding habitats of *Aedes aegypti* in north and south of Pakistan.

	North	**South**	**Total**
Tyres	2483	871	3354
Domestic	691	1162	1853
Total	3174	2033	5207

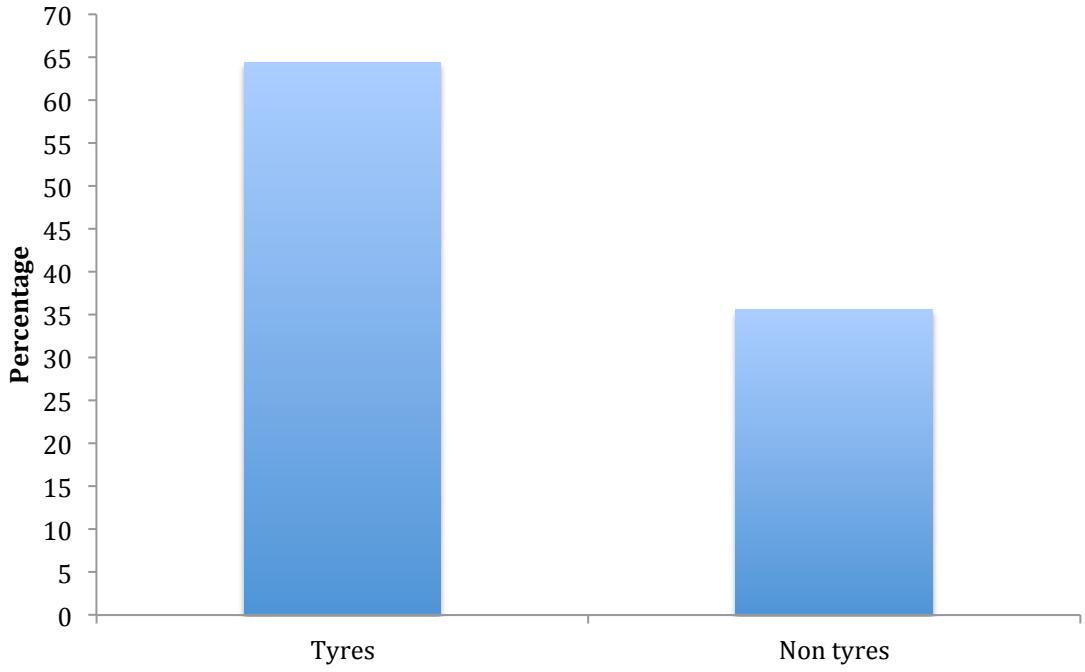

Figure 3.2: Percentage of mosquitoes collected from tyres and non-tyre containers during both surveys.

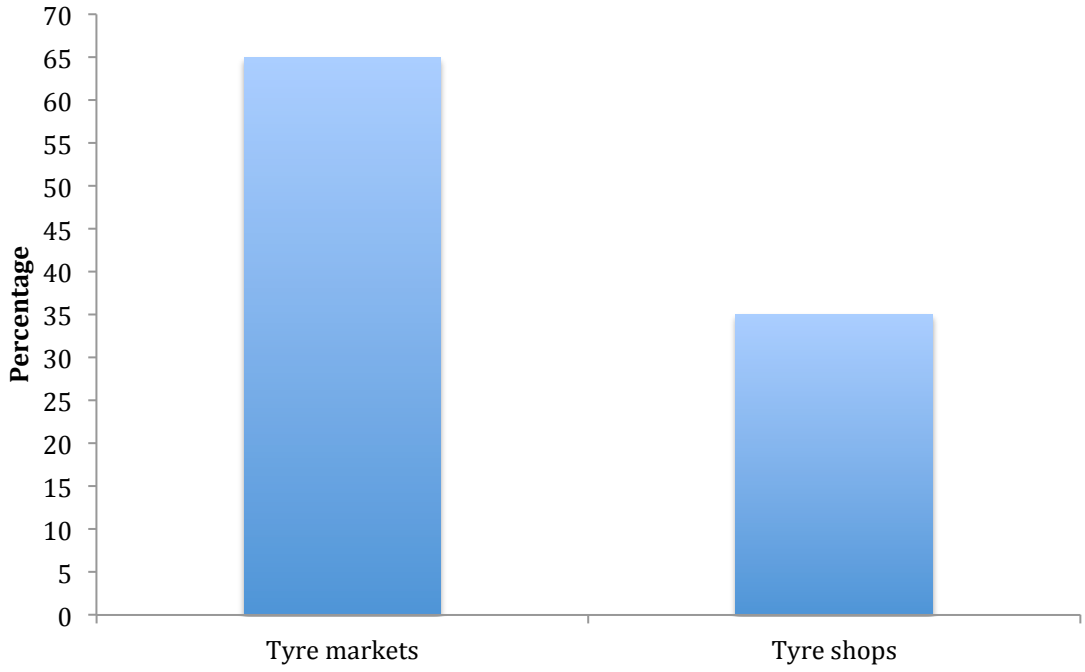

Figure 3.3: Percentage of mosquitoes collected from main tyre markets and independent tyre shops during both surveys.

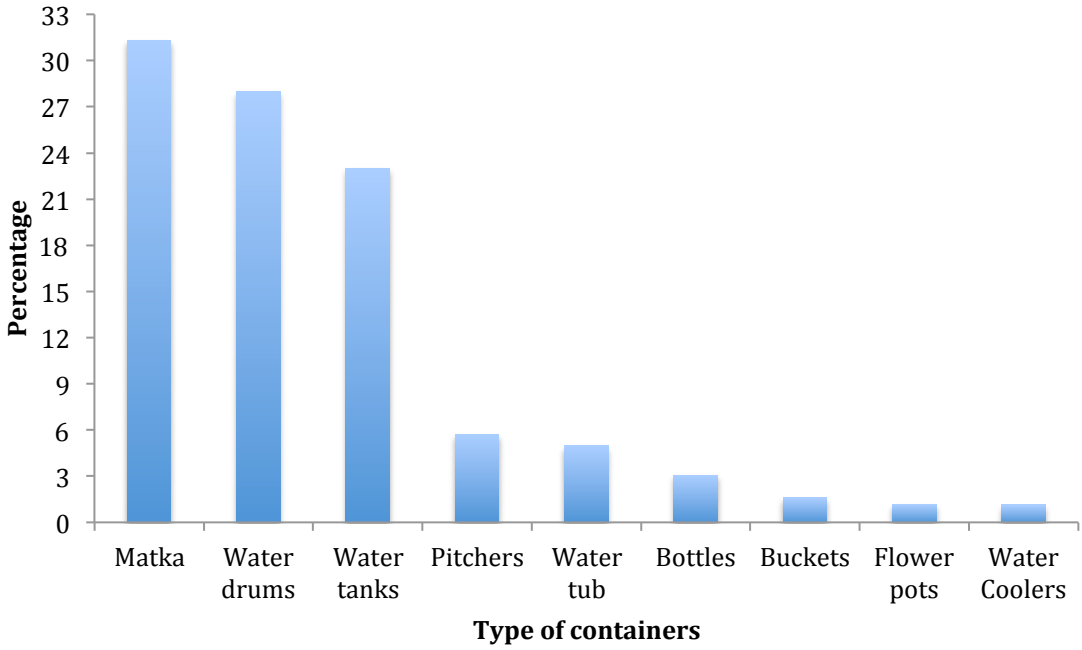

Figure 3.4: Percentage of mosquitoes collected from different types of non-tyre containers during both surveys.

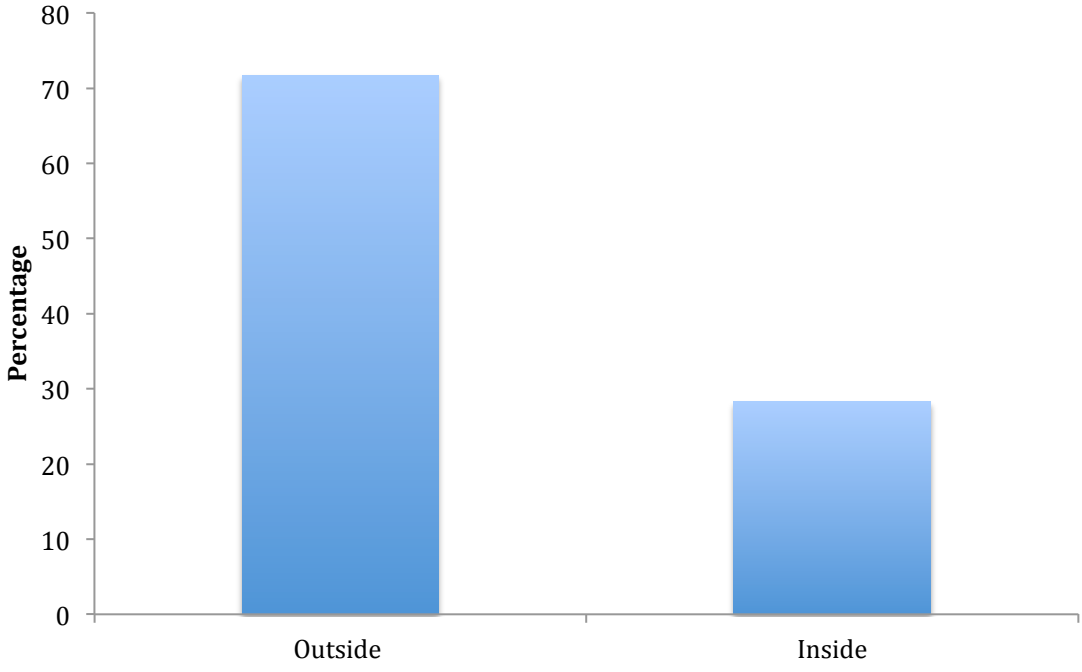

Figure 3.5: Percentage of mosquitoes collected form inside and outside houses during both surveys.

culicifacies and *Ae. unilineatus* were also found in Karachi, *Culex pipiens quiquifasciatus* was found sharing breeding habitat with *Ae. aegypti* in almost every city whereas *Ae. albopictus* was found in Attock, Lahore, Haripur, Hasanabdal, Taxilla and Rawalpindi in the same container having *Ae. aegypti* larvae. *Aedes pseudotaeniatus* species was collected from Attock and Taxilla, in Attock it was found in water tanks whereas in Taxilla in tyres. Larvae from the containers having *Ae. albopictus* and *Ae. unilineatus* were again identified with a better binocular in the lab to minimize the chance of error especially in processing the samples for the population genetics work.

3.4 Discussion

The emergence of a continuous dengue epidemic in Pakistan during the last few years has made it an important health problem within the country. However, no adequate data regarding the occurrence, ecology and distribution of its primary vector i.e. *Aedes aegypti* is available. As a consequence an entomological survey was urgently needed to determine the ecological and geographical distribution of this vector in Pakistan. Here I have described a detailed survey carried out over two years. The initial survey examined a wide geographical area while the second concentrated on the major dengue endemic areas.

These surveys were conducted with two main objectives, first to examine the distribution of *Aedes aegypti* in Pakistan and secondly to gain information on the breeding habitats i.e. type of containers in which this mosquito is breeding in the study area. During the survey the mosquito was collected from a total of 13 different cities in Pakistan. After the reemergence of the mosquito in the early 1980s this is the first report of *Aedes aegypti* from Peshawar and Lahore. The mosquito had never previously been reported from Hyderabad, Multan, Faisalabad, Sheikhupura, Gujranwala, Rawalpindi, Taxilla, Hasanabdal, Haripur and Attock. As such this survey is the first time *Aedes aegypti* has been collected from these areas. The reemergence of *Aedes aegypti* in Pakistan started from Karachi when it was first reported in 1983 (Kamimura et al. 1986). Now this mosquito is well established and distributed in the city of Karachi (Tariq and Zafar 2000, Tariq et al.

2010). Previous studies showed that the distribution of *Aedes aegypti* was either restricted to the port city of Karachi (Kamimura et al. 1986, Tariq and Zafar 2000, Tariq et al. 2010) or to the northern border town of Khyber agency (Suleman et al. 1996) and it has not been collected in any of the surveys in Punjab (Aslamkhan and Salman1969, Reisen et al. 1982, Reisen 1978, Reisen and Boreham 1976) and Khyber Pukhtoonkhwa (Suleman et al. 1993, Suleman and Khan 1993). The present study has shown that *Aedes aegypti* has now expanded its range from the port city of Karachi and is well established in most parts of the country.

In Pakistan a variety of containers were found to be used by *Aedes aegypti* to oviposit its eggs ranging from big water tanks to small plastic containers and bottles. However the maximum numbers of larvae were collected from rain filled tyres. Suleman et al. (1996) had identified tyres as an important breeding point for *Aedes aegypti* in Pakistan. He also described tyres as a potential source for the passive dispersal of *Aedes aegypti*. Not only in Pakistan but also in other countries tyres have been found as an important ovipositing container for *Aedes aegypti.* For example, Dutta et al. 1998 found 54% of the 647 rain filled tyres were positive for *Aedes aegypti* larvae along the national highways and major trunk roads of northeast India. In India tyres have also been reported as an important breeding point in Delhi (Sharma et al. 2005) and Rachni City, Jharkhand State (Singh et al. 2008). Studies conducted in Cibu City, Phillipines (Mahilum et al. 2005), Trinidad (Chaddee 2004), American Samoa (Burkot et al. 2007) and Argentina (Stein et al. 2002, 2007) also reported tyres as a preferable ovipositon site for *Aedes aegypti.* In the current study the number of *Aedes aegypti* contributed by tyres to the environment could not be estimated because most of the tyres in the open air were piled up to the height of ≤ 5 meters or they were dumped in a way that only the top tyres were accessible.

Since tyres were generally outside and exposed to rainfall followed by sun drying, they provide the perfect conditions for *Aedes* embryonic development. As a consequence the majority of the collection was made from them but other containers were also positive for *Aedes aegypti*. These domestic artificial containers ranged from 336 cubic feet water tanks to small bottles but most of the larvae were collected from matkas (closely followed by drums). Other containers containing

larvae were drums, pitchers, water coolers, water tubs, buckets, plant pots and plastic bottles. Big containers like water tanks and drums were cleaned only intermittently by the inhabitants, which allowed the mosquito to oviposit. Other domestic containers were rarely found positive for larvae and larvae were present only when the inhabitants store containers without cleaning and drying them or when they throw them outside or on the roofs of houses. No larvae were collected in this survey from natural containers like tree holes or rock pools. However, *Aedes aegypti* larvae have been reported from natural containers like tree holes, rock pools, coconut shells and banana leaf axils in India (Mahadev et al. 2004, Singh et al. 2008), Phillipines (Mahilum et al. 2005), Thailand (Preechaporn et al. 2006), Trinidad (Chaddee 2004), America (Burkot et al. 2007) and Argentina (Stein et al. 2011). Studies conducted in India (Sharma et al. 2005, Mahadev et al. 2004, Singh et al. 2008), Thailand (Preechaporn et al. 2006) Phillipines (Mahilum et al. 2005), Trinidad (Chaddee 2004), Argentina (Stein et al. 2007, Stein et al. 2011), Costa Rica (Olger et al. 2004), Brazil (Medronho 2009) Fiji (Kay et al. 1995) and American Samaon (Burkot et al. 2007) also reported the presence of *Aedes aegypti* larvae in similar artificial containers as reported here. However our study suggests that in Pakistan *Aedes aegypti* is more likely to breed in domestic containers rather then natural containers (Suzuki & Sone, 1978; Samarawickrema et al. 1993).

Aedes aegypti was collected from indoor as well as outdoor containers but by far the largest number of collection were carried out from outdoor containers placed in shady places. A major reason for the collection from these outdoor containers is that no care has been taken for cleaning these containers. The availability of water also affects the indoor or outdoor breeding of *Aedes aegypti* in subtle ways. In areas where the availability of water was plentiful and the inhabitants don't have to store the water in containers, mosquitoes get less opportunity to breed inside houses. On the other hand in areas with scarcity of water such as in south of Pakistan people use containers including drums and plastic tanks to store water. This was confirmed statistically using chi-square test, which showed that in south this mosquito was getting a better chance to breed in domestic containers rather than in tyres due to availability of domestic containers for laying eggs. In Goa India, Mahadev et al. (2004) observed that the maximum numbers of larvae were collected from outdoor containers due to their exposure to rain during

wet season. Similarly in present study maximum numbers of larvae have been collected from outdoors because of the exposure of these containers to rain and precautionary measures taken by the inhabitants regarding indoor containers. Due to occurrence of epidemics people have begun to take care of the water storage containers. This management minimizes the chances of mosquitoes laying eggs in these containers, and generally if any larvae were collected in one visit then after that the inhabitants started to keep the water storage containers clean. Overall our observations show that *Aedes aegypti* can breed outdoors as well as indoors throughout Pakistan, but that it is overwhelmingly outdoors apart from in areas with water shortages.

The water in the containers from which *Aedes aegypti* larvae have been collected varied considerably. Larvae were present in drinking water as well as turbid water, and found in water containing fallen leaves as well as rancid old water. In Karachi larvae were also collected from brackish water. Wigglesworth (1933) showed that *Aedes aegypti* larvae were not affected by salinity up to 0.9% and if the salinity of water was increased gradually the larvae could adapt to up to 1.75%. In Karachi the maximum salinity was found to go up to 0.8% (Mahmood et al. 1997), which still falls within the limit described by Wigglesworth (1933). In most of the containers *Aedes aegypti* was the only species breeding. In some containers *Anopheles* as well as *Culex* species were also found with *Aedes aegypti* and *Aedes albopictus* were collected from containers, which were near vegetation and many trees that always remained outdoors.

Since breeding is occurring in artificial containers a control strategy should be adapted, depending on the type of containers in which the mosquito is breeding. During and after the rainy season when the mosquito population increases fogging with insecticide with knock down effects is used in the dengue-effected areas. This method only decreases the population of adults but doesn't effect the larval and pupal population allowing subsequent re-emergence. Two main types of artificial containers were identified in this study, commercially sold commodities such as in particular tyres and domestic containers in and around houses. Tyres are the main source of larval breeding in Pakistan and main focus of the government should be on tyre markets during and after rainy season. Larvicides could be used to kill any

larvae in the tyres and this should be done on a regular basis especially after the rainy season until the end of breeding season (December when the temperatures falls to less than 10ºC; a temperature at which larvae are unable to survive (Horsfall 1955)). The continuous use of insecticides could result in the development of resistance within the population (Kaunter et al. 1997), to reduce this risk simple kerosene oil, waste oil or engine oil could be used in addition in the tyres. Recently Djouaka et al. (2007) showed that these oils not only kill the larvae by suffocation but also have a toxic effect on the larvae and as a result they are killed. Experiments have been carried out on resistance strain of *Anopheles gambiae* which showed 100% control by using these oils (Dajouaka et al. 2007). They can have a major impact by not allowing the water to stand in tyres or by adding a small quantity of kerosene oil or engine oil to tyres. Any strategy formulated by the government to reduce the larval population in the areas will not be successful without the help of the local community.

Insecticides have restricted use in domestic containers due to their toxic effects on human health. In this case involvement of the community is the only possible solution to decrease the mosquito population in the area. In particular action that destroys the habitats used by mosquitoes for laying eggs by not discarding containers in the open air or washing small containers thoroughly before storing them and always keep them upside down. Covering the containers used for water storage and thoroughly cleaning them at least once a week is also likely to have a major impact. Covering large containers like drums and water tanks is effective at preventing the laying of eggs. Mechanical control like collecting larvae from drums or other big containers and then throwing those larvae on the earth may also be effective. Big water tanks which are difficult to cover completely and could not be cleaned regularly could be made free of larvae by releasing small local fish like *Poecelia reticulata, Cyprinus carpio, Tilapia mosambicus and Opheocephalus* sp. These fish have been found as a very good source of Biological control in Karachi (Tariq et al. 2011).

It can be concluded that control of this mosquito in Pakistan is not possible without the help of the community and strategies like those in place in the Philippines (Mahilum et al. 2005) should be adopted. These surveys were

conducted to examine the distribution of *Aedes aegypti* in Pakistan and to determine the type of containers in which they are breeding. However a large-scale spatial and temporal study is required to better understand the ecology of *Aedes aegypti* in Pakistan. This will help in formulating better control strategies for this increasingly important vector mosquito in Pakistan with the aim of ultimately controlling dengue.

Chapter 4

POPULATION GENETIC STRUCTURE OF *AEDES AEGYPTI* IN PAKISTAN

4.1 INTRODUCTION

Dengue is an emerging vector borne disease in Pakistan, which is likely to become a much bigger health problem in the coming years (Chapter 2). In Pakistan this mosquito is not only expanding its range but also was found to be breeding in almost all types of containers, especially in tyres (Chapter 3). Tyres have been identified as breeding habitat for *Aedes aegypti* in Brazil (Neto 1997), Trinidad (Chadee 2004), India (Mahadev et al. 2004), Philippines (Mahilum et al. 2005) and American Samoa (Burkot et al. 2007). In India tyres have been reported to play an important role in dispersal and abundance of *Aedes aegypti* on important commercial transport routes (Dutta et al. 1998). Introduction of *Aedes albopictus* into various countries, including the USA, has been attributed to the tyre trade bringing the mosquito in the form of desiccated eggs (Knudsen 1995). International tyre trade and the ability of *Aedes* eggs to desiccate and then hatch when in contact with water has facilitated its establishment in many new countries on different continents (Neto 1997). In Pakistan, mosquitoes collected in 1993 from Landi Kotal, Peshawar, were breeding in tyres stored in warehouses present in the area and for the first time the spread of this mosquito in Pakistan was associated with the tyre trade (Suleman et al. 1996).

Vector-borne diseases like dengue can be controlled by reducing the population of its vector in the affected areas. Insecticide use can serve this cause very efficiently but its extended use could favour insecticide resistance in mosquito populations (Pasteur and Raymond 1996). These resistant individuals could give rise to a population genetically quite different from the eradicated one. Knowledge of the population genetic structure can help to infer dispersal and colonisation patterns and so to model spread of disease or of insecticide resistance. Isoenzyme markers have been used in a number of studies to investigate the genetic structure

of *Aedes aegypti* and its relation with dengue transmission (Mousson et al. 2002). These markers were very helpful in estimating the population structure on a large spatial scale, as in French Guiana (Failloux et al. 2002) and Brazil (Ayres et al. 2004). In some studies more than one genetic marker type has been used to access the population structure of *Aedes aegypti*, as in the use of Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length polymorphism (RFLP) in Trinidad (Yan et al. 1999) and AFLP, microsatellites and isoenzymes in Cambodia, although in this study the F_{ST} values based on the three marker types were different they revealed the same population structure (Paupy et al. 2004). More recently, microsatellites have become the markers of choice in population genetics studies due to their high polymorphism, co-dominance and broad genome distribution (Chambers et al. 2007). Microsatellites have been used not only to determine the genetic structure of *Aedes aegypti* in Vietnam (Huber et al. 2002), Rio de Janerio (Costa-Ribeiro 2006), Southeast Asia (Hlaing et al. 2010) and Australia (Endersby et al. 2009) but also to describe the influence of human transportation on the gene flow of this mosquito in Southeast Asia (Huber et al. 2004).

The aim of this study was to investigate the population structure of *Aedes aegypti* in Pakistan and specifically to test two hypotheses: **i)** *Aedes aegypti* started its colonization in Pakistan from Karachi and moved towards the north, leading to the prediction that expected heterozygosity would decrease with distance from Karachi, and **ii)** Movement of this mosquito in Pakistan is facilitated by the tyre trade, which predicts, **a)** genetic differentiation, F_{ST}, will be low between towns with good road connections and higher between towns with weaker transport links, and **b)** there will be isolation by distance on a larger spatial scale than expected from active dispersal. To test these hypotheses mosquitoes were collected from 13 different cities of Pakistan as described in chapter 3.

4.2 MATERIALS AND METHODS

4.2.1 DNA extraction

DNA was extracted from half of each larva. Those larvae preserved in 70% ethanol were dried first and then cut into two and transferred to 1.5ml flip-top tubes containing 250 µl Digsol buffer (20mM EDTA, 120 mM NaCl, 50mM Tris and 20% SDS, pH 8.0) and 10µl Proteinase K (10mg/ml). Samples were placed in a rack and wrapped in a tissue then shaken at 55°C. After an incubation of 3 hours the liquid turned a straw colour due to suspended protein in solution. To precipitate the proteins, 300µl 4M ammonium acetate was added to each sample and was vortexed several times for 15 minutes at room temperature and centrifuged at 13000 revolutions per minute (rpm) for 10 minutes. The clear liquid containing the DNA was transferred to another 1.5 ml flip-top tube to discard the protein present in the form of a pellet at the bottom. 1 ml of 100% ethanol was added to each sample to precipitate the DNA and then again centrifuged for 10 minutes at 13,000 rpm. Ethanol was poured off taking care not to lose the DNA pellet.

Next, 500 µl 70% ethanol was added to each sample and tubes were inverted several times to rinse the pellet. Each tube was again subjected to centrifugation at 15,000 rpm for 5 minutes to reduce the loss of the DNA pellet in case it was dislodged from the tube during rinsing. Ethanol was then poured off in a smooth movement taking care not to lose the pellet and tubes were made to stand upside down on clean tissue paper for approximately one hour. Once the tube were fully dry, 50µl low T.E (T_{10} $E_{0.1}$) (10mM Tris, 0.1mM EDTA, pH 7.5-8.0) was added to each sample and placed in a water tub having a temperature of 37°C for 30 minutes. During this time tubes were subjected to flicking after every 10 minutes. Samples were stored at 4°C for short term and at -20°C for long term.

4.2.2 Population Genetic Analysis Using Microsatellites

Microsatellites were selected as genetic markers to determine the population structure of this mosquito in Pakistan. Different microsatellite markers have been

identified in *Aedes aegypti* (Huber et al. 1999, Ravel et al. 2001, Chambers et al. 2007 and Slotman et al. 2007) with different numbers of alleles. Thirteen polymorphic microsatellite markers (Table 4.1), with six different dinucleotide repeats; (AT)n, (CT)n, (GT)n, (AC)n, (AG)n, and (GC)n reported by Slotman et al. (2007) were used in this study of the genetic structure of *Aedes aegypti* in Pakistan.

4.2.2.1 Preparation of Primer solution for PCR

Fluorescently labeled primers were ordered from Operon (www.operon.com). These primers were labeled with the ROX set of dyes i.e. 6-FAM (blue), HEX (green) and NED (yellow). In the first step a 5μM primer stock solution was prepared in a 1.5 ml flip-top tube by mixing 50μl of original primer with 950μl of Low T.E. This was done to all reverse and forward primers and stored at -4°C. Primers normally work at the concentration of 0.2μM, so the PCR stock solution was used to prepare 0.2μM primer solution by adding 1μl each of forward and reverse primers and then the level of solution was raised to 25μl by adding Low T.E.

Thirteen polymorphic markers were used in this analysis. Setting up a singleplex reaction was time consuming and costly so multiplex PCR was selected for amplifying these markers. Primers were divided into two multiplex sets and two singleplex, for AG2 and AT1. The multiplexes consisted of one set of six primer pairs and a second set of five primerpairs (Table 4.1). In this case, primer mix was prepared by adding 1μl each of reverse and forward primers that makes 12μl in set 1 and then raising the level of solution to 25μl by adding 13μl of Low T.E., similarly 10μl of primers in set 2 and then raising the level of solution to 25μl by adding 15μl of Low T.E. These primer mixes were then used for PCR. In single plex 1μl each of forward and reverse primers for AG2 and AT1 were mixed in 23μl of Low T.E to make 0.2μM primer mix. Primers along with number of dinucleotide repeats, dye, primer sequence and NCBI Accession numbers are shown in Table 4.1.

4.2.2.2 Multiplexing pre-PCR and PCR

A 2μl Qiagen Multiplex reaction was set up to amplify these 13 markers. 1μl of DNA extracted earlier from each specimen was pipetted out in two 96-well microtitre plates in similar sequence and dried by keeping it open to the air at room temperature for about 30-60 minutes. A mastermix was prepared for each set by mixing 1μl Qiagen mastermix (Taq DNA Polymerase, PCR buffer, $MgCl_2$ and dNTPs) and 1μl primer mix where all primers were at 0.2μM concentration. The overall quantity of this master mix was 10% more than required for each plate.

Each 96-well microtitre plate contained DNA from 86 specimens and 10 were replicas of some of the samples that were extracted from the remaining half of the larvae. 2μl of the master mix from each set was added to the dried DNA in each plate. The plates were labeled for primers set, date and plate number and placed in the PCR machine with the following temperature profile:

95°C for 15 minutes

35 cycles of

94°C for 30 sec
55°C for 90 sec
72°C for 60 seconds

60°C for 30 minutes

4.2.2.3 Genotyping of post-PCR product

In population genetic studies, microsatellite alleles are scored according to their fragment size. At the time of multiplexing, fluorescent labeled primers were used so that they can be identified on the basis of the colour of dye while genotyping them on ABI3730 48-well sequencer. The PCR microplate was removed from the thermo cycler once the reaction was complete. The microplate

Table 4.1. Set of primers used in multiplex and two primers amplified in singleplex.

Name	Repeats	Dye	Primer Sequence 5' →3'			Slotman Size Range	Accession No.
Set 1							
AC4	$(CA)_8$	6-FAM	For	GCGAATCGGTTCCCATAGTA		128–130	CH477271.1
			Rev	CTTTATCGATCGACGCCATT			
AG4	$(GA)_8$	6-FAM	For	AAAACCTGCGCAACAATCAT		147–169	CH478013.1
			Rev	AAGGACTCCGTATAATCGCAAC			
CT2	$(CT)_9$	6-FAM	For	CGCAGTAGGCGATATTCGTT		184–192	CH477294.1
			Rev	ACCACCACCAACACCATTCT			
AG1	$(AG)_9$	HEX	For	AATCCCCACACAAACACACC		113–129	CH477258.1
			Rev	GGCCGTGGTGTTACTCTCTC			
AC5	$(CA)_{12}$	HEX	For	GGATTGTTCTTAACAAACACGAT		149–163	CH477303.1
			Rev	CGATCTCACTACGGGTTTCG			
AG5	$(AG)_9$	HEX	For	TGATCTTGAGAAGGCATCCA		170–180	CH477395.1
			Rev	CGTTATCCTTTCATCACTTGTTTG			
Set 2							
AC7	$(CA)_{14}$	6-FAM	For	TCGGCAAATTACCACAAACA		129–143	CH477308.1
			Rev	CATTGGACTCGCTATAACACACA			
AG3	$(AG)_8$	6-FAM	For	CGCCAAAACTGAAAACTGAA		164–178	CH477258.1
			Rev	AAGGGCGGTGATGACTTTCT			
AC1	$(CA)_8$	6-FAM	For	TCCGGTGGGTTAAGGATAGA		193–209	CH477799.1
			Rev	ACTTCACGCTCCAGCAATCT			
AC2	$(CA)_9$	HEX	For	AATACAACGCGATCGACTCC		176–190	CH477835.1
			Rev	AACGATTAGCTGCTCCGAAA			
AG7	$(GA)_{10}$	NED	For	CGTGCGAGTGAATGAGAGAC		153–185	CH477233.1
			Rev	ATCCTCTCATCAGCTTCTAATAAA			
Singleplex							
AT1	(AT)9	HEX	For	CGTCGACGTTATCTCCTTGTT		141-165	CH477273.1
			Rev	GGACCGGAAAGACACAGACA			
AG2	(AG)14	HEX	For	TCCCCTTTCAAACCTAATGG		98-166	CH478315.1
			Rev	TTTGCCCTCGTATGCTCTCT			

containing amplified fragments of primers were used for genotyping.

The PCR product was diluted 1:1000 by adding double distilled water. The diluted PCR product was prepared according to the standard procedure for genotyping in an ABI sequencer. “GeneScan 500ROX” size standard, a ROX red dye-labeled size standard, was used for sizing of fragments on an ABI gene sequencer. A mixture of Hi-Di formamide and ROX size standard was prepared by mixing 5µl of ROX size standard to 1ml of formamide. Formamide is a loading solution used for denaturing of DNA. Formamide is highly dangerous so all steps having formamide were performed in a fume hood. 1µl of diluted post-PCR product was added to a special type of loading plate and 9.5µl of formamide/size standard mixture was added to each well and was covered with a clean rubber septum.

DNA was denatured at 95°C for 3 minutes using the PCR machine and then placed on icy water for about 3 minutes, this denatured the DNA and due to the temperature shock it did not re-nature. The plate was then placed in a special plate cover and placed in the ABI sequencer for genotyping. Raw data from the ABI sequencer was transferred to a computer containing software GeneMapper v3.7. This software is used to score the alleles. The alleles were binned and scored and values for each locus were exported in text file format. The raw data were then arranged and used for analysis as described below.

4.2.3 Statistical Analysis

4.2.3.1 Arrangement of Data

Raw data retrieved from GeneMapper v3.7 were arranged in order to use it for different statistical analyses. Each specimen was given a specific identity in the form of a number and given a longitude and latitude value noted at the time of collection of that specimen. The data were arranged by considering population structure at three different levels or as continuous isolation by distance and checked for different parameters. In the first level the population was arranged according to the city from where they were collected, dividing the genotypic data into 13

different populations. In the second level the data were divided according to the patch within the city thus making 42 populations and then data were divided according to containers from where these were collected making 101 populations. Population genetic analysis was performed on these three data sets to know which best explains the population structure.

4.2.3.2 Error Rate

A total of 850 samples were selected for genetic analysis. Among these samples 102 were replicated by using the remaining half of the larva to estimate error rate. Error rate was estimated by the following formula

$$\text{Error rate} = \frac{\text{No of Alleles different in replicas}}{\text{Total no of Alleles Scored}} \times 100$$

Alleles were checked for any difference between the replicas and then that number was divided by total number of alleles scored. Each locus has two different alleles if heterozygous or two similar alleles if homozygous. Total number of alleles scored was calculated by multiplying the number of replicas with number of loci and then multiplying it with two, as genotype data were diploid. So the number of alleles scored was

$$\text{Total number of Alleles Scored} = \text{No. of replicas} \times \text{No. of loci} \times 2$$

$$\text{Total number of Alleles Scored} = 102 \times 12 \times 2$$

$$= 2448$$

$$\text{Error rate} = \frac{\text{No of alleles different in replicas} \times 100}{2448}$$

4.2.3.3 Checking for Null Alleles

The mutation rate in the flanking region sequences of microsatellites is non-negligible and mutations may prevent the primer annealing to the template DNA of the microsatellite locus during PCR amplification, resulting in null alleles. Preferential amplification of short alleles due to irregular DNA quality or quantity

or slippage during PCR amplification could be the other possible causes of occurrence of microsatellite null alleles (Chapius and Estoup 2007). Genotyping errors due to non-amplified alleles (null alleles) and PCR artefacts cause large allele dropout and stuttering resulting in deviation from Hardy-Weinberg equilibrium (HWE) and biased spatial and temporal population genetic estimates (Oosterhout et. al. 2004). To minimize these errors, null alleles were checked by using the programs CERVUS (Marshall et al. 1998; downloadable at http://www.fieldgenetics.com/pages/ aboutCervus_Overview.jsp) and MICRO-CHECKER (Oosterhout et. al. 2004; downloadabale at http://www.microchecker.hull.ac.uk/).

CERVUS was developed for the analysis of co-dominant markers assuming that markers are autosomal and the species under investigation is diploid. CERVUS calculates the frequency of each allele for each locus in a population along with other summary statistics like HW and presence of null alleles. Microsatellite data from a diploid population can also be tested by using MICRO-CHECKER, which helps in detecting various genotyping errors which not only estimates the null allele frequency at certain locus using a series of algorithms but also compares it to null allele frequencies obtained using methods of Chakraborty (Chakarborty et al. 1992) and Brookfield (Brookfield 1996) and adjusts genotype and allele frequencies allowing the data to be used in other population genetics analysis packages like FSTAT and GENEPOP.

4.2.3.4 Hardy-Weinberg Test and Linkage Disequilibrium

Genotypes for a certain locus are in HWE proportion when they are combined at random due to random mating. Alleles at different loci are in a state of linkage equilibrium when they are in random association and if they are not, they are said to be in linkage disequilibrium (Hartl and Clark, 1997). Linkage disequilibrium results in allele frequencies being insufficient to describe genetic variation. To interpret genetic diversity in a population, it is important to know whether all loci are randomly associated and in HWE. Data obtained after genotyping were checked for linkage disequilibrium and HWE by using modified

version of GENEPOP v4.0 (Raymond and Rousset 1995; downloadable from http://kimura.univ-montp2.fr/~rousset/Genepop. htm).

GENEPOP can perform three different tests for HWE having the same null hypothesis that gametes combine at random. The HWE test used by Haldane (1954), Weir (1996), Guo and Thompson (1992) and others was adopted in this analysis. In this test, the P-value is the sum of the probabilities of all tables having the same marginal allelic counts with lower or equal probability under the null hypothesis. The data were checked for Hardy-Weinberg equilibrium by dividing the data into 42 different populations according to the patches within the cities from where samples were collected.

GENEPOP tests the null hypothesis of linkage equilibrium, which is that "Genotypes at one locus are independent from genotypes at the other locus". Pair-wise linkage disequilibria were checked by dividing the data into 42 different populations according to the patches within the cites from where samples were collected.

GENEPOP performs more than one test of HWE and linkage disequilibrium and returns the P-values. In such cases, the chances of Type I errors increase. Bonferroni and Sequential Bonferroni-type correction procedures decrease the type I errors but at the cost of type II errors. Benjamini and Hochberg false discovery rate (FDR) (α/m, 2α/m, 3α/m…….. i α /m; where α= 0.05 and m is the total number of tests performed) procedure was used to control type I errors, simultaneously reducing type II errors (Verhoeven et al. 2005).

4.2.3.5 Genetic Distance Based Analysis

The total variance was divided into component variances due to presence of different hierarchical population levels. In the presence of several levels of hierarchial sampling it would be convenient to test for the effect of each level in the hierarchy independently of the effect of lower levels (Goudet, 2005). It was done by using HIERFSTAT, a package of R statistical software (R Development Core Team

2004) developed by Jerome Goudet (Goudet, 2005). This package can be used to estimate variance components at each level and hierarchical F-statistics for any number of hierarchical levels using Yangs's algorithm. Using HIERFSTAT the total variance was divided among four sampling levels in a nested manner (Cities nested in patches, patches nested in samples (containers), sample nested in individuals). The significance of the different levels of differentiation was tested by 1000 permutations for each analysis.

Inbreeding due to population fragmentation can be used to measure the degree of differentiation among various population fragments. Inbreeding coefficients within and among populations are directly related to differentiation among subpopulations. Wright's (1921) fixation index has been used in this analysis to measure genetic differentiation. F_{ST} is the " inbreeding due to differentiation among subpopulations relative to the total population" or in other words it is the fixation index of subpopulations relative to total combined population (Frnakham et al. 2002, Hartl and Clark 1997). F_{ST} analysis was carried out to document the differentiation among different populations in Pakistan. SPAGeDi 1.3 (Hardy and Vekemans 2002; downloadable at http://ebe.ulb.ac.be/ebe/Software.html) was used to measure population differentiation according to the Weir and Cockerham (1984) method. The average number of breeders per subpopulation was estimated using an equation proposed by Pudovkin et al. (1996) and implemented as equation 2 in Balloux (2004). The following equation was used to calculate the effective breeding population size

$$N_{eb} = 1/(-2F_{IS}) + ½(1-F_{IS})$$

Along with population differentiation, F_{ST} /(1- F_{ST}) was also calculated to estimate isolation by distance by plotting this value against log of distance (Rousset, 1997). Jackknifing of data was used to calculate the significance of population differentiation, with 10,000 permutations using SPAGeDi.

4.2.3.6 Correlation Analysis

Correlation analysis was used to detect the process of colonization of *Ae. aegypti* in Pakistan. Spearman rank correlation was used to test the relationship between expected heterozygosity and distance. Expected heterozygosity was calculated using CERVUS where as distance was taken in kilometers from Karachi designated as point of origin for distance measurement. Spearman rank correlation analysis was carried out using the online Free Statistics Software (Wessa 2011, downloads at http://www.wessa.net/).

It is difficult to assess a relationship and test its statistical significance between two matrices by simply evaluating the correlation coefficient between the two sets of distances. The Mantel test is used to deal with this problem by subjecting the columns and rows of one of the matrices to random permutations many times and recalculating the correlation after each permutation to assess the significance of any departure from a zero correlation (Sokal and Rohlf, 1995). ZT is a free software tool (Van de Peer, Y. 2002, downloaded at http://www.psb.ugent.be/~erbon/mantel/), which performs simple and partial Mantel tests. The simple Mantel test looks for correlation between two matrices only, with the null hypothesis of no correlation between them, whereas partial the Mantel test investigates the correlation between two matrices while controlling the effect of a third matrix, thus trying to remove any false correlations (Van de Peer, 2002).

Three types of matrices were produced for correlation analysis using Simple Mantel and Partial Mantel tests. A matrix of genetic distance was created using SPAGeDi 1.3. A unique type of matrix was created by using the information of transport and roads connections between pairs of cities. The transport and road links between two cities are given in table 4.2. Each category was allotted a number (table 4.3 and 4.4) and then the two numbers for each city were added and combined into a matrix (table 4.5), which was taken as road connection between the cities. The road connection matrix was checked for different scoring methods and it was found that the results were not affected either the scores decline linearly or non-linearly. A geographical distance matrix was created by calculating

geographical distance between two cities shown as italicized in table 4.5. Simple and Partial Mantel tests were used to correlate these matrices.

4.2.3.7 Model-based Structure Analyses

Patterns of population genetic structure of *Aedes aegypti* in Pakistan were investigated using multilocus Bayesian approaches to data from 13 different populations genotyped at 11 microsatellite loci from nuclear DNA. Pritchard et al. 2000 developed a software STRUCTURE based on Bayesian clustering algorithms for inferring population genetic structure. STRUCTURE probabilistically assigns individuals to clusters on the basis of their multi-locus genotypes. Populations are grouped by minimizing HW and linkage disequilibrium within clusters without taking into account pre-defined populations (Pritchard et al. 2000). STRUCTURE estimates the most likely number of genetic subpopulations or clusters (K) by estimating the log probability of the data Pr ($X|K$) for each value of K (Pritchard et al. 2000).

Ten independent runs of K = 1-14 were performed assuming an admixture model with separate α (alpha) for each population and correlated allele frequencies with 1,000,000 Markov chain Monte Carlo (MCMC) iterations and a burnin period of 500,000. The number of genetic clusters or subpopulations (K) was estimated using STRUCTURE v2.3.3 (Pritchard et al. 2000). Ten independent runs of K = 1-14 were performed, assuming an admixture model with separate α for each population and correlated allele frequencies with 1,000,000 Markov chain Monte Carlo (MCMC) iterations and a burnin period of 500,000. Mean estimated log-likelihood of K was used to choose the optimal K (Evanno et al. 2005).

The STRUCTURE algorithm may be poorly suited for estimating the number of genetic structures in the presence of isolation-by-distance (Pichard and Wen 2003, Evanno et al. 2005). Therefore ΔK statistic was used on the basis of rate of change of the successive K values to estimate the uppermost level of structure in the data set, as recommended by Evanno et al. (2005). Evanno et al. calculate ΔK first by calculating the difference between successive likelihood values of K, $L'(K)$ given as

Table 4.2: Transport Categories between different cities.

	Peshawar	**Attock**	**Hasanabdal**	**Haripur**	**Taxill**	**Rawalpindi**	**Gujrnawala**	**Sheikhupura**	**Lahore**	**Faisalabad**	**Multan**	**Hyderabad**
Attock	Main Road Side Road Link Road Transport											
Hasanabdal	Main Road Better Transport	Link road side road main road Less transport										
Haripur	Main Road Side Road Better Transport	Link road Side road Main road Side road less transport	Side road Better transport									
Taxilla	Main Road Best Transport	Link road Side road Main road Normal transport	Main road Best transport	Main road Side road Best transport								
Rawalpindi	Motorway Main Road Excellent Transport	Link road Side road Main road Good transport	Main road Best transport	Side road main road better transport	Main road Best transport							
Gujranwala	Main Road Better Transport	Side road Link road Main road Less transport	Main road good transport	Side road Main road Good transport	Main road Good transport	Main road Better transport						

	Peshawar	Attock	Hasanabdal	Haripur	Taxilla	Rawalpindi	Gujrnawala	Sheikhupura	Lahore	Faisalabad	Multan	Hyderabad
Sheikhupura	Main Road Side Road Good Transport	Link road Side road Main road Side road Less transport	Main road Side road Normal transport	Side road Main road Side road Less transport	Main road Side road Normal transport	Main road Side road Good transport	Main Road Side Road Better transport					
Lahore	Motorway Main Road Best Transport	Link road Side road Main road Less transport	Main road Motorway Better transport	Side road Main road Good transport	Main road Better transport	Motorway Main road Excellent transport	Main road Excellent transport	Main road Better transport				
Faisalabad	Motorway Main road Better transport	Link road Side road Main road Less transport	Main road Motorway Best transport	Side road main road Normal transport	Main road Good transport	Motorway Main road Better transport	Main road Better transport	Side road Better Transport	Main road Motorway Better transport			
Multan	Main road Good transport	Link road Side road Main road No transport	Main road Less transport	Side road Main road Less transport	Main road Normal transport	Main road Better transport	Main road normal transport	Main road Less transport	Main road Better transport	Main road Good transport		
Hyderabad	Main road Normal transport	Link road Side road Main road No transport	Main road less transport	Side road main road less transport	Main road Normal transport	Main road Less transport	Main road less transport	Main road Less transport	Main road Less transport	Main road less transport	Main road Normal transport	
Karachi	Main road Best transport	Link road Side road Main road No transport	Main road less transport	Side road main road less transport	Main road normal transport	Main road Good transport	Main road less transport	Main road Less transport	Main road good transport	Main raod good transport	Main road better transport	Main road excellent transport

Table 4.3: Score for road quality used for driving the road connection matrix.

Road quality	Score
Main Road and Motorway	10
Motorway or Main Road	9
Main Road and Side Road	8
Side Road	7
Main Road and Link Road	6
Main Road, Side Road and Link Road	5
Link Road, Side Road, Main Road and Side Road	4

Table 4.4: Score for transport links used for driving the road connection matrix.

Transport links	Score
Excellent	10
Best	9
Better	7
Good	5
Normal	3
Less	1
No regular Transport	0

Table 4.5: Pairwise matrix of geographical distance in kilometres (italicized) and road connections prepared from Table 4.3 and Table 4.4.

	Peshawar	Attock	Habdal	Haripur	Taxilla	Rpindi	Gwala	Spura	Lahore	Fabad	Multan	Habad	Karachi
Peshawar	-	*83*	*108*	*127*	*120*	*146*	*320*	*341*	*378*	*321*	*423*	*1004*	*1100*
Attock	6	-	*50*	*40*	*56*	*73*	*238*	*261*	*297*	*250*	*444*	*981*	*1086*
Hasanabdal	16	6	-	*30*	*14*	*41*	*232*	*264*	*297*	*271*	*418*	*1025*	*1132*
Haripur	15	5	14	-	*33*	*44*	*231*	*271*	*300*	*285*	*410*	*1018*	*1126*
Taxilla	18	8	18	17	-	*27*	*219*	*251*	*283*	*261*	*412*	*1022*	*1130*
Rawalpindi	20	10	18	15	18	-	*194*	*228*	*259*	*244*	*409*	*1018*	*1128*
Gujranwala	16	6	14	13	14	16	-	*52*	*69*	*133*	*339*	*941*	*1066*
Sheikhupura	13	5	11	7	11	13	15	-	*39*	*92*	*292*	*891*	*1018*
Lahore	19	6	17	13	16	20	19	16	-	*120*	*310*	*899*	*1028*
Faisalabad	17	6	19	11	14	17	16	14	17	-	*206*	*812*	*935*
Multan	14	5	10	9	12	16	12	12	16	14	-	*614*	*732*
Hyderabad	12	5	10	9	12	10	10	10	10	10	12	-	*145*
Karachi	18	5	10	9	12	14	10	10	14	14	16	19	-

Legend: Habdal = Hasanabdal, Rpindi = Rawalpindi, Gwala = Gujranwala, Spura = Sheikhupura, Fabad = Faisalabad, Habad = Hyderabad.

$$L'(K)=L(K)-L(K-1)$$

In the second step $L''(K)$ was calculated by finding the difference between the successive values of $L'(K)$, given as

$$L''(K)=L'(K+1)-L'(K)$$

Mean absolute value of $L''(K)$ was divided by its standard deviation to get the value of ΔK.

$$\Delta K=m(|L''(K)|)/s[L(K)]$$

The maximum value of ΔK should be located at the real K. The height of this modal value of ΔK was described by Evanno et al. as an indicator of the strength of the signal detected by structure. Arcglobe (http://www.arcgis.com/home/group.html?owner=esri&title=ESRI%20Maps%20and%20Data); a GIS software was used to map the information on assignment of individuals to each group defined by STRUCTURE.

4.3 RESULTS

4.3.1 PCR And Genotyping Results

A total of 13 microsatellite loci were amplified in two primer set combinations for a total of 850 individuals of *Ae. aegypti,* 464 specimens from the first survey and 386 from the second survey. A maximum number of eight larvae were taken from each container whereas the minimum number of larvae was 3. Additionally, 102 individuals were replicated randomly to assess the error rate while scoring the genotypes. All 850 individuals were scored successfully at all 13 loci. Numbers of alleles for different loci, their size ranges and comparison with previous studies is shown in Table 4.6.

Table 4.6: Loci with number of alleles and size range**.**

Name of locus	Current study in Pakistan		Thaung study in South East Asia (Hlaing et al. 2010)		Slotman study Slotman et al. 2007	
	No. of alleles	Size range	No. of alleles	Size range	No. of alleles	Size range
AC4	2	113-115	4	111-117	2	128-130
AC5	16	130-152	9	131-148	6	149-163
AG1	6	100-110	7	100-112	7	113-129
AG4	8	130-155	9	130-156	7	147-169
AG5	6	152-166	8	153-167	6	170-180
CT2	4	168-181	8	168-182	3	184-192
AC1	5	179-196	7	179-196	5	193-209
AC2	5	169-177	6	165-175	5	176-190
AC7	9	112-129	10	112-131	5	129-143
AG2	29	98-166	14	100-130	10	115-178
AG3	5	147-156	10	146-164	6	164-178
AG7	10	137-167	14	136-182	10	153-185
AT1	13	141-165	10	139-158	8	156-174

Table 4.7: Loci showing errors while scoring alleles both in samples and its replica.

Locus	Alleles in samples	Alleles in replica
AC4	113, 113	113, 115
AG1	100, 104	104, 104
AG3	150, 154	154, 154
CT2	173, 173	168, 173
AC4	113, 115	113, 113
AG1	100, 106	100, 100
AG4	130, 153	130, 151
AG7	140, 140	140, 143
AG7	139, 150	152, 152

4.3.2 Error Rate

A total of 2448 alleles were scored for 12 loci in 102 individuals and only 9 alleles, in 9 individuals, disagreed with the original scoring making the estimated error only 0.36%. No particular locus was over-represented among these scoring errors (Table 4.7). The thirteenth locus, AC5 had alleles with a difference of only one nucleotide and showed a very high error rate in genotyping. This locus was removed from subsequent analyses.

4.3.3 Null Alleles

A locus was considered unsuitable for further analysis if it had an estimated frequency of null alleles of more than 0.15 by all methods of estimating null alleles. The data were divided into two sets on the basis of sampling year because MICRO-CHECKER can only analyze data up to 750 individuals The results showed a high percentage of null alleles for locus AG5, i.e. 0.24, in the 2009 data set and 0.19 in the 2010 data set. This locus also showed high null allele frequency by the Chakraborty and Brookfield method as shown in Table 4.8.

CERVUS also gives percentage of null alleles at each locus. CERVUS could analyze big data sets so the data set was checked for null alleles by combining the new and old data set as well as separately. Only one locus, AG5, was considered positive for null alleles, showing an estimated null allele frequency of more than 0.15. The results were almost similar to those from MICRO-CHECKER. The locus showing high null allele frequency by MICRO-CHECKER was also giving null allele frequency of more than 0.15. Table 4.8 compares results of MICRO-CHECKER and CERVUS for each locus. The estimated null allele frequency for locus AG5 was much higher than for any other locus and so AG5 was excluded from subsequent analyses.

4.3.4 Hardy-Weinberg Test and Linkage Disequilibrium

The microsatellite data were checked for HWE at each locus by using CERUVUS and GENEPOP. Both results showed that all the loci were in HWE in

almost all populations. Loci that deviated from HWE in some of the populations may be due to population structure within the sample analyzed, which as at the patch level. As this deviation was not consistent across populations or loci, all were retained for further analysis. Table 4.9 shows the HWE results for 11 loci in all 42 patches.

Similarly to HWE, random mating results in random association of alleles at different loci and thus they are in a state of linkage equilibrium; if they are not then they are said to be in linkage disequilibrium. To determine that all loci can be treated independently, they should be in a state of linkage equilibrium. GENEPOP was used for checking pair-wise linkage equilibrium. The data were divided into 42 different populations according to the patch from which they were collected.

A total of 2310 pair-wise tests for linkage equilibrium were conducted, out of which 72 pairs showed significant deviations after correction with the Benjamini and Hochberg (1995) FDR method. As these linkage disequilibria were detected between different locus pairs and not in the same loci in all samples, it is likely that they are due to structure within patches among containers, rather than to close linkage. Therefore, all 11 loci were used in all subsequent analyses.

4.3.5 GENETIC STRUCTURE ANALYSIS

Two surveys were conducted with a separation of one year and samples from both surveys were used for genotyping. In populations of small size having multiple generations in a year, allele frequency can vary with the passage of time, which could effect the proper estimation of genetic structure. To check that the population of *Ae. aegypti* in Pakistan is large enough that its allele frequency may have not varied due to the year difference between the two data sets, allele frequencies were calculated by using CERVUS and were graphed to see if there is any large differences in allele frequencies (Appendix 2 to 12). The comparison of allele frequency showed very little difference between the two data sets showing that the population was large enough to minimize any abrupt change in allele frequency due to genetic drift, mutation or migration.

4.3.5.1 Hierarchical F-Statistics

The Hierarchical F-Statistics (HIERFSTAT), which divide the total variance into components due to among population, within groups among populations and within population differences were used to test the effect of year difference between the two data sets. HIERFSTAT showed that only 0.93% of the total variation is contributed to the data set due to year effect (Table 4.10) with hierarchical *F*-statistic key value $F_{year/city}$ = 0.009 (P<0.001) describing variation that is attributed to the time span difference of one year between the two data set which was too low to affect the overall estimate of population genetic structure. HIERFSTAT and comparison of allele frequencies showed that the population of *Aedes aegypti* was large enough to neutralize any significant change in allele diversity due to genetic drift, migration or mutation revealing that the effect of an year difference between the two data sets were very low and the two data sets were combined for further analysis of population genetics.

HIERFSTAT was also used to detect the basic population structure among the individuals in the combined data set from the two years of sampling. Four levels of sampling hierarchy were checked with HIERFSTAT, but at individual within container level $F_{individual/sample}$, the *F*-statistics value was negative so this hierarchial level was removed and only three levels of sampling hierarchy were used to partition the variance at different sampling levels (Tables 4.11 and 4.12). *F*-statistics describing variation at three different sampling levels was calculated by HIERFSTAT. $F_{city/total}$ gives variation that is attributed to the cities from where collection was done which was 0.0607 (P=0.001), $F_{patch/city}$ describes the variation at patches within the cities giving a value of 0.055 (P=0.001), $F_{individual/patch}$ gives variation at individuals within patches having a value of 0.033 (P=0.001).

The variance component analysis shows that the maximum variation of 85.8% came from within individuals whereas among individuals within containers contributed nothing to the overall variance. Cities contributed 6.07% of the total variance whereas among patches and within patches contributed 5.18% and 2.96% of variance to the overall variation. Individuals separately contributed more than

Table 4.8: Null allele frequency at all loci as calculated by MICRO-CHECKER and CERVUS. High null allele frequencies are shown by underlining them.

Loci	CERVUS			MICRO-CHECKER							
				Old Data Set				New Data Set			
	All Data	New Data	Old Data	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
AC1	0.0703	0.0487	0.0877	0.0783	0.0864	0.0587	0.0587	0.0458	0.0442	0.0322	0.0322
AC2	0.1045	0.0953	0.1106	0.0973	0.1096	0.0794	0.0794	0.0853	0.0953	0.071	0.071
AC4	0.0425	0.0361	0.0475	0.044	0.0475	0.0281	0.0281	0.0339	0.0361	0.0213	0.0213
AC7	0.0426	0.0717	0.0152	0.0148	0.0164	0.0101	0.0101	0.0637	0.0731	0.0442	0.0442
AG1	0.0897	0.1187	0.0624	0.058	0.0655	0.0513	0.0513	0.1041	0.1202	0.0909	0.0909
AG2	0.0757	0.068	0.0777	0.0716	0.0782	0.0669	0.0669	0.0634	0.0666	0.0585	0.0585
AG3	0.0874	0.0833	0.0816	0.0738	0.0782	0.0575	0.0575	0.0756	0.0765	0.059	0.059
AG4	0.0795	0.048	0.0993	0.0872	0.0968	0.0671	0.0671	0.0454	0.0427	0.034	0.034
AG5	0.2956	0.2404	0.3414	0.2424	0.3422	0.2144	0.2144	0.189	0.2403	0.1718	0.1718
AG7	0.0684	0.0754	0.0512	0.0482	0.0514	0.0414	0.0414	0.0695	0.0755	0.0626	0.0626
AT1	0.0332	0.0378	0.026	0.0253	0.0294	0.0261	0.0261	0.0363	0.0388	0.0344	0.0344
CT2	0.1036	0.0998	0.0974	0.0829	0.0935	0.0509	0.0509	0.0867	0.0975	0.0598	0.0598

Table 4.9: HWE results for all 42 populations at 11 loci. Tests, which were significant after Benjamini and Hochberg false discovery rate (FDR), are denoted by (***). (P=Peshawar, R=Rawalpindi, T=Taxilla, L=Lahore, S=Sheikhupura, G=Gujranwala, F=Faisalabad, M=Multan, A=Attock, K=Karachi, H.R=Haripur, H.L=Hasanabdal, H.D=Hyderabad).

	AC1	**AC2**	**AC4**	**AC7**	**AG1**	**AG2**	**AG3**	**AG4**	**AG7**	**AT1**	**CT2**
P1	N.S	***	N.S	N.S	***	N.S	N.S	N.S	N.S	N.S	N.S
P2	N.S	N.S	N.S	N.S	***	N.S	N.S	N.S	N.S	N.S	N.S
P3	N.S	N.S	N.D	N.D	N.S	N.S	N.S	N.S	N.S	N.S	N.S
R4	N.S	N.S	N.S	N.S	N.S	***	N.S	N.S	N.S	***	N.S
R5	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
T6	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
L7	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
L8	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
L9	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
L10	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
S11	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
G12	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
F13	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
F14	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
F15	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
M16	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
A17	N.S	N.S	N.S	N.S	N.S	***	N.S	N.S	N.S	N.S	N.S
A18	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
K19	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
K20	N.S	N.S	N.S	N.S	N.S	N.S	N.S	***	***	***	N.S
K21	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
HR22	N.S	***	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	***
HR23	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
HL24	N.S	N.S	N.S	N.S	N.S	***	N.S	N.S	N.S	N.S	N.S
A25	N.S	N.S	***	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
T26	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
R27	N.S	N.S	N.S	N.S	N.S	***	N.S	N.S	N.S	N.S	N.S
R28	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
S29	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
L30	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
L31	N.S	***	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
M32	N.S	***	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
HD33	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
K34	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	***	N.S	N.S
K35	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
K36	N.S	N.S	N.S	N.S	N.S	***	N.S	N.S	N.S	N.S	N.S
K37	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
K38	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	***	N.S
K39	N.S	N.S	N.S	N.S	N.S	***	N.S	N.S	N.S	N.S	N.S
K40	N.S	N.S	N.S	N.S	N.S	***	N.S	N.S	***	***	N.S
K41	N.S	N.S	N.S	N.S	***	***	N.S	N.S	N.S	N.S	N.S
K42	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	***	N.S

Table 4.10: HIERFSTAT table showing variance components and *F*-statistics value to know the effect of separation of year on the two data sets.

Source of Variation	**Variance Component**	**Percentage of Variation**	**Hierarchical *F*-statistics**	***F*-Statistics Values**	**Significance *P* < 0.05**
Among Cities	0.4364	5.34%	$F_{city/total}$	0.053	*** (*P*=0.001)
Year within Cities	0.0761	0.93%	$F_{year/city}$	0.009	*** (*P*=0.001)
Patches within Year	0.4006	4.91%	$F_{patch/year}$	0.052	*** (*P*=0.001)
Individuals within Patches	0.2985	3.65%	$F_{individual/patch}$	0.041	*** (*P*=0.001)
Within Individuals	6.9623	85.17%			
Total	8.1739				

Table 4.11: HIERFSTAT table showing variance components and *F*-statistics at different hierarchical sampling levels.

Source of Variation	**Variance Component**	**Percentage of Variation**	**Hierarchical *F*-statistics**	***F*-Statistics Values**	**Significance *P* < 0.05**
Among Cities	0.4449	5.98%	$F_{city/total}$	0.06	*** (*P*=0.001)
Patches within cities	0.1569	2.11%	$F_{patch/city}$	0.02	*** (*P*=0.001)
Samples within Patches	0.5702	7.66%	$F_{sample/patch}$	0.08	*** (*P*=0.001)
Individuals Within Samples	-0.1339	0%	$F_{individual/sample}$	-0.02	*** (*P*=0.001)
Within Individuals	6.2729	84.25%			
Total	7.4449				

Table 4.12: HIERFSTAT table showing variance components and *F*-statistics at different hierarchical sampling levels after removing the container level of sampling.

Source of Variation	**Variance Component**	**Percentage of Variation**	**Hierarchical *F*-statistics**	***F*-Statistics Values**	**Significance *P* < 0.05**
Among Cities	0.4440	6.07%	$F_{city/total}$	0.0607	*** (*P*=0.001)
Patches within cities	0.3785	5.18%	$F_{year/city}$	0.055	*** (*P*=0.001)
Individuals Within Patches	0.2163	2.96%	$F_{individual/patch}$	0.033	*** (*P*=0.001)
Within Individuals	6.2729	85.8%			
Total	7.3117				

groups at different levels thus showing presence of a weak population structure at different levels at which collection of this mosquito was done in Pakistan.

4.3.5.2 F-statistics

Two components of F-statistics were used in this analysis to measure the genetic structure, F_{ST} and F_{IS}. F_{ST} was used in this analysis to measure the genetic structure. F_{ST} value represents the differentiation among subpopulations (populations within groups) relative to the total population (groups) and F_{IS} or inbreeding coefficient is the measure of the proportion of the variance contained in an individual of a subgroup, both of which were calculated for the overall population as well as for each city. F_{ST} for the overall population was calculated in three different ways on the basis of level of collection, this was done first by dividing the main population into 13 subpopulations, i.e. the city from which these mosquitoes were collected, and secondly by dividing the population into 42 different subgroups, according to the area from which they were collected within a city, and finally according to the container of collection.

F_{ST} value at the sample level was much higher (F_{ST}=0.15) than at patch (F_{ST}=0.106) or city level (F_{ST}=0.0779) (Table 4.13). In all three cases, the F_{ST} was significant ($P<0.05$) showing that these subgroups within these populations are significantly different from each other and these values were not obtained by chance. High F_{ST} values at the sample level indicate that population structure is not only present at the overall city level but also present within cities. F_{ST} for populations present within 13 cities were also calculated. Of these 13 cities, the F_{ST} for populations present within 8 cities were significantly different from zero ($P<0.05$) with F_{ST} ranging between 0.06 for Rawalpindi to 0.23 for Peshawar indicating a higher degree of genetic differentiation of the individuals within Peshawar as compared to other cities. However the F_{ST} value obtained from within city population comparison of Faisalabad, Gujranwala, Hasanabdal, Haripur and Hyderabad were not genetically significantly different ($P>0.05$). F_{ST} values of Gujranwala and Hyderabad were negative (-0.0212) which means there was no difference among sub populations relative to overall population present in these cities. Faisalabad also has a very low F_{ST} value (0.0089), which was not significant.

A positive F_{IS} means that the number of heterozygous individuals in the population is less than expected, usually due to inbreeding. Negative F_{IS} reveals greater than expected heterozygosity. Positive and significant F_{IS} values of 0.07 and 0.03 (P<0.05) were observed at city and patch levels of collection showing an excess of homozygotes due to structure within these levels but at sample level a negative and significant F_{IS} of -0.0181 was observed showing excess of heterozygote. F_{IS} was also calculated within cities. Karachi (F_{IS}= -0.0137), Lahore (F_{IS}= -0.0854), Sheikhupura (F_{IS}= 0.0997), Attock (F_{IS}= -0.065) and Peshawar (F_{IS}= -0.1162) had significant (P<0.05) F_{IS}, with only Sheikhupura having a positive F_{IS} value, the remaining cities showing an excess of heterozygotes. Negative F_{IS} at sample level is most likely due to the small effective size of the breeding population contributing to each container which leads to chance differences in allele frequency between males and females and so an excess of heterozygotes among larvae. The effective breeding population size contributing to each sample calculated according to Balloux (2004) equation 2 [$N_{eb} = 1/(-2F_{IS}) + ½(1-F_{IS})$] was estimated to be between 20.27 and 46.37 based on the 95% confidence interval of the F_{IS} estimate.

A matrix of pair-wise F_{ST} values between the populations present in cities is shown in table 4.15. A total of 78 pair-wise comparisons were calculated, all of which were significantly different from zero (P<0.05). F_{ST} values range from 0.013 for Rawalpindi and Haripur to 0.17 for Hyderabad and Hasanabdal. Hyderabad followed by Karachi showed high pairwise F_{ST} values in all comparisons; which might be due to their geographical separation from other cities. Two patterns were observed in pairwise comparison. One was an increase in F_{ST} values with increase in geographical distance and secondly a unique and different pattern of F_{ST} values were observed related to the transport links between two cities. Both of these relations are tested below by correlation and partial correlation using Mantel tests.

Isolation by distance (IBD) was estimated by plotting F_{ST} /(1- F_{ST}) against the log of distance in metres. This was done at city (Figure 4.1), patch (Figure 4.2) and individual level (Figure 4.3). Karachi and Hyderabad are located in the south of Pakistan and there was a more than 600 kilometres gap having no specimen collection between these cities and the north, so Karachi and Hyderabad were removed from the whole data set and the impact on the results was checked for

IBD. In all these cases the IBD slope was positive and significantly different from zero ($P < 0.05$) showing IBD in the population of mosquitoes in Pakistan. In container level and patch level IBD analysis, the intercept was negative due to homozygote deficiency. IBD was also calculated at city level and only Karachi (Figure 4.4) had significant IBD. In other cities IBD was not significant ($P > 0.05$).

4.3.5.3 Correlation Between Genetic Distance, Geographical Distance and Road Connections

Pair-wise F_{ST} analysis showed some relationship between genetic distance and geographic and road connections. Simple Mantel tests and Partial Mantel tests were used to correlate matrices of the three different distances. Simple Mantel tests were used to correlate the pair-wise F_{ST} matrix with each of the other two matrices separately and the Partial Mantel test for comparing two matrices keeping the effect of the third matrix constant.

The simple Mantel test showed a positive correlation ($\rho = 0.48$, $P = 0.006$) between genetic distance and geographic distance showing an increase in genetic differentiation with increase in distance. The closer the populations are the less differentiated they are, and vice versa. These results were in agreement with isolation by distance and confirm the presence of IBD between populations. On the other hand, a strong negative correlation ($\rho = -0.62$, $P = 0.0002$) was revealed between genetic and road distances showing a decrease in F_{ST} with the increase in quality of road connection (Figure 4.5). The effect of road distance was found to be stronger then the effect of geographical distance and to test whether these relationships were independent of each other or not, a Partial Mantel test was used. The Partial Mantel test also showed a strong negative correlation ($\rho = -0.57$, $P = 0.01$) between genetic and road distance independent of the effect of geographical distance and similar positive results ($\rho = 0.38$, $P = 0.04$) for genetic and geographical distance independent of road distance showing that these relationships are not affecting each other. However the effect of road distance was stronger than the geographical effect as shown by the values of rho.

Table 4.13: F_{ST} values at different levels of population structure.

POPULATION	F_{IS}	F_{ST}	SE	F_{IS}	F_{ST}	Heterozygosity
13 Populations	0.0706	0.0779	0.0102	***	***	-
42 Patches	0.0333	0.106	0.0091	***	***	-
Sample	-0.0181	0.15	0.0082	***	***	-
Karachi	-0.0137	0.1061	0.0059	***	***	0.6784
Multan	0.0587	0.1358	0.0235	Ns	***	0.6
Faisalabad	-0.002	0.0089	0.0155	Ns	Ns	0.6547
Lahore	-0.0854	0.0754	0.01	***	***	0.6345
Sheikhupura	0.0997	0.0855	0.0128	***	***	0.5721
Gujranwala	0.0505	-0.023	0.0084	Ns	Ns	0.5669
Rawalpindi	-0.0056	0.0597	0.0071	Ns	***	0.5999
Haripur	-0.0115	0.0847	0.011	Ns	Ns	0.5612
Attock	-0.0659	0.1401	0.0214	***	***	0.4992
Peshawar	-0.1162	0.2346	0.0263	***	***	0.5824
Hasanabdal	-0.0847	0.0386	0.0302	Ns	Ns	0.5365
Hyderabad	0.0381	-0.0002	0.0284	Ns	Ns	0.6728
Taxilla	-0.0086	0.1395	0.0279	Ns	***	0.6091

Table 4.14: Pairwise matrix of F_{ST} between different cities, non-significant values are underlined.

	Peshawar	Attock	Hasanabdal	Haripur	Taxilla	Rawalpindi	Gujranwala	Sheikhupura	Lahore	Faisalabad	Multan	Hyderabad
Peshawar	0											
Attock	0.1109	0										
Hasanabdal	0.1406	0.1368	0									
Haripur	0.0691	0.0847	0.087	0								
Taxilla	0.078	0.0633	0.045	0.0261	0							
Rawalpindi	0.0426	0.0821	0.0741	0.0131	0.0266	0						
Gujranwala	0.101	0.1076	0.072	0.0445	0.0311	0.0435	0					
Sheikhupura	0.091	0.1272	0.1011	0.0323	0.046	0.0287	0.0479	0				
Lahore	0.0501	0.0775	0.0795	0.0233	0.0324	0.0178	0.0519	0.0391	0			
Faisalabad	0.0513	0.1138	0.069	0.0538	0.0409	0.0326	0.0593	0.0547	0.0394	0		
Multan	0.1005	0.1645	0.1167	0.0641	0.0652	0.062	0.0742	0.082	0.0591	0.0573	0	
Hyderabad	0.0908	0.1563	0.1726	0.1133	0.1049	0.0997	0.1152	0.1277	0.0632	0.0564	0.0681	0
Karachi	0.0687	0.1316	0.1383	0.1155	0.098	0.0844	0.0987	0.1131	0.0668	0.0527	0.0695	0.0257

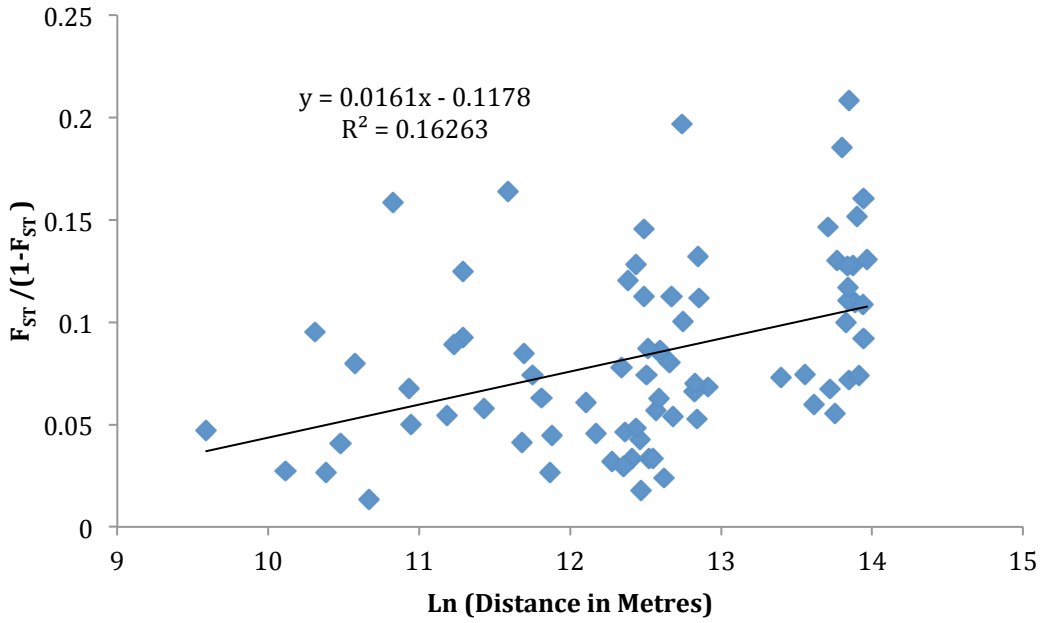

Figure 4.1. Graphs showing Isolation by distance at the city level of sampling hierarchy.

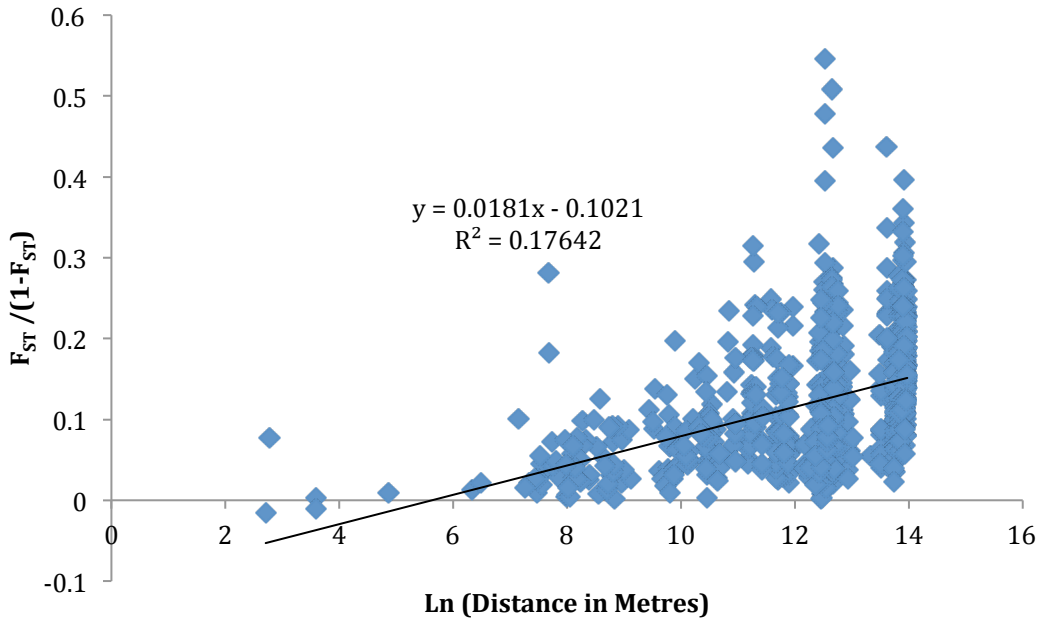

Figure 4.2. Graph showing isloation by distance at patch level of sampling hierarchy.

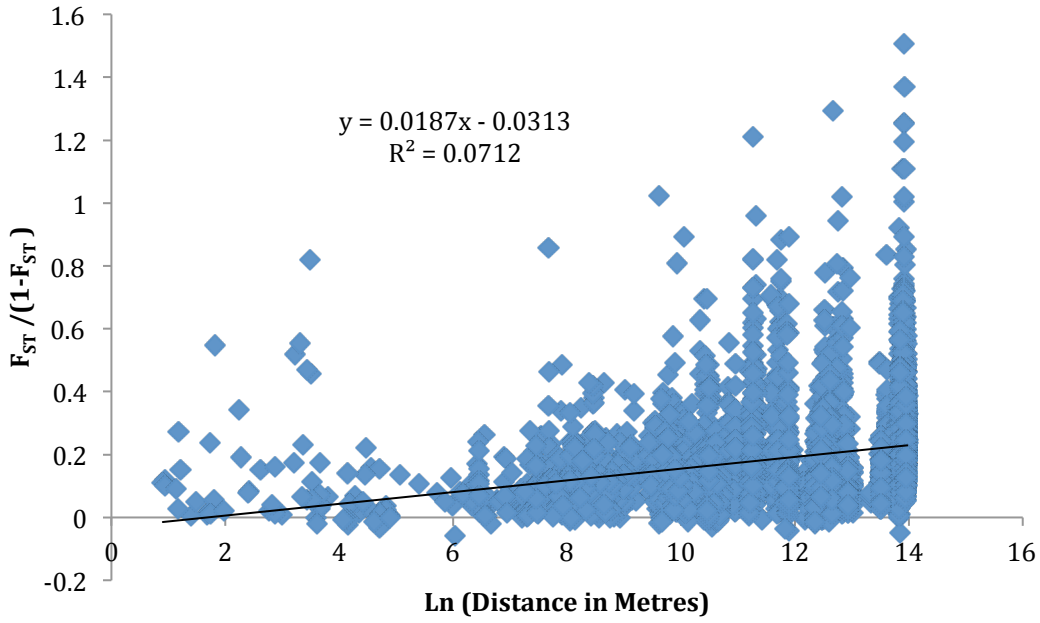

Figure 4.3. Graph showing isloation by distance at container level of sampling hierarchy.

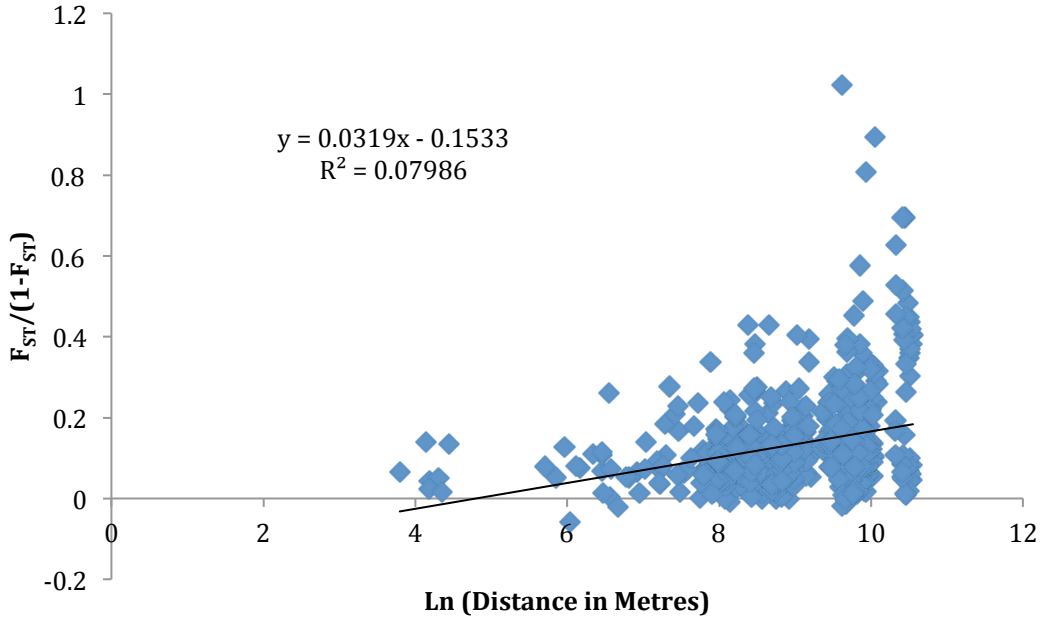

Figure 4.4. Graph showing isloation by distance within Karachi.

4.3.5.4 Correlation Between Genetic Diversity and Geographical Distance

Variation in genetic diversity (expected heterozygosity) can be explained by the process of colonization if there is recent spread in a population. Expected heterozygosity is the possible number of heterozygotes for a randomly selected locus when it is in Hardy-Weinberg Equilibrium. Heterozygosity was compared with the distance of each city from Karachi in kilometres (Table 4.15). Spearman's rank correlation was used to test the strength of correlation between the two data sets. The correlation was negative and quite strong ($r = -0.72$, $P = 0.01$) which was significantly different from zero showing that the heterozygosity decreases as we move away from Karachi to Peshawar.

4.3.5.5 Model Based Structure Analysis

Using STRUCTURE, the number of clusters, *K,* was not easy to determine. The most probable value of *K* was decided then by using *ΔK* statistics. In STRUCTURE gave the log-likelihood values approached an asymptote with increasing K, a pattern indicating isolation by distance rather than distinct subpopulations (Frantz et al. 2006) (Figure 4.6). Using the Evanno et al. method based on rate of change in the log probability of the data between successive cluster levels, ΔK was highest for K=2 (Figure 4.7). STRUCTURE showed that the number of clusters of *Ae. aegypti* may be 2 and plotting the assignment of each cluster at K=2 on a map showed a gradual decrease of one cluster and increase of another cluster from the northern part of the country to southern part (Figure 4.8 but this pattern may also be generated by isolation by distance due to which it is over estimating the number of genetic clusters of this mosquito in Pakistan so keeping all this in view the most probable number of genetic cluster of *Aedes aegypti* in Pakistan is one.

Table 4.15. Expected heterozygosity of each city and distance in kilometers taking Karachi as point of origin.

CITY	DISTANCE FROM KARACHI	HETEROZYGOSITY
Peshawar	1100	0.5824
Attock	1021	0.4992
Hasanabdal	994	0.5365
Taxilla	981	0.6091
Haripur	974	0.5612
Rawalpindi	957	0.5999
Gujranwala	785	0.5669
Faisalabad	781	0.6547
Sheikhupura	760	0.5721
Lahore	727	0.6345
Multan	677	0.6000
Hyderabad	98	0.6728
Karachi	0	0.6748

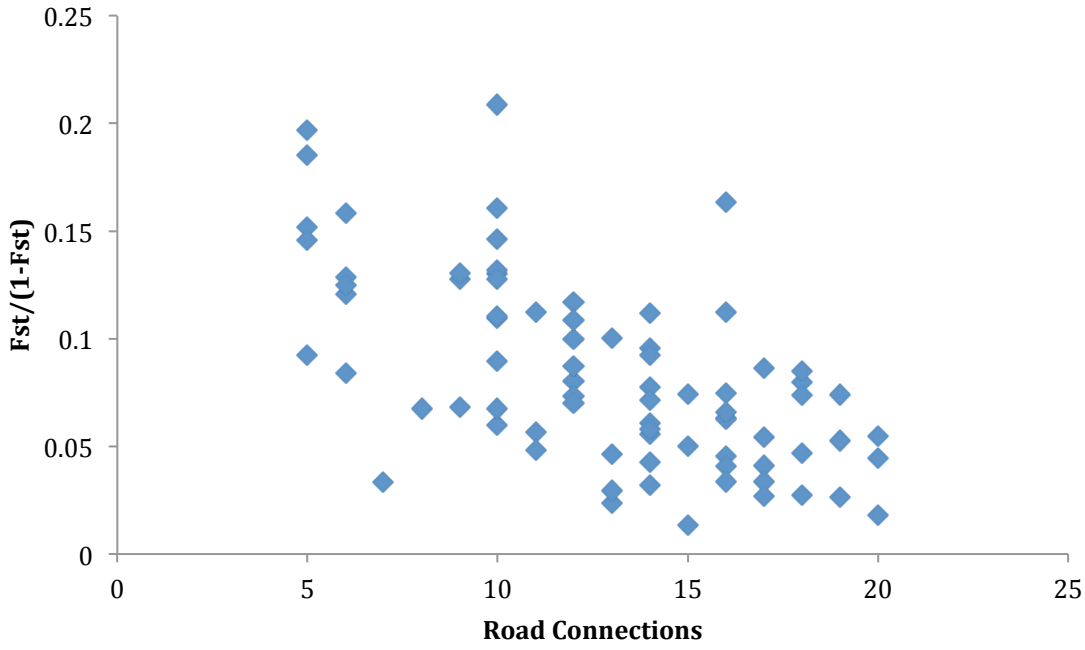

Figure 4.5. Graph showing relationship between Genetic Distance and Road Connections.

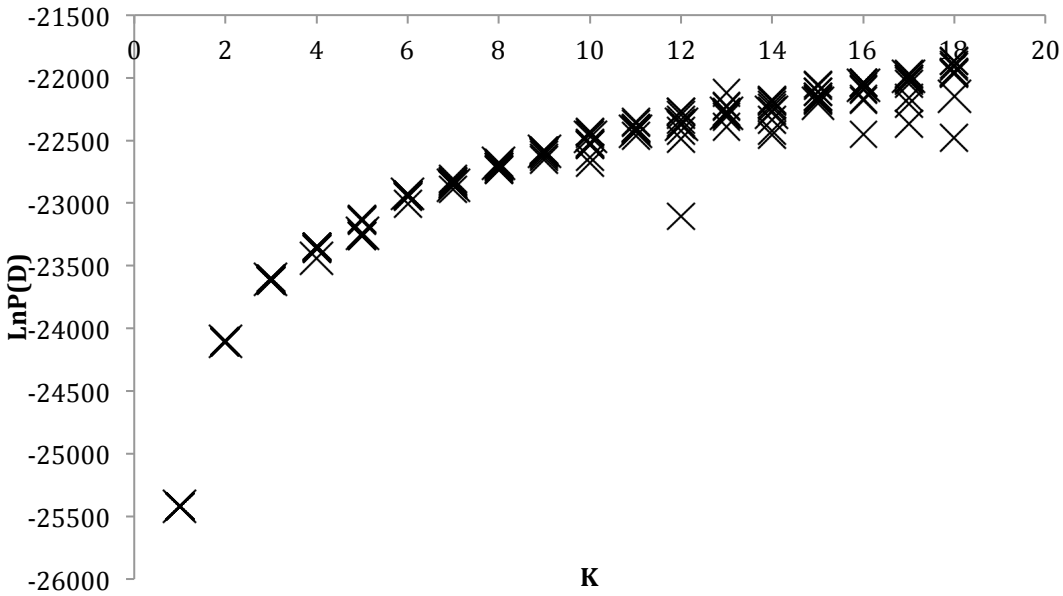

Figure 4.6: Inference of the number genetic clusters (K) of mosquitoes in Pakistan using STRUCTURE. Ln (X|K) - log-likelihood values, of 10 independent runs.

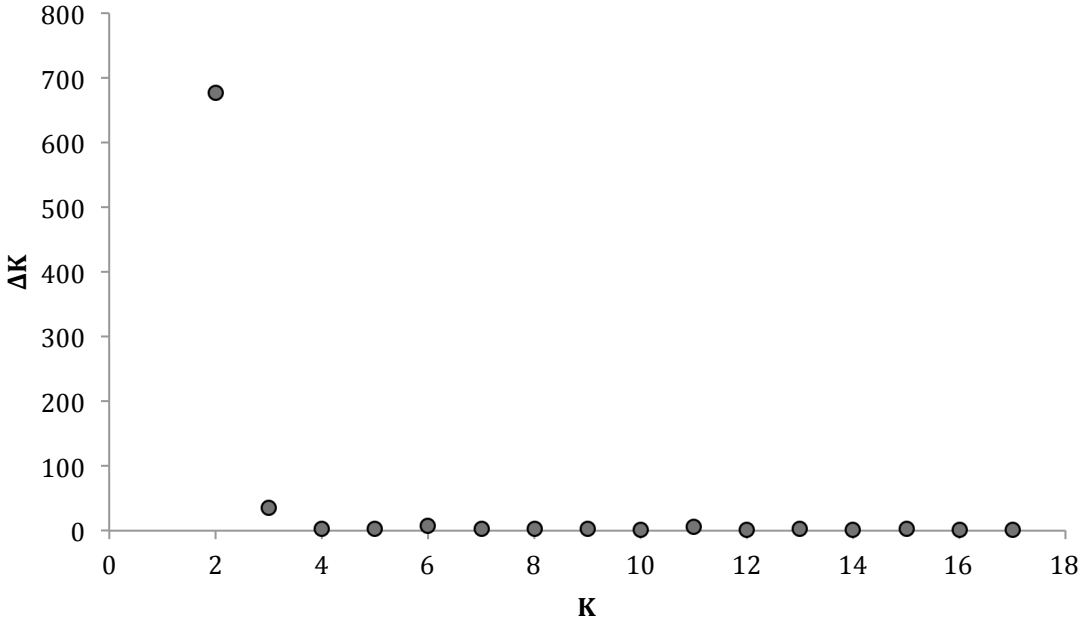

Figure 4.7: ΔK - the rate of change in log probability between successive values of K.

Figure 4.8: Proportion of mosquitoes assigned to each STRUCTURE cluster at K=2 for each city.

Legend: 1 = Peshawar, 2 = Attock, 3 = Hasanabdal, 4 = Haripur, 5 = Taxilla, 6 = Rawalpindi, 7 = Gujranwala, 8 = Sheikhupura, 9 = Lahore, 10 = Faisalabad, 11 = Multan, 12 = Hyderabad, 13 = Karachi.

4.4 DISCUSSION

Aedes aegypti is considered to be the primary vector of dengue, which has emerged during the last few years as an important vector-borne disease in Pakistan, affecting thousands of people with deaths in every year (Hakim et al. 2011). No adequate control strategy is in place in Pakistan for this disease and no predictions have been made regarding the future spread of dengue in the country. More research on the disease and its vector is urgently needed.

This study was conducted to test two hypotheses concerning the status of the primary vector of dengue, *Aedes aegypti,* in Pakistan. First, we tested the hypothesis that the recent spread of this mosquito started from Karachi and moved towards the north. The second hypothesis was that movement of this mosquito in Pakistan is facilitated by the tyre trade. Our microsatellite analysis demonstrated that significant genetic differentiation among the subpopulations is present in Pakistan. Genetic diversity decreased towards the north. The genetic distances among samples were positively correlated with geographical distance, at a large spatial scale. In addition, areas connected by better road facilities were less differentiated than those lacking good road connections. These observations are consistent with both of our hypotheses and further suggest that the dispersal of this mosquito occurs over large distances, probably because of passive transport associated with the movement of people and goods.

All of our population structure analyses were consistent with a pattern of isolation by distance (IBD) in Pakistan, rather than the presence of distinct mosquito populations. IBD was detected at a large spatial scale but not within cities, except Karachi. The significant IBD in Karachi may be due to the spread of samples over a larger area than in other cities. Positive slopes for the relationship between F_{ST} /(1 -F_{ST}) and log of geographic distance were observed at all levels of the sampling hierarchy but the slope decreased at higher levels, as expected for a balance between gene flow and genetic drift (Rousset 1997). Hierarchical analysis of F statistics was in agreement with the pattern of IBD. The maximum variation was within individuals but there was highly significant variation at each higher level: containers, patches and cities. All 78 pair-wise F_{ST} comparisons between

cities were also significant. These patterns are expected from a general increase in genetic distance with geographical distance, rather than the existence of distinct but internally homogeneous sub-populations.

The genetic pattern of isolation by distance observed in this study could either be due to equilibrium between drift and gene flow (Slatkin 1993) or to recent colonization (Ibrahim et al. 1996). The slope of the IBD relationship was low which suggests very long distance dispersal if the population is at equilibrium. Since the effective density of mosquitoes is not known, no dispersal estimate is possible but applying the formula for neighbourhood size (slope $b=1/4\pi\sigma^2D$, where σ^2 is the variance in parent-offspring distance and D is effective density {Rousset 1997}) shows that our observed slope (0.016) is consistent with σ=0.1km if D=20 individuals/km^2 or σ=0.5km if D=500 individuals/km^2. Alternatively, Ibrahim et al. (1996) have shown that isolation by distance over scales much greater that the dispersal distance can be generated by colonization and can persist for many generations (~600 in their stimulations). Other evidence is needed to separate these two possibilities (see below).

Some information on mosquito density in our study area can be gained from considering the numbers of adults contributing to the larval population in sampled containers. Relatively small breeding populations of animals tend to have an excess of heterozygotes relative to Hardy-Weinberg proportions due to differences in allele frequencies between the two sexes (see Allendorf and Luikart 2006, p 136-138). It is probably due to this effect that we observed a negative F_{IS} at the container level and also a negative intercept of the IBD regression. The average size of the breeding population contributing to each container was calculated according to Balloux (2002) [$N_{eb} = 1/(-2F_{IS}) + ½(1-F_{IS})$], assuming equal sex ratio, and found to be between 20.27 and 46.37 individuals. This estimate suggests a high density of mosquitoes around each container and that this mosquito is very abundant in Pakistan, at least in urban areas. This conclusion is supported by the small changes in allele frequency observed between two sample periods one year apart because high effective population size would result in only a small effect of genetic drift. The higher the density, the greater the dispersal distance needed to account for the observed isolation by distance so this argues against the equilibrium explanation.

The dispersal of *Aedes aegypti* is driven by the availability of oviposition sites (Edman et al. 1998) and a female distributes its eggs among several water-filled containers (Reiter et al. 1995). Huber et al. (2002) showed that the availability of oviposition sites to lay eggs and dense human population for blood feeding tends to limit the mosquito's dispersal resulting in genetic differentiation. In Ho Chi Minh City centre, Vietnam, the abundant breeding sites and dense human population were associated with genetic differentiation, whereas in the outskirts the population was more genetically differentiated as compared to the city centre because the oviposition sites were not as abundant as in the city centre, indicating that the gene flow was not high enough to reduce genetic differences at the corresponding distances.

Similar large-scale population structure has been reported for *Aedes aegypti* in other regions. Hlaing et al. (2010) found that weak isolation by distance was present only at a large spatial scale and not within collection sites in Southeast Asia and took this to suggest extensive dispersal rather than due to limited dispersal within continuous populations. Few studies have directly tested for a positive correlation between genetic and geographic distances. Huber et al. (2004) reported isolation by distance in mosquito populations from Ho Chi Minh City, Phnom Penh and Chiang Mai. The value of slope observed in this study was approximately equal to our value showing a similar degree of IBD. Isolation by distance was observed in mosquitoes collected from Australia, Vietnam and Thailand by Endersby et al. (2009) but no regression slope was reported. Comparing their F_{ST} values with ours at the same spatial scale shows that this relationship was weak compared to that in Pakistan. Within the Australian region the correlation between genetic and geographic distance was significant but much weaker than among all three regions. The patterns were attributed to passive movement of eggs and immature stages of *Aedes aegypti* in containers moved by humans. Fonseca et al. (2010) observed not only a significant negative correlation between genetic and geographic distances among *Aedes japaonicus* in USA at smaller distances due to limited flight distance and the juxtaposition of two genetically unique populations bordering each other but also a significant positive correlation across large distances was reported due to movement of this mosquito on large spatial scale helped along by human mediated transport.

Bayesian methods implemented in STRUCTURE were used to estimate the number of genetically distinct populations of *Aedes aegypti* present in Pakistan. STRUCTURE revealed two genetic clusters with a tendency for one cluster to be more abundant in the north and many geographical regions being occupied by both clusters. It has been reported that STRUCTURE can detect spurious discontinuities in the presence of isolation by distance, regardless of whether spatial or non-spatial priors are used so in this study only non-spatial priors were tested for estimating the number of genetic clusters of *Ae. aegypti* in Pakistan. Continuous spatial variation is interpreted by the algorithm as the presence of discontinuous clusters even in the absence of any barrier to gene flow (Frantz et al. 2009). The other evidence for isolation by distance in our data, and the continuous change in proportions of the two STRUCTURE clusters, suggest that this is the situation here and that there are no genuinely distinct sub-populations in our sample area. However, it appears that effects other than simple geographical distance may be required to explain the greater differentiation of some regions, especially Attock.

A strong negative correlation was observed between genetic distance and quality of road connections, independent of the effect of geographical distance: mosquitoes in cities connected through better road connections were less differentiated than those in cities with poor transport links. This strongly suggests that human-aided passive dispersal of *Aedes aegypti* makes an important contribution to population structure in our study area. Since passive dispersal can cover distances well above the natural dispersal range, this is in agreement with IBD over large spatial scales. Mark-release-recapture studies have shown that the natural flight range of *Aedes aegypti* in normally 50 – 100 m around its larval habitat (Reiter et al. 1995) although estimates vary from 10 m to 800 m (McDonald 1977, Tripis and Hausermann 1986). However, *Aedes* mosquitoes can disperse passively, through human transportation, in the form of desiccated eggs or immature stages in containers (Solani et al. 2010). Desiccated eggs can live for several months and hatch into larvae on contact with water (Christopher 1960). Decrease in genetic differentiation due to passive dispersal has been observed repeatedly in studies on *Aedes aegypti* and it has been considered an important and efficient means for colonization over long distances, such as more than 1000 km in a relatively short period (Duenas et al. 2009).

In Pakistan, a possible relationship between movement of tyres and spread of *Aedes aegypti* was first described by Suleman et al. (1996) when this mosquito was reported from tyre warehouses in a previously *Aedes aegypti*-free area. In our study, *Aedes aegypti* was collected from used tyres in almost all collection cities, so used tyres are certainly acting as an important breeding site for this mosquito. In north-eastern Argentina, Stein et al. (2002) reported that used tyres and car batteries exposed to rainfall act as important breeding habitats for *Aedes aegypti*. It has also been reported that gravid *Aedes aegypti* females prefer dark or shady containers for oviposition (Nelson 1986), making tyres likely to be preferred habitat. Used tyres having *Aedes aegypti* eggs, immature stages and gravid females are likely to be transported from one city to another city for trade purposes thus acting as a means for long-range passive dispersal of this mosquito into and around Pakistan. Cities with better road connections have more trade so the eggs, larvae and adults are transported from one city to another city either in tyres or other water containers, which results in gene flow among mosquito populations and reduces genetic differentiation between them. In northeast India, a survey has been conducted along the national highway and trunk roads to detect breeding of *Aedes aegypti* in used/waste tyres piled outdoors. The results suggest that tyres have been playing an important role in dispersal and abundance of *Aedes aegypti* on commercial transport routes in this area (Dutta et al. 1998).

Huber et al. (2004) reported low genetic differentiation between *Aedes aegytpi* samples from Ho Chi Minh City; Vietnam, and Phnom Penh; Cambodia, that has been attributed to passive dispersal of this mosquito through human transportation. These two cities are 250 km apart but are well connected by aircraft, vehicles and waterways. Three daily flights, many buses and navigation on Mekong River by ships connect Ho Chi Minh City and Phnom Penh, which presumably contribute to the accidental transportation of eggs, immature stages and adults of *Aedes aegypti*. In Arizona, southern USA, roadway systems also correspond to the observed patterns of population differentiation. The lowest levels of genetic differentiation were found among areas connected by a better roadway, although this relationship was not assessed statistically (Merrill et al. 2005). As in our study, Hlaing et al. (2010) reported that in southern and northern Thailand some geographically distant sites were still genetically similar because they were

connected through main roads. In addition, they also observed high genetic heterogeneity in *Aedes aegypti* samples from port cities (Colombo in Sri Lanka, Yangon in Myanmar and Songkla in Thailand) or major cities (Phnom Penh, Cambodia). They suggested that this pattern was observed because there is some long-distance dispersal of *Aedes aegypti* aided by major human transportation routes from multiple sources (Hlaing et al. 2010). Thus passive dispersal can explain not only the genetic similarity between cities connected by good road connections in Pakistan but also high genetic diversity in Karachi.

Returning to the question of whether the observed isolation by distance in Pakistan reflects a stable balance between dispersal and genetic drift or a consequence of colonization history, we note that long-distance passive dispersal can help to accommodate the equilibrium hypothesis. On the other hand, high mosquito densities predict finer-scale population structure. Colonization could explain the IBD pattern in the data set and is supported by two further sources of evidence. First, the historical records for this mosquito suggest that it was first present, in the south of Pakistan only, in early 1980's (Kamimura et al. 1985) because it was coincidently eradicated with malaria mosquito due to malaria eradication programmes after 1950 (Suleman et al. 1996)). Second, we observed a negative correlation between heterozygosity and geographical distance from Karachi, which is consistent with loss of genetic diversity during range expansion. Together, these observations support the idea of rapid colonization from south to north during the last three decades. These sources of evidence suggest that the IBD pattern observed is unlikely to be due to drift and gene flow in equilibrium but rather may be due to the process of colonization and range expansion, which also seems to have played a major role in shaping large-scale population structure of *Aedes aegypti* elsewhere, as observed in Southeast Asia by Hlaing et al. (2009).

Colonization events involve very few individuals compared to source populations. As a result, founder effects become apparent and the populations farthest from the original colonization source will have depleted genetic diversity detectable as lower heterozygosity (Nei et al. 1975). A correlation between geographic distance and genetic diversity has been used in a number of studies as evidence for colonization history. For example, Tabachnick and Powell (1979)

observed low heterozygosity in Asia, as compared to New World, East and West African populations of *Aedes aegypti,* suggesting recent colonization of this mosquito into Asia. Wilcock et al. (2001) reported a significant northward decrease in expected heterozygosity in northwest European caddis fly populations. This was attributed to post glacial colonization of caddis fly from the south (Wilcock et al. 2001). Similar patterns are common in Europe e.g butterfly species like *Coenonympha hero* from Sweden (Casel and Tammaru 2003), *Polyommatus coridon* from central western Europe (Schmitt et al. 2002) and bog fritillary butterfly in Czech Republic (Neve et al. 2009) displayed a significant negative correlation between heterozygosity and distance along the route of colonization from source population during the colonization process.

The correlation between geographic distance and expected heterozygosity of 51 human populations has been used as an excellent predictor of likely colonization routes from East Africa pointing to the history of colonization of the world by humans (Prugnolle et al. 2005). A strong negative linear correlation between genetic diversity and geographical distance showed that populations geographically most distance from Ethiopia had the lowest genetic variability suggesting that the world was colonised by individuals from a single original population in Africa. This pattern of erosion of genetic diversity along the colonisation routes could only arise through successive bottlenecks of small amplitude as the range of humans increased, with the populations farthest away from East Africa being furthest from mutation-drift equilibrium (Prugnolle et al. 2005).

Loss of genetic diversity with colonization has also been observed in more recent colonization events. *Drosophila buzzatti* is native to Argentina, South America, but colonized Europe and Australia 200 and 65 years ago, respectively. Microsatellite analysis showed that genetic diversity was much larger in the native population than in colonizing ones and a decrease in heterozygosity was observed in the colonizing population (Frydenberg et al. 2002). *Drosophila subobscura* is native to a wide range from North Africa to Scandinavia and was first observed in South and North America in the early 1980's. Microsatellite analysis showed a decrease in genetic diversity in the Americas compared to Europe. The much lower genetic diversity suggested that a severe bottleneck had occurred during the

colonization process (Pascual et al. 2007). Bonizzoni et al. (2004) reported decreased heterozygosity using microsatellite markers among medfly in southern Australia recently colonized from western Australia where this fly is present since 1940's. This decrease in heterozygosity was typical of populations subjected to founder effects and genetic drift following a colonization process. Colorado potato beetle invaded Europe from North America in the early 20th century and expanded almost throughout Europe in about 30 years. AFLP markers revealed a decrease in genetic diversity in European populations compared to American populations (Grapputo et al. 2005). A hydrozoan *Cordylophora* in the Great Lakes of Chicago, USA was reported in 1990 but its colonies throughout the Chicago harbors were not reported until approximately 10 years later. Microsatellite analysis showed a gradual decrease in microsatellite diversity in this hydrozoan from south to north in Great Lakes of Chicago due to population expansion after the first report in 1990 (Darling and Folino-Rorem 2009).

Our data suggest that the expansion of *Aedes aegypti* in Pakistan began from a relatively large and polymorphic population like that of Karachi but the founders of progressively distant city populations repeatedly originated from already colonized populations that were themselves already subject to genetic drift. Thus, genetic diversity decreases from Karachi towards Peshawar. The IBD pattern on a large spatial scale may be a reflection of this colonization process rather than regular large-scale dispersal of this mosquito in Pakistan.

The colonization process and long-range dispersal of *Aedes aegypti* in Pakistan is facilitated by road connections between cities. This passive dispersal could be either the movement of adults, eggs or immature stages in containers along the travelling and commerce routes via human mediated transportation. These findings suggest that long-range passive dispersal of mosquitoes could have far-reaching implications for the spread of dengue in Pakistan. Eggs, immature stages and adults may carry dengue virus from one city to another very quickly through human transport and so could result in the spread of dengue in Pakistan even to areas that are separated by hundreds of miles from each other. Alternatively, passive dispersal could establish vector populations in new areas resulting in rapid

expansion of dengue epidemics when infected humans bring the disease into the area.

Conventional control methods like insecticides treatment or removal of larval habitats in and around houses after a dengue outbreak could be effective in removing infective *Aedes aegypti* population from an area (WHO 2006). Sterile insect technology (SIT) could be effective in areas with limited dispersal (Lewis and Van Den Driessche 1993). In this technique large number of sterile males are released, which could mate with local females and reduce population size thus generating a travelling wave of extinction (Thomas et al. 2000). Long-range passive dispersal limits the successive implementation of these methods due to influx of mosquitoes from other areas. A major problem in insecticide treatment is insecticide resistance (Gubler et al. 2002) but stable zone strategy would make it difficult for the spread insecticide resistance gene. In this strategy the area treated with insecticides should be relatively small as compare to scale of dispersion in order to allow the reinvasion of fitter mosquitoes carrying non-resistant genotypes into the treated area thus preventing the establishment of the resistance gene (Lenormand and Raymond 1998). Long-range dispersal of *Aedes aegypti* in Pakistan could also facilitate the strategy of introducing and driving disease refractory genes (James et al. 2006) or intracellular bacterium like *Wolbachia* (Hoffmann et al. 2011) through large geographical areas. Thus long-range passive dispersal suggests that, along with local control practices, strategies like introducing disease-refractory genes or *Wolbachia* in a population and stable zone strategies could be formulated to control the spread of dengue as well as its vector on a countrywide scale in Pakistan.

Chapter 5

DENGUE DETECTION IN MOSQUITOES

5.1 INTRODUCTION

In this chapter we set out to detect the prevalence of dengue virus in larvae collected from different parts of Pakistan. Surveillance of the prevalence of dengue-infected mosquitoes is important for a number of reasons. Most obviously it may provide an early warning system for the risk of transmission of dengue disease in an area (Kow et al. 2001). Currently the occurrence of dengue viral infection in the human population is used as the stimulus to initiate vector control measures. However there is inevitably a delay in this and therefore vector control may prove to be insufficient for suppressing the epidemic (Lee and Rohani 2005). Direct monitoring of the viral prevalence in the mosquito population may therefore substantially improve the effectiveness of control methods. In addition, vertical transmission of dengue virus within populations of mosquitoes may be used within an active epidemiological surveillance program in order to identify the circulation of dengue virus in a region (Cecilio et al. 2009). Information regarding the ability of vectors to transmit dengue virus transovarially is therefore potentially useful in applying a more effective campaign against dengue infection and its vector (Rohani et al. 2009). This is another reason for the early detection of dengue virus infection in mosquitoes before its introduction into the human population so that effective control could be taken to constrain any outbreak (Lee and Rohani 2005).

In Chapter 4 we described the passive movement of Aedes aegypti in Pakistan most probably through tyres. This confirms the findings of Suleman et al. (1996) that tyres are spreading this mosquito in Pakistan. One of our main aims in this chapter is to determine the prevalence of dengue virus in larvae collected from tyres compared to those collected from other containers. It is clear that mosquitoes are being spread by human mediated transport probably the tyre trade, but it is important to determine the prevalence of dengue in the tyre population relative to

the general mosquito population. We will then be able to better assess whether the tyre trade acts to move dengue virus as well as the vector population.

The question of survival of dengue during winter or dry season has always puzzled the investigators. Two possibilities exist to answer this puzzle, either infected mosquitoes survive throughout the interim period or there is transfer of virus from infected mosquitoes to its progeny (Hutamai et al. 2007). *Aedes aegypti* once infected with virus remains infected throughout life, the median survival time is 1-2 week but the longest life span recorded to date is 174 days (Kyle and Harris, 2008). However survival of mosquitoes for such a long time is very rare and therefore vertical transmission could be the method by which the virus over winters and survives the dry period (Cecillo et al. 2009). As a consequence it may also play an important role in the persistence of this virus in nature (Rosen 1987). Transovarial transmission of all four strains of dengue had been observed experimentally in *Aedes* species during early 1980's but at that time its occurrence in nature remained doubtful (Khin and Than 1983). Later studies showed that this phenomenon could happen in nature also (Joshi et al. 1996, Thenmozhi et al. 2000, Gunther et al. 2007) but still it needs a lot of investigation to establish the role of vertical transmission in viral persistence in nature (Zeilder et al. 2008). The final aim of this chapter is to determine the rate of vertical transmission by detecting dengue virus in adults as well as larvae collected from Karachi.

5.2 MATERIALS AND METHODS

5.2.1 Pooling and lysis of mosquitoes

A total of 4799 mosquitoes, collected from five cities, were used for the detection of dengue. Mosquitoes were batched in groups of six in racked 96 well format collection microtubes (Qiagen). One 3mm stainless steel bead and 250µl of Eagles minimum medium (Invitrogen) with a final concentration of 100 units of Penicillin and Streptomycin each per ml was added to each tube. Collection tubes were covered with caps and then subjected to lysis at 60Hz for 90 seconds by using a Qiagen tissue lyser II. Collection tubes were centrifuged at 100g using a plate

centrifuge to collect all the liquid at the bottom of tubes (minimizing contamination of the samples, upon removing the caps). 60µl of lysate from each well was used to form 204 pools by combining 4 batches so that each pool contained lysates from 24 mosquitoes. Lysates in each pool were loaded onto a QIA shredder homegenizer, and the liquid was allowed to flow through by gravity. This is in order to remove any remaining coarse material before proceeding to RNA extractions. The flow through was then collected and used for RNA extraction.

5.2.2 RNA Extraction

RNA was extracted from the homogenized lysate by using Qiagen RNeasy 96 kit, using QIAvac 96 Vacuum Manifold according to the supplier protocol as described below.

The waste tray was placed inside the QIAvac base. The top plate was placed securely over the base and the RNeasy 96 plate was placed in the QIAvac top plate, making sure that the plate is seated tightly.

The vacuum pump was attached to the QIAvac 96 manifold. 250µl of Buffer RLT was added to each microplate well containing the 240µl mosquito lysate, mixed well and then incubated for 30 to 45 minutes. After this 250µl of 70% ethanol was added and mixed by pipetting up and down 3 times. The samples (740µl) were transferred into the wells of the RNeasy 96 plate and subjected to vacuum until the mixture was transferred to the waste tray.

The RNeasy 96 plate was treated with RNase-Free DNase set (Qiagen) to remove any trace of DNA by pipetting 80 µl of the DNase I incubation mix directly onto the RNeasy membrane into each well and sealing the plate with an AirPore Tape Sheet. It was left for 15 minutes at room temperature. The AirPore Tape was removed from the RNeasy 96 plate and 1 ml of Buffer RW1 was added to each well and incubated for 5 minutes. After this the RNeasy 96 plate was again subjected to vacuum. Once the buffer was sucked out 1 ml of Buffer RPE was added to each sample, and the vacuum source was switched on. This step was repeated once more.

The bottom side of the RNeasy 96 plate was strucked several times on a stack of paper towels (~4 cm high) until no further liquid is released onto the paper towels. Any residual RPE buffer from the collars and nozzles of each well was absorbed by using paper towels. The RNeasy 96 plate was placed back in the vacuum apparatus and vacuum was applied for 10 min and then dried to remove any ethanol. Once the membrane is dried the waste tray was replaced with an elution microtube rack containing 1.2 ml elution microtubes assembled on top of the Elution Microtube Adapter.

In order to elute, 60µl of RNase-free water was pipetted out and placed directly onto the membrane in each well which was allowed to stand for 1 min and then the vacuum source was switched on until transfer is complete. This step was repeated again to ensure complete recovery of RNA. The elution volume was approximately 30 µl less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume. The elution microtubes were sealed with the caps provided and then stored at–70°C.

5.2.3 cDNA Synthesis

Aliquots of extracted RNA were used for cDNA synthesis. RNA was converted into cDNA by using the SuperScript III first strand synthesis system kit (Invitrogen) and hexanucleotide mixture (Roche). 10 µl of RNA from each pool were transferred into PCR microplates and RNA was heat denatured at 72°C for 10 minutes. Plates were immediately incubated on melting ice to prevent renaturation of RNA.

A mastermix for the Reverse transcription was prepared as follows:

Reagents	Volume
5x RT Buffer	4µl
0.1 M DTT	1µl
SuperScript III RT	1µl
10x Hexanucleotide Mixture (Roche)	2µl
DNTP solution (10mM: 2.5 mM each)	2µl (0.25mM each)
Total Volume	**10**µl

Ten microliters of the mastermix were dispensed into each of the 96 wells of the PCR microplate. The plates were incubated at 25°C for 10 minutes in order to maximize the annealing of the hexanucleotides to RNA targets. The plates were then incubated at 42°C for 1 hour after which the cDNAs were stored at -20°C until further usage.

5.2.4 Running Controls

To ensure that RNA extracts from the adult mosquito or larvae pools was converted to cDNA, primers designed from the mosquito 18's ribosomal RNA (Genbank accession number AB085210) were used for PCR. Primers 18S417 (5'-ACGGGGAGGTAGTGACGAGAAATA-3') /18S920 (5'-TAATACTAATGCCCCCAACTACTT-3') were used for the detection of cDNA as described previously (Hoffmann et al. 2004). The KOD hotstart DNA polymerase was used in the PCR. The reaction mixtures contained:

Reagents	Volume
10x PCR Buffer	5µl
dNTP mixture	5 µl
25 mM $MgSO_4$	3 µl
18S417 primer (10 µM)	2.5µl
18S920 primer (10 µM)	2.5µl
KOD Polymerase	1µl
cDNA	3µl
H_2O	xxx µl
Total Volume	**50µl**

PCR reactions were run in 96 well PCR microplates using the following cycling parameters

95°C for 15 minutes (1 cycle)

45 cycles of, 94°C for 30 sec, 60°C for 1 minute, 68°C for 1 minute and 68°C for 10 minutes (1 cycle).

5.2.5 Analysis of PCR products Using Agarose Gel electrophoresis

a) Preparation of the gels

The sides of 20cmx20cm plastic gel trays were covered with masking tape and three 41-well combs placed in position and the tray placed on a flat surface. 1.5% agarose gel was prepared by adding 3.75g agarose to 250ml 1xTAE in a 500ml flask. The solution was heated in a microwave until it become clear and transparent with no traces of agarose particles. 2.5µl of ethidium bromide (10 mg/ml) per 50ml of gel was added to the solution and cooled under the tap water whilst rotating the

flask. The semi-cooled solution was added in the already prepared plastic gel tray and left for approximately 30 minutes. When set the combs were removed and placed in an electrophoresis tank containing 1xTAE buffer.

b) Preparing Samples To Load Onto the Agarose Gel

I pipetted out 5µl 2x bromophenol blue loading buffer in wells into a 96-well Terrasaki plate. Next I diluted the PCR product by adding 10 µl of double distilled water and then added 5µl of this diluted PCR product to the well containing the loading buffer sample. All the samples were loaded to the gel with each row containing 0.5µg of Lambda DNA size standard (Promega). The gel was run at 100Volts (approximately 7.5 volts/cm), (DNA is –ve so goes toward the +ve electrode). The PCR products were viewed under ultraviolet light and a picture taken to compare the samples against a size standard to determine the quality and quantity of DNA.

5.2.6 Virus Detection

Presence of virus in the mosquitoes was detected by using panflavivirus primers designed from sequences of the NS5 gene of all sequenced flaviviruses (Mourea et al. 2007). Primers PF1S (5'-TGYRTBTAYAACATGATGGG-3')/PF2R-bis (5'-GTGTCCCAICCNGCNGTR-3') were designed within the highly conserved region of the NS5 and were found to amplify a 269-272 nucleotide sequence (depending on which flavivirus is tested) from any flavivirus.

The cDNAs prepared using the hexanucleotide primers were used as targets for the PF1S/PF2R-bis primers. The QuantiTect® SYBR® Green PCR (Qiagen) was used for real-time PCR. A typical 25µl reaction was prepared for each cDNA (generated initially from an RNA extract of a pool of 24 mosquitoes or larvae). The real time PCR were run on a Viia7 real time PCR machine (Life tech: previously Applied Biosystems) with following volumes:

Reagents	Volume
Qiagen 2x Mastermix	12.5µl
PF1S primer (0.55µM)	1.4µl
PF2R-bis primer (0.55µM)	1.4µl
cDNA	5µl
H_2O	4.7µl
Total Volume	**25µl**

The real-time PCR plate was covered with an optical plastic cover and placed in the Viia7 with the following temperature profile: 95°C for 15 min and 45 cycles of 94°C for 15 sec, 60°C for 30 sec and 72°C for 45 sec. Plasmid (rDENV-4-rLGTswap 3'UTR) in Tumban et al. 2011 was used as a positive control for the real time PCR.

5.3 RESULTS

5.3.1 cDNA Preparation and Efficiency of ribosomal RNA as Control

A total of 4799 mosquitoes comprising 851 adults and 3948 larvae collected from five cities of Pakistan were used for the detection of dengue virus. A total of 816 batches were made by combining 6 larvae, which were further divided into 204 pools by combining six batches. Among these 26 pools were those of Adults, 10 pools of adults reared from pupae collected in different cities and 168 pools of larvae collected from tyres and other types of containers in which this mosquito is breeding in Pakistan. The post PCR amplified product of 18's ribosomal RNA primer was mounted on agarose gel and after electrophoresis it was analyzed using an UV transilluminator. A 481 base pair single band for the amplified fragment of 18's ribosomal RNA was clearly visible in all pools (Figure 5.1 to 5.3), suggesting

that the method used for extracting RNA and cDNA formation was working properly.

5.3.2 Detection of Virus in Mosquitoes

Simple real time PCR was used for amplification of any viral cDNA. Following analysis of amplification plots (figures 5.4 to 5.6), 66 samples: 31 out of 92 pools in the first plate, 24 out of 92 in the second plate and 11 out of 20 pools in the third plate produced a positive signal. These samples were tested again (figure 5.7) using the same protocol including a melt curve (60°C to 95°C) at the end of the reaction. Melt curves are useful whenever positive signals are obtained with syber green real time PCR assays to distinguish true positives from false positives which are due to the formation of primer dimers. The suspected samples were found to be negative as indicated by the profiles of the melt curve. This is the energy required to break the base-base hydrogen bonding between DNA strands and is dependent on their length (Figure 5.8 to 5.10). All suspected samples were negative for viral genetic material as indicated by the melt curves (figure 8). Melting temperatures of PCR amplicons derived from the plasmid controls (Figure 5.9) peaked at ~80°C (as previously found for other flaviviruses (Moureau et al. 2007). The melting temperatures of the suspected samples all peaked around ~71°C indicating that these are most likely primer dimers as previously reported (Macpherson et al. 2006). The melt curve shows that there was primer dimmer formation, to which SYBR green was accumulating and giving florescence. These results conclude that no virus was detected in any of the pools of mosquito.

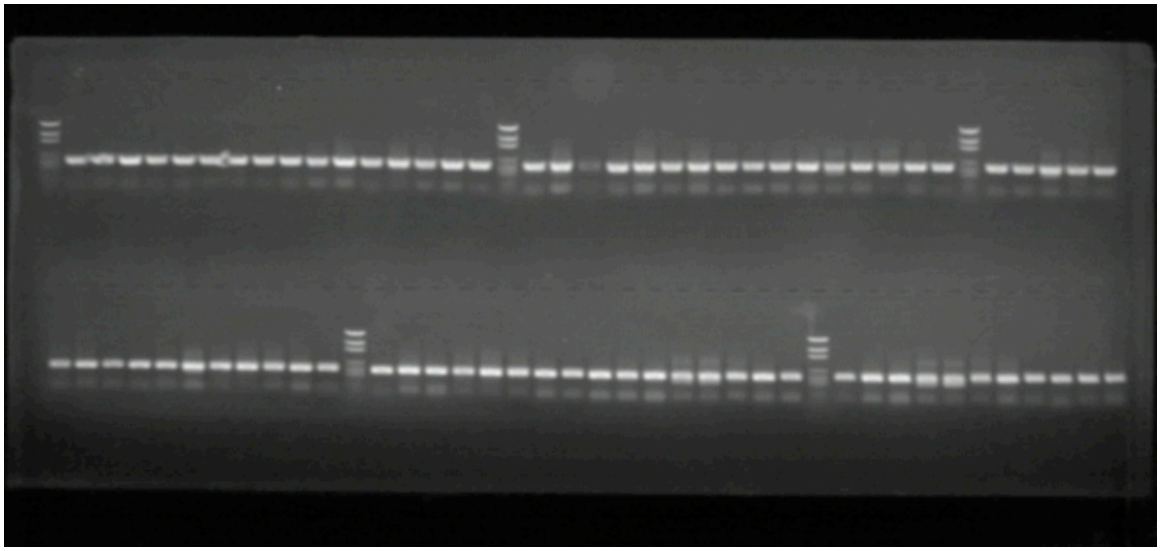

Figure 5.1: Agarose gel showing a 481 base pair single band amplified using primers 18S417/18S920 for the detection of cDNA of 18s ribosomal RNA in 75 Pools of mosquitoes with each row containing 0.5µg of Lambda DNA size standard (Promega) in lane 1, 18, 35 in first row and lane 12 and 30 in second row.

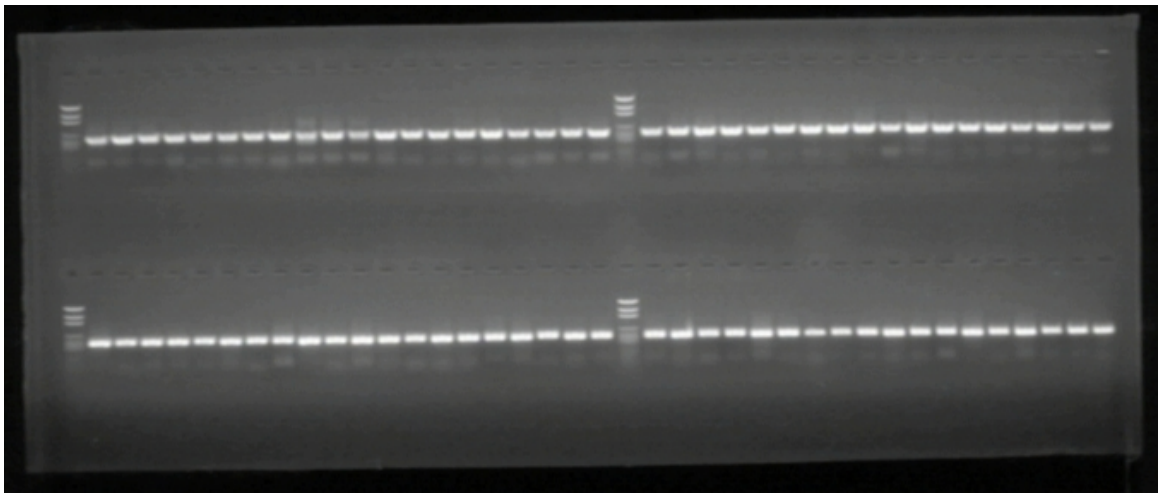

Figure 5.2: Agarose gel showing a 481 base pair single band amplified using primers 18S417/18S920 for the detection of cDNA of 18s ribosomal RNA in 76 Pools of mosquitoes with each row containing 0.5µg of Lambda DNA size standard (Promega) lane 1 and 22 of both rows.

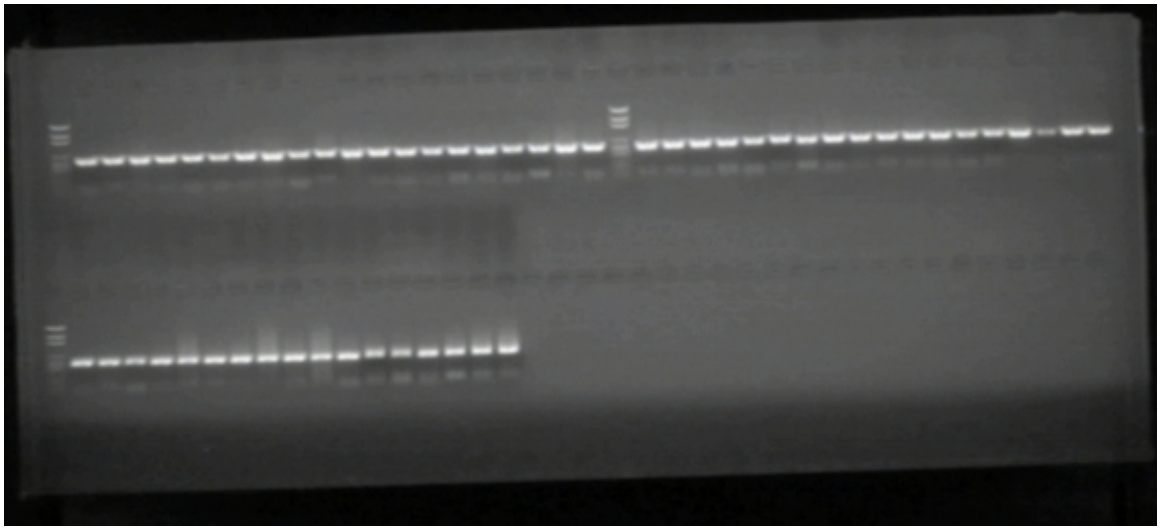

Figure 5.3: Agarose gel showing a 481 base pair single band amplified using primers 18S417/18S920 for the detection of cDNA of 18s ribosomal RNA in 55 Pools of mosquitoes with each row containing 0.5µg of Lambda DNA size standard (Promega) in lane 1 and 23.

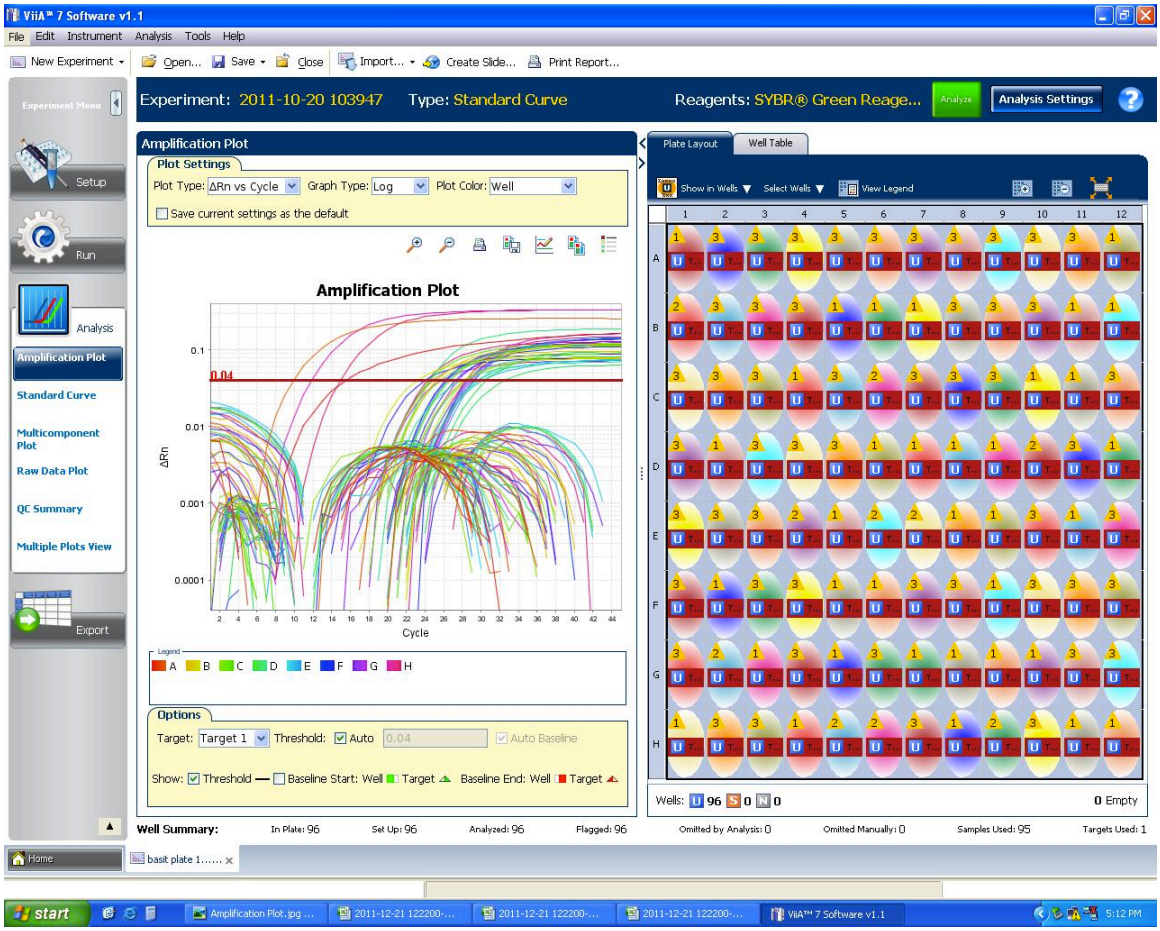

Figure 5.4: Screen shot of real time PCR amplification plot of plate 1. The positive samples are marked as 1 in the plate layout on right side or in the form of curve moving above the thresh hold line (horizontal straight red line with the value 0.04) in the Amplification plot on the left side of the screen shot. Corner wells (A1, A12, H1 and H12) were having the plasmid as a positive control for the real time PCR.

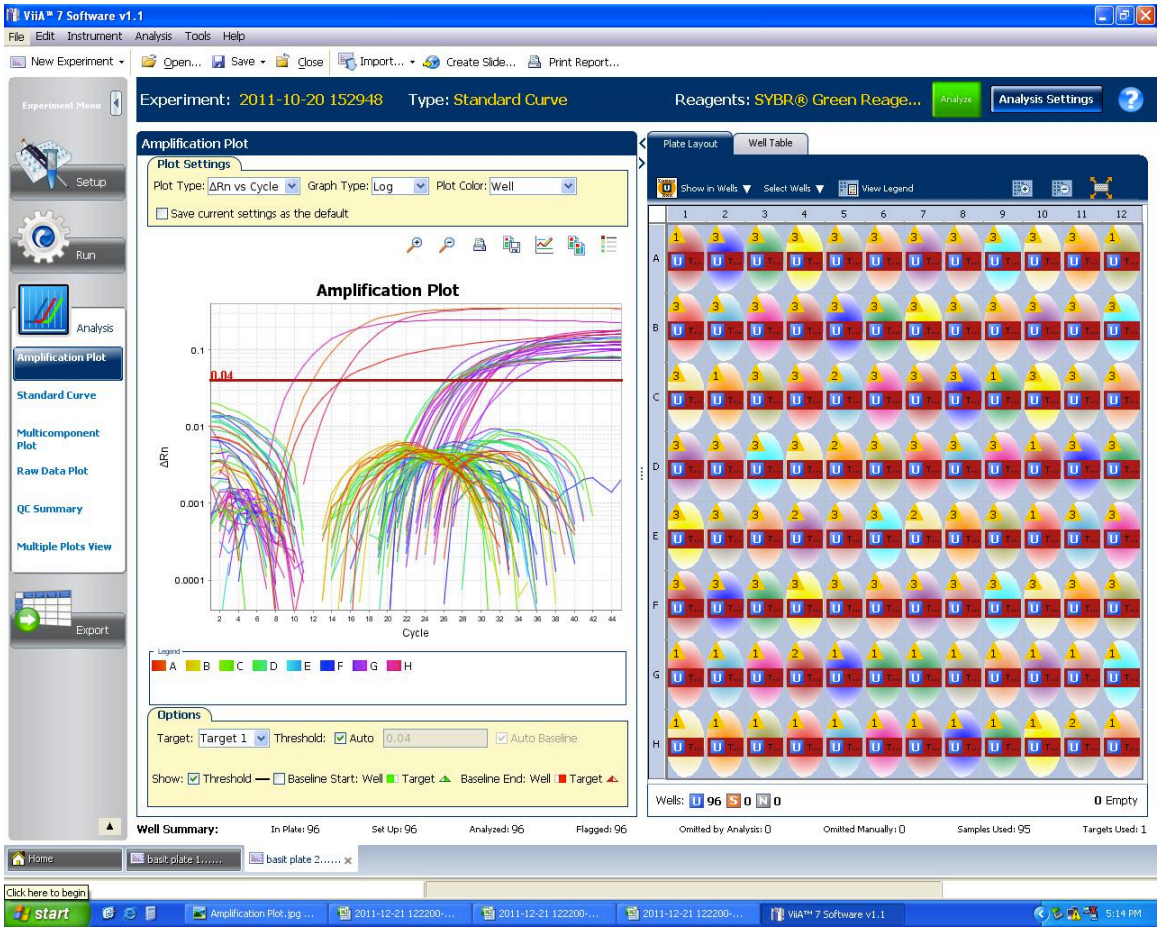

Figure 5.5: Screen shot of real time PCR amplification plot of plate 2. The positive samples are marked as 1 in the plate layout on right side or in the form of curve moving above the thresh hold line (horizontal straight red line with the value 0.04) in the Amplification plot on the left side of the screen shot. Corner wells (A1, A12, H1 and H12) were having the plasmid as a positive control for the real time PCR.

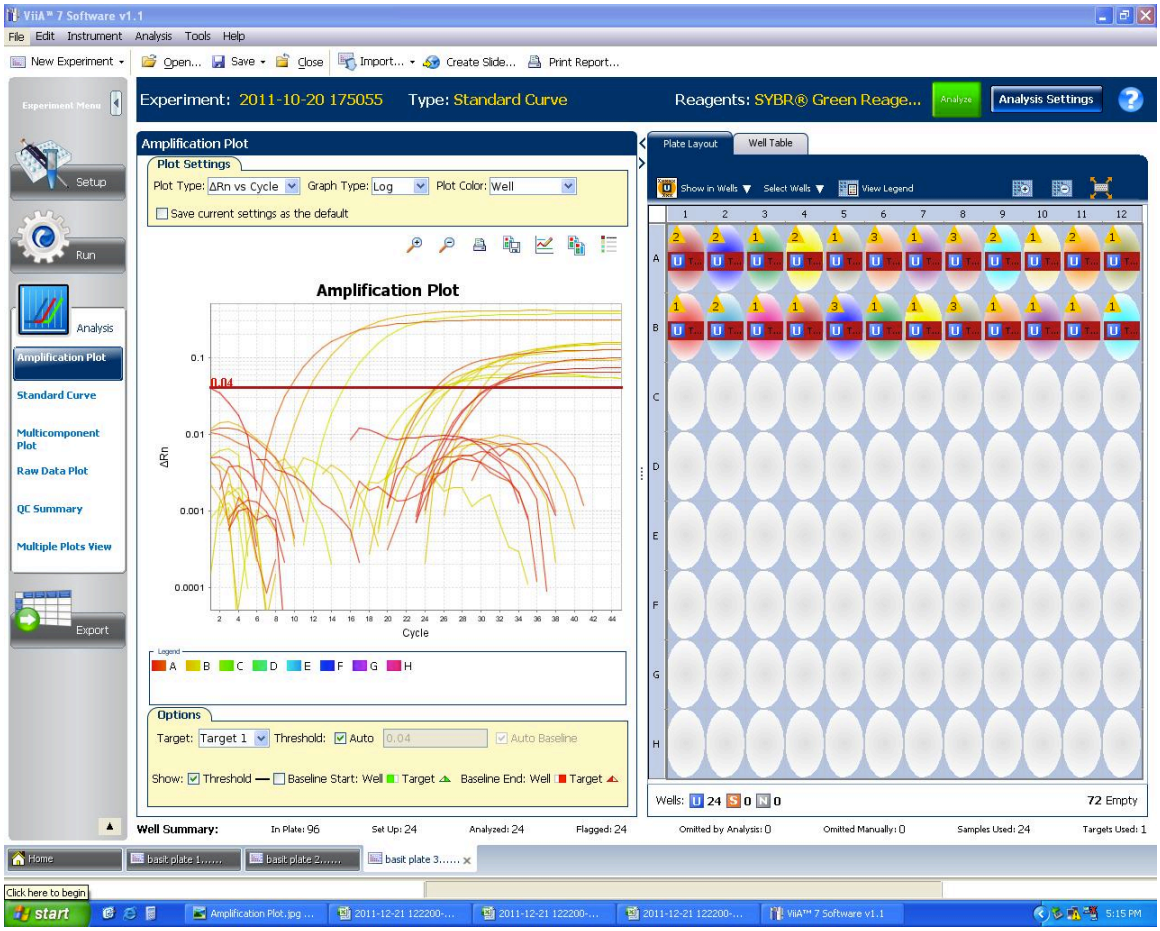

Figure 5.6: Screen shot of real time PCR amplification plot of plate 3. The positive samples are marked as 1 in the plate layout on right side or in the form of curve moving above the thresh hold line (horizontal straight red line with the value 0.04) in the Amplification plot on the left side of the screen shot. Corner wells (A1, A12, H1 and H12) were having the plasmid as a positive control for the real time PCR.

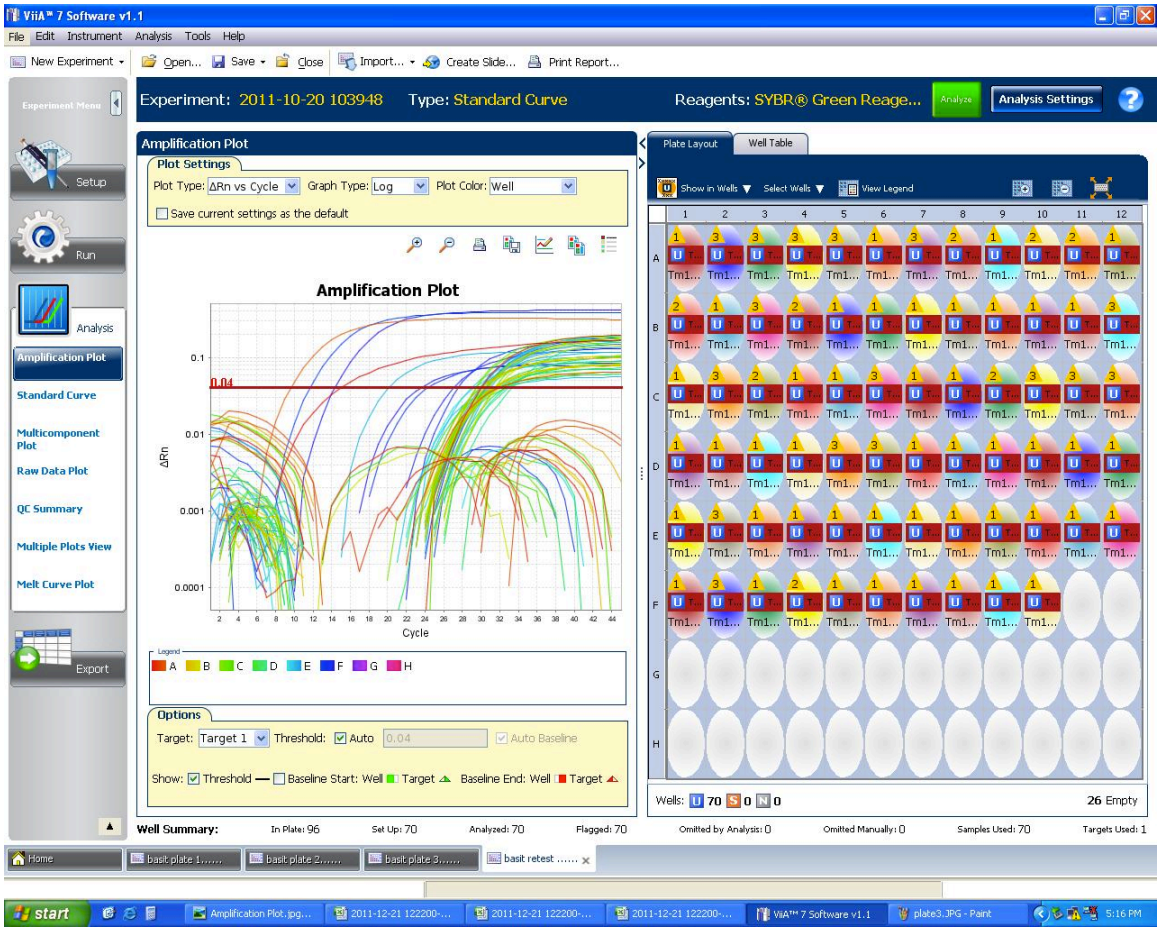

Figure 5.7: Screen shot of real time PCR amplification plot of repeats for all suspected samples. . The positive samples are marked as 1 in the plate layout on right side or in the form of curve moving above the thresh hold line (horizontal straight red line with the value 0.04) in the Amplification plot on the left side of the screen shot. Corner wells (A1, A12, F1 and F10) were having the plasmid as a positive control for the real time PCR.

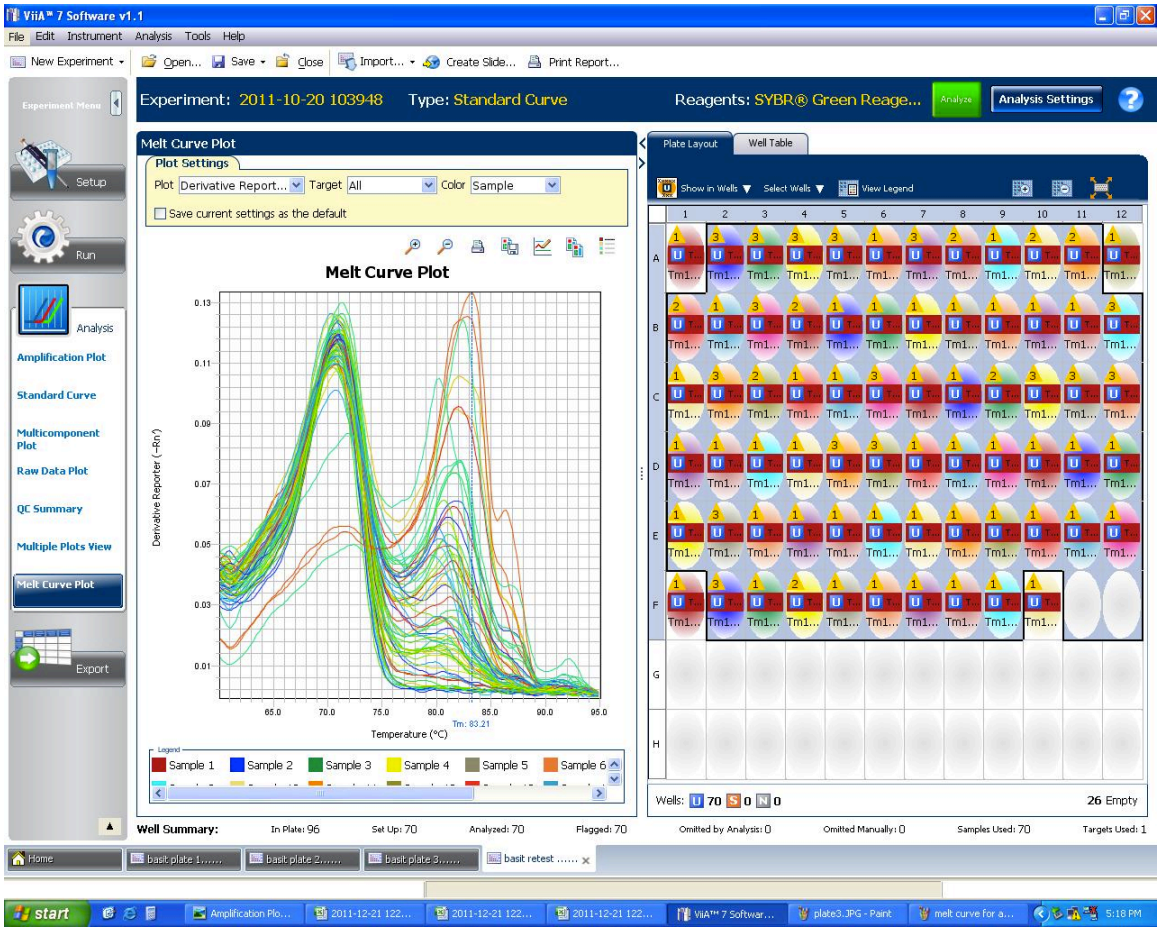

Figure 5.8: Melt curve for amplicons from suspected samples. The melting temperatures of the suspected samples are peaked around ~71°C indicating as shown in the graph of screen shot that these are most likely primer dimers.

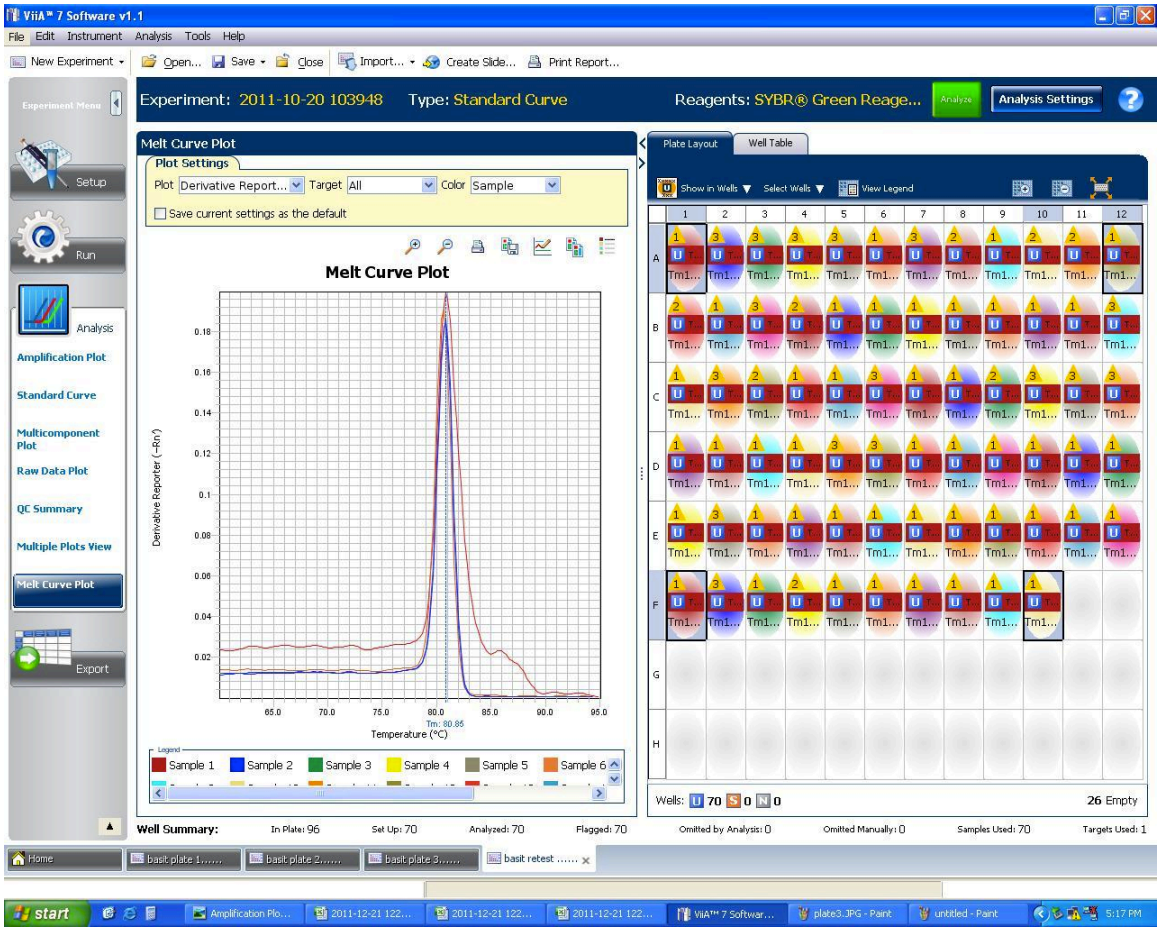

Figure 5.9: Melt curve for amplicons from plasmid controls peaked at 81°C (Moureau et al. 2007) which is the melt curve for dengue serotype 4 virus integrated into the plasmid used as positive control.

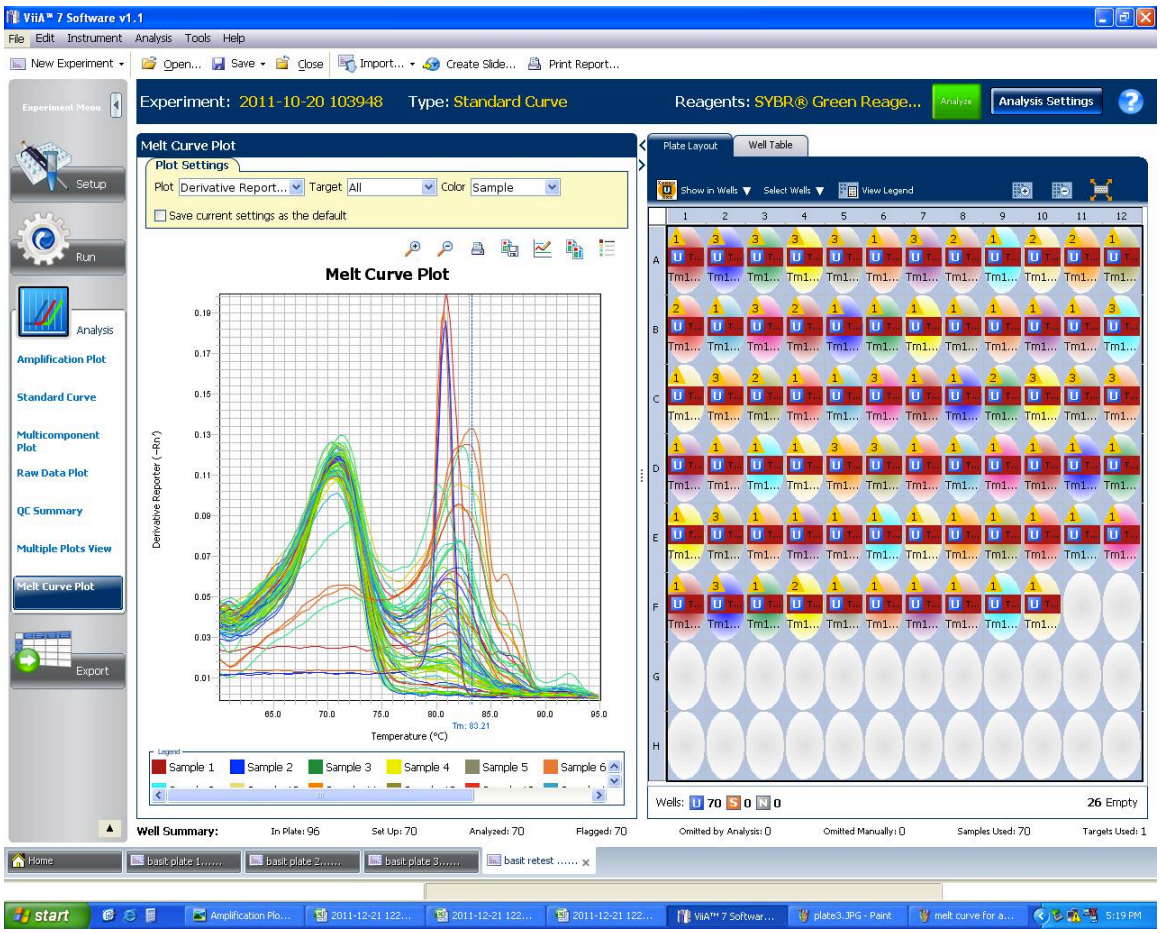

Figure 5.10: Melt curve for amplicons from plasmid controls superimposed on those of suspected positive samples.

5.5 DISCUSSION

The role of long-range passive dispersal of *Aedes aegypti* in shaping its population genetic structure in Pakistan has been discussed in the previous chapter along with the importance of the tyre trade. The work in this chapter was conducted in part to compare the prevalence of dengue virus in larvae collected from both tyres and other containers. A total of 26 pools of adults, 10 pools of adults reared from immature stages and 168 pools of larvae, each consisting of 24 individuals were tested for dengue virus. Amplification of 18s ribosomal RNA of the mosquito suggested that the RNA isolation was shown to be working properly but despite the high sensitivity of real time PCR no virus was detected in any of the pools. Khin and Than (1983) reported a minimum infection rate (MIR) of 1:2,067 whereas Hull et al. (1984) reported a MIR of 1:1,855 in field collected *Aedes aegypti* larvae. In the current study the number of larvae used were almost double that of MIR reported by both these studies but still no virus was detected suggesting a relatively infrequent occurrence of vertical transmission of dengue in Pakistan. One implication of this is that our failure to detect dengue virus in larvae collected from tyres suggest that tyres are only playing an important role in the spread of *Aedes aegypti* in Pakistan but less role in the spread of the dengue virus itself. Secondly this study also showed that transovarial transmission of dengue may be relatively low in the Pakistani vector population.

In spite of the occurrence of dengue epidemics in Pakistan continuously for the last six years and the collection of our samples from the dengue-affected area, no virus was detected in any of the pools of larvae. These results are in agreement with other studies using IFA (Romero-Vivas et al. 1998) and different PCR techniques including Chow et al. (1998), which failed to detect dengue virus among 53 pools of *Aedes aegypti* larvae collected from Singapore where each pool consist of 1 to 10 larvae. In another study conducted in Manaus, Brazil Pinheiro et al. (2005) were also not able to detect dengue virus among 59 pools of *Aedes aegypti* larvae, each pool consisting of 4 to 49 specimens. This is despite the fact that the collection was made from households where residents were suspected of suffering from dengue virus. In the same study they reported 17.1% of the female mosquitoes positive for dengue virus but failed to detect dengue virus in any larvae. Similarly

other studies conducted in Indonesia (Akbar et al. 2008) and Brazil (Zeilder et al. 2008) also detected no virus in field-collected larvae. In the later study the collection was carried out using ovitraps and then rearing the larvae up to 4th instars, a total of 44 pools consisting of 1,172 larvae were tested but none of them were positive for dengue virus. Hutamai et al. (2007) also failed to detect dengue virus in 9,825 *Aedes aegypti* larvae collected from Chiang Mai and Lampang provinces of northern Thailand. They used a different molecular technique known as nucleic acid sequence based amplification assay, which has been shown to be specific to dengue virus and it does not show cross reactivity with non-dengue related viruses or other flaviviruses (Wu et al. 2001, Usawattanakul et al. 2000a,b). Similar to this study all these studies showed that the transovarial transmission of dengue virus in vector population is very rare and if it occurs it is happening at such a low rate that it is not detectable in the field collected larvae.

Molecular techniques like reverse transcriptase polymerase chain reaction (RT-PCR) and real time PCR detect viral sequence in the samples. As such they are considered to be the most powerful tools for detecting the presence of virus in mosquitoes (Zeilder et al. 2008). As discussed above studies generally find that the transovarial transmission of dengue virus is very low when it is measured through field collected larvae or adult males using molecular techniques like PCR. However lab based studies often detect a high rate of dengue virus (48.5% in *Aedes albopictusi* and 27.3% in *Aedes aegypti*) among the progeny of experimentally infected mosquitoes (Castro et al. 2004). Ibanez et al. (1997) were the first to detect the presence of dengue virus in field collected adult males. Males can only get the virus through transovarial or sexual transmission so this may have been the first example of vertical transmission in nature using RT-PCR. They detected virus in only one pool of adult males but they don't report the exact number of pools of adult males used. In another study using type specific PCR Kow et al. (2001) reported 1.33% of the *Aedes aegypti* and 2.15% of the *Aedes albopictus* field collected males to be vertically infected with dengue virus. The only study, which has definitively reported the presence of dengue virus in field-collected larvae using PCR, is that of Cecilio et al. (2009). In this study they collected eggs by using ovitraps in Brazil and 2,241 *Aedes aegypti* larvae were divided into 163 pools and 1,241 larvae of *Aedes* albopictus in 72 pools, of these pools 76 of *Aedes aegypti* and

35 pools of *Aedes albopictus* were found positive with MIR of 1:45.82 and 1:35.45 for the two species respectively. The reasons for obtaining such a high rate of vertical transmission could be the high prevalence of dengue disease in that area as more than 324,000 cases of dengue were reported during the collection period (Cecilio et al. 2009). In the year of our study the epidemic started later than expected at the end of October (it normally it starts in the start of August). The low occurrence of dengue cases in Pakistan as compared to Brazil and the absence of dengue disease during the period of collection could be the reason of failure to detect dengue virus in the larvae collected from Pakistan.

Many studies have reported vertical transmission of dengue virus in mosquitoes using various immunological techniques such as immuno florescent assays (IFA) (Khin and Than 1983, Hull et al. 1984, Thenmozhi et al. 2000, Fouqe et al. 2004, Thnmozhi et al. 2007, Angel and Joshi 2008) complement fixation tests (CFT) (Khin and Than 1983, Hull et al. 1984) and Enzyme Linked Immunosorbant Assays (ELISA) (Thenmozhi et al. 2007, Arunachalam et al. 2009). Some of these studies also reported a very low MIR regarding detection of virus in larvae such as Khin and Than (1983) in Rangoon, Burma (0.048%) and Hull et al. in the Port of Spain, Trinidad (0.054%) using IFA and CFT and Fouqe et al. (0.036%) by only using IFA. Other studies using ELISA and IFA reported low rates of vertical transmission in the vector population such as Thenmozhi et al. (2000) who reported the presence of virus among 4 out 607 (0.66%) pools of field collected males and larvae from Vellore district of Tamil Nadu India. In another study by the same author in 2007 using the same techniques in Kerala, India 0.8% of the mosquitoes checked for vertical transmission were dengue positive. Arunachalam et al. (2008) reported a higher MIR of 28:1000 (2.8%) among the male mosquitoes collected from Tamil Nadu, India using ELISA. Angel and Joshi (2008) reported a very high rate of vertical transmission of up to 8.4% in *Aedes vittatus*, 11.2% in *Aedes aegypti* and 15.9% in *Aedes albopictus*. They used ELISA to detect dengue virus in laboratory-reared adults collected as larvae in Rajisthan, India. This is the only study reporting such a high rate of vertical transmission of dengue virus in vector populations. All these studies used serological tests, which is based on antigen antibody reaction. In the Flaviviridae a strong antibody cross reactivity occurs amongst its members. Due to this antibodies of dengue have a wide reactivity with

other flaviviruses (Santos et al. 2008), which makes it difficult to interpret the antibody response with regard to presence of dengue unless the area is free of other flavivirus infections (Laue et al. 1999). On the other hand detection of dengue virus using molecular technique like PCR is highly indicative of the presence of virus itself in the mosquito sample. Whether it's a serological technique or molecular technique to detect vertically transmitted virus in mosquitoes, all these studies show that the efficiency of transmission is so low that it is not effective in maintaining endemic persistence (Adams and Boots, 2010).

In most of the studies transovarial transmission of dengue was detected either in adults reared from field-collected larva (Khin and Than 1983, Hull et al. 1984, Thenmozhi et al. 2000, Thenmozhi et al. 2007, Angel and Joshi 2008) or field collected adult males (Ibanez et al. 1997, Kow et al. 2001, Arunachalam et al. 2008). Gaunter et al. (2007) failed to detect dengue virus in larvae collected from Oxaco, Mexico but when larvae from the same batch were reared to adults and kept alive for 14 days dengue virus was detected in 9.3% of the pools tested. He suggested several factors such as the S-phase of the cell cycle in mosquito cells (Helt and Harris 2005), cellular proteins involved in dengue virus replication (Monroy et al. 2007) and conditions like larval growth (Sumanochitrapon et al. 1998) may be responsible for these differences. In this study the failure of detection of dengue virus in any of the pools could be due to the assaying at the larval stage (Gaunter et al. 2007).

The absence of large dengue epidemics during the study period could also be the cause of absence of dengue in adults collected from Karachi. Most of the studies conducted for detection of virus have used larval collection for estimating the prevalence of dengue virus in the vector population. Using adult mosquitoes for detection of dengue virus is often hampered by the fact that the method of collection of sufficient number of adult mosquitoes since it is tedious, slow and not cost effective, on the other hand large numbers of larvae could be collected simply by using ovitraps (Lee and Rohani 2005). Very few studies have used adult collection for dengue surveillance. These studies were conducted either for long period covering one or more dengue epidemics (Chow et al. 1998, Chung and Pang 2002) or only during the dengue epidemic periods from houses and surroundings of

dengue patients (Urdenata et al. 2005, Pinheiro 2005). Chow et al. (1998) detected dengue virus in adult mosquitoes collected during 1995 and 1996 covering two dengue epidemics in the area. Dengue virus was detected six weeks earlier then the occurrence of thedengue epidemic and during dengue epidemics. In another study conducted from 1997 to 2000 in Singapore also by Chung and Pang (2002) dengue virus was detected in field-collected adult *Aedes*. Dengue virus was detected in adults only during the epidemic periods. Some studies have focused on the surroundings of dengue patients for entomological collections and then detecting dengue virus in the mosquitoes. Various studies conducted in Manaus, Amazonas (Pinheiro et al. 2005), Venezuela (Urdaneta et al. 2005), Mexico (Garcia-Rejon et al. 2008) and Brazil (Guedes et al. 2010) used the strategy of collecting adult mosquitoes during the dengue epidemic period from the surroundings of dengue patients. In all these studies the dengue infection rate varied from 1.5% to 6.9%. In current study adults were collected from a dengue endemic area of Karachi but during the study period there were relatively low numbers of dengue cases and this may explain why the virus was not detected in adult mosquitoes.

In this study dengue virus prevalence was checked in larvae without considering the infection rate of adults in the area. There is no information available regarding the dengue infection rate in adults in the mosquito populations of Pakistan so it is difficult to estimate the infection rate of vertically infected larvae so investigating the presence of dengue in adults will not only give an insight into the rate of infection of adults population but will also help in estimating the rate of vertical transmission of dengue virus in the population by looking for dengue virus in larvae along with adults. Therefore a detailed study of the dengue infected areas of Pakistan is required focusing both on adults as well as larvae not only to check the prevalence of larvae in mosquito populations but also to estimate the rate of vertical transmission of dengue virus in local mosquito populations.

My work suggests that vertical transmission of dengue virus in vector mosquitoes occurs at very low frequency in nature so we were unable to detect it in the larvae. Addressing the questions of the spread of this disease in Pakistan and the role of tyres in it, it is clear that human movement may play an important role in the spread of the disease in Pakistan. This was observed in the 2006 epidemic when

people travelling from Karachi to Lahore and then to Lahore to other parts of Punjab carry this virus along with it and then suffers from dengue in those areas. Most recently according to the provincial health department of Khyber Pukhtoonkhwa in 2011, 296 of the dengue cases reported in Khyber Pukhtoonkhwa province, 92 had a history of travelling to the dengue-affected areas of the Punjab. These virus carrying people are the most likely cause of the spread of this disease in Pakistan. The low prevalence of the virus in larvae suggests that tyres are spreading the vector but it's difficult to establish its role in the spread of dengue virus across Pakistan.

Very low transovarial transmission of Dengue virus in Pakistani vector populations suggest that this mechanism might be occurring at a very low frequency in nature, and its role in viral persistence in Pakistan may not be very extensive (Zeilder et al. 2008) so there may be other strong sources of reintroduction of the virus into the Pakistani population such as the international movement of people (Zeilder et al. 2008) or local low level asymptomatic circulation (Adams and Boots, 2010). People from Pakistan travel to other dengue endemic countries such as Malaysia, Indonesia, Brazil and India and it is possible that they may bring virus on their return. The presence of the vector and good climatic conditions such as monsoon rains may help the virus to transfer from these infected people to mosquitoes and then to other people. During the epidemic period the cases reported have been described as a tip of the iceberg and about 50 to 90% of all the dengue virus infections are asymptomatic (Kyle and Harris 2008). The presence of vector mosquito throughout the year in Karachi (Siddiqui and Naqvir, 2008) due to its moderate climatic conditions and local low level of asymptomatic or subclinical cases could maintain the virus in Pakistan. Favorable climatic conditions resulting in the increase of *Aedes aegypti* could then give rise to an epidemic. People travelling from Karachi to other parts especially during the rainy season could also result in the spread of the epidemic in Pakistan.

Chapter 6

DETECTION OF FLAVIVIRUS LIKE SEQUENCES IN THE MOSQUITO GENOME

6.1 INTRODUCTION

There are two types of interaction between the virus and infected cells: autonomous replication of the virus within the cell and the integration of the viral genome into the host cell chromosome. Integration of the viral genome into that of the host cell has been known for a long time in the temperate bacteriophages, oncogenic viruses and the related slow viruses (Zhdanov 1975). The integration of the genome of classic infectious viruses such as the measles virus and arbovirus into the nuclear DNA of avian, mammalian and human cells has also been reported as long ago as 1974 (in Zhdanov 1975) however the potential evolutionary mechanism involved in the capture of genomic information from non-retroviral RNA viruses by eukaryotic cells still lacks a complete explanation (Crochu et al. 2004). Zhdanov (1975) suggested that prior to the integration into the host genome, RNA is copied into DNA by intracellular reverse transcriptase activity. Zhdanov and co-workers reported DNA forms of numerous RNA viruses such as Measles virus (family Paramyxoviridae; Zhdanov and Parganovich 1974), Sindbis virus (family Togaviridae; Zhdanov and Azadova, 1976), Lymphocytic Choriomeningitis virus (LCMV, family *Arenaviridae*; Gaidamovich et al. 1978) and Tick-bone encephalitis virus (family *Flaviviridae*; Drynov et al. 1981). Among these studies, however, only the integration of the LCMV sequences as DNA in mice over 200 days after infection has been confirmed by Klenerman et al. (1997).

It has been shown *in vitro* that DNA synthesis from viral RNA was carried out by the endogenous activity of the reverse transcriptase but the way in which the viral sequences then integrate into the cellular genome has not been investigated (Crochu et al. 2004). The possibility of horizontal transfer of genetic information from RNA virus to eukaryotes has been supported by a limited number of observations. For example, Morvan et al. (1999) detected DNA sequences of Ebola

virus (family Filoviridae) in the spleen of an infected rodent and Malik et al. (2000) detected regions of high similarity of Phleboviruses (family Bunyaviridae) in the genome of *Caenorhabditis elegans*.

The genus *Flavivirus* of the family *Flaviviridae* is composed of a large number of single stranded positive sense RNA viruses that exhibit a wide range of geographic distributions alongside a diverse host relationship. The majority of the viruses in this genus are arthropod borne with mosquitoes or ticks acting as vectors to transmit them to a vertebrate host. However some of the members of this genus have no known arthropod vector making them exclusively vertebrate viruses (Sang et al. 2003). Finally Cell fusing agent virus (CFAV) and Kamiti river virus (KRV) are two members of the genus, which have been classified as an insect virus with no vertebrate host. CFAV was isolated from *Aedes aegypti* cell culture but it replicates in nature in *Aedes albopictus* (Stollar and Thomas 1975) whereas KRV was isolated from *Aedes macintoshi* collected in the central province of Kenya (Sang et al. 2003). Crochu et al. (2004) reported the integration of viral sequences similar to CFAV into *Aedes albopictus* and Kamiti river virus in *Aedes aegypti.* They called these sequences Cell Silencing Agent (CSA) and CSA2. The NS5 like sequences in *Aedes aegypti* was different from that of *Aedes albopictus* because it does not include the functional motifs of a polymerase. This was the first example of flavivirus like sequences detected in the double stranded DNA of any mosquito. Crochu et al. (2004) also demonstrated that the virus takes the form of DNA integrated into the cellular genome itself. They also discussed the evolutionary implications of this integration by showing that the split between *Aedes w-albus, Aedes albopictus* and *Aedes aegypti* is approximately 32-44 million years ago where as the most recent common ancestor of CFAV, KRV and CSA is probably around 3500 years ago and certainly less than 350,000 years ago. Thus the split from the common ancestor of flaviviruses is significantly much later than the split of the *Aedes* species. *Aedes aegypti* is a mosquito of African origin and both CFA and KRV were isolated from African mosquitoes whereas *Aedes albopictus* is an Asian mosquito. This shows that two independent integration events occurred in *Aedes aegypti* and *Aedes albopictus* since the common ancestor of these mosquito existed long before the existance of these flaviviruses (Crochu et al. 2004).

This study uses the same method as that of Crochu et al. (2004) to detect CSA2 sequences in mosquitoes collected in Pakistan and to discuss their evolutionary implications. In particular I examine whether the same sequences exist in mosquitoes in Pakistan or whether there are differences among these sequences and then to establish a phylogenetic relationship between those isolated from Pakistan and those of Crochu et al. (2004).

6.2 MATERIALS AND METHODS

DNA extracted from mosquito samples as described in Chapter 4a was used for the detection of flavivirus DNA sequences integrated into the *Aedes aegypti* genome. In total, 32 DNA samples were randomly selected for this analysis.

6.2.1 Preparation of Primers solution for PCR

Flavivirus like NS5 DNA sequences in the genome of *Aedes aegypti* were detected by using the primer set PF1S (5'-TGYRTBTAYAACATGATGG-G-3') /PF2R-bis 5'-GTGTCCCAICCNGCNGTR-3'). A 10μM primer stock solution of each primer was prepared and stored at -20°C until needed. PCR reactions in 50 μl volumes each contained a final concentration of 0.5 μM of each primer, 0.25 mM of each dNTP. Reactions were run in a 96 well PCR microplate using the KOD hot start DNA polymerase as described in section 2.5.5. Cycling parameters were as follows:

95°C for 15 minutes (1 cycle), 40 cycles of 94°C for 30 sec, 50°C for 45 sec, 68°C for 35 seconds and 68°C for 10 minutes (1 cycle).

6.2.2 Sequencing of PCR Product

Agarose gel electrophoresis of DNA showed a clean PCR product revealing a single band of the expected size. When compared to a DNA standard, DNA quantities were estimated to be > 20ng/μl. An ExoSAP technique was used for the clean up. This method degrades and dephosphorylates single stranded DNA i.e.

unincorporated primers and dNTPs by using a mixture of shrimp alakaline phosphotase (SAP) and exonuclease I. Following ExoSAP, both the quality and quantity of DNA were adequate as analysed by gel electrophoresis. 2μl volumes of each DNA sample were used for the cycle sequencing reaction setup.

a) ExoSAP Protocol

First, 66 samples with at least one sample from each collection patch were selected for cycling sequencing. Next 5μl of PCR product was transferred to a new microtitre plate and 2μl of ExoSAP was added to each well containing the DNA, was mixed well and covered with silicon PCR seal. The plate containing the DNA was incubated at the following temperature using a PCR thermal cycler with hot lid on: 37°C for 15 minutes then 80°C for 15 minutes to deactivate the enzymes. Finally the samples were collected as soon as possible and stored at -4°C.

b) Cycle Sequencing Reaction Set-Up

A Greiner 96-well plate was used for setting up the cycle sequencing reaction as this plate will also be used for the final implementation on the ABI-3730 sequencer. The following recipe was used for the clean-ups in each well:

Reagents	**Vol/rxn (μl)**
BigDye Terminator Mix v3.1	0.8
Sequencing Dilution buffer (5x)	1.5
Primer (1μM)	1.6
DNA template	2.0
Double distilled (dd) H_2O	4.1
Total Volume	**10.0μl**

Sequencing reactions were set up using the forward primer or the reverse

primer. Sequencing reactions were carried out using a thermal cycler with a hotlid. Cycling parameters were as follows: 96°C for 1 minute (1 cycle), followed by 30 cycles of 94°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes.

c) Ethanol/EDTA/Sodium Acetate Precipitation

The following procedure was carried out to precipitate the DNA in the 96-well plates. Firstly, 2μl of 125mM EDTA, 2μl 3M sodium acetate (pH 5.2), 10μl ddH_2O and 52.5μl 95% ethanol were added to each well containing the DNA. Double distilled H_2O was added before ethanol to prevent precipitation of DNA by ethanol and then both plates were incubated in the dark for 15 minutes. After incubation the plates were centrifuged in a plate centrifuge at 3000g for 15 minutes. After centrifuging the plates were banged upside down on a paper towel to remove most of the liquid. This was done with care in order not to lose the DNA pellet. Next the plate was placed upside down on a paper towel in the plate centrifuge and spun for 30 seconds at 190g in order to remove any liquid left in the wells. The pellets were then washed using 76.5μl of 70% ethanol. The plates were again spun at 3000g for 5 minutes, and then ethanol was removed by banging the plates upside down on paper towel. At the end the plates were again placed upside down on a paper towel in the plate centrifuge for 30 seconds at 190g in order to dry the DNA pellets.

The plates were allowed to dry briefly and then 10μl of Formadide was added to each well. In the empty wells water alone was used. DNA was denatured at 95°C for 3 minutes using the PCR machine and then placed on icy water for about 3 minutes. This denatured the DNA and due to the temperature shock it does not re-nature. The plate was then placed in a special plate cover and placed in a ABI3730 48-well sequencer for sequencing. Raw data from the sequencer was transferred to a Linux operating system and a special Perl Script written by University of Edinburgh for the Sheffield Molecular Genetic Facility at the University of Sheffield was modified and then used for getting the final sequences in Fasta format. The Fasta format file was then aligned using Clustal X (Larkin et al. 2007). Neighbour-joining polygenetic trees were generated using the P-distance

algorithm and genetic relatedness was reported as % identity.

6.3 RESULTS

The presence of Cell Silent Agent 2 (CSA2) was examined by using NS5 degenerative primers PF1S/PF2R-bis. All 32 samples were amplified using these primers that had been described by Moureau et al. 2007 giving a single band of 269-272 nucleotides long (Figure 6.1). All the samples were subjected to sequencing and a 165 base pair sequence from all samples was used for building the phylogenetic tree. The nucleic acid identity between the various sequences of the wild caught mosquitoes/larvae were 100% while the identity values with the sequence that was originally identified in the A20 cell line (derived from fertilized eggs of *Aedes aegypti*) were 98.2%. This integrated NS5 sequence is most closely related to that of the Kamiti River Virus (KRV), an insect flavivirus of the Cell Fusing Agent (CFA) group within the genus *Flavivirus*. Nucleotide identities between the NS5 of KRV and that of the A20 were 75% while nucleotide identity between NS5 of KRV and those of mosquitoes from Pakistan were 76.8%.

Sequence analysis indicates that all NS5 sequences derived from the Pakistani mosquitoes have a C→T change at position 1390, an A→G at position 1405 and a T →C at position 1512 (relative to the sequence derived from the A20 cell line: Genbank accession number; AY347953). Neighbour joining phylogenetic trees using the Nei-Gojobori method (Nei and Gojobori 1986) (to account for synonymous and non-synonymous changes) incorporating a P-distance algorithm showed the A20 sequence roots all other NS5 sequences that are included in this study with KRV as an out group (Figure 6.2).

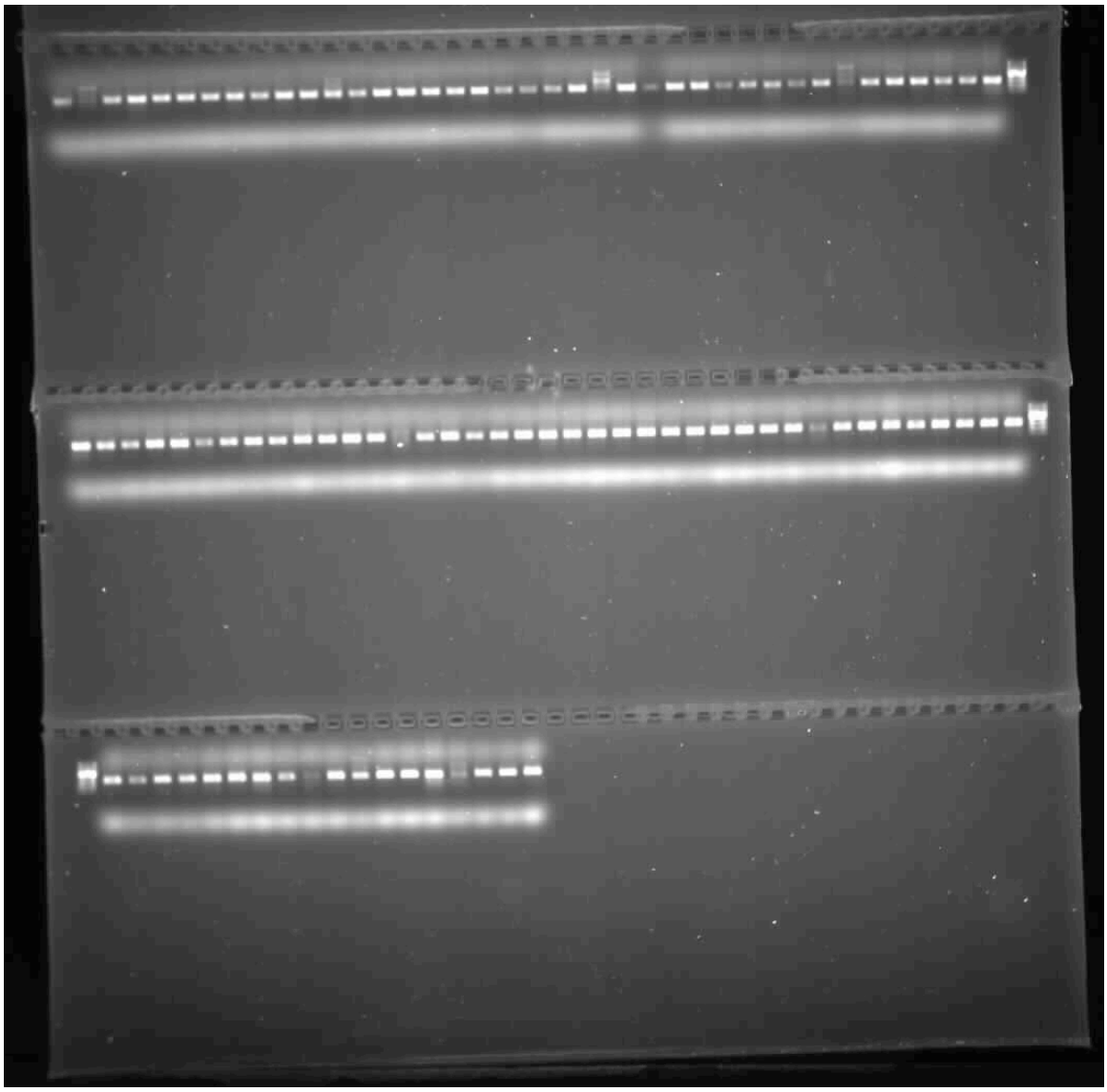

Figure 6.1. Flavivirus like NS5 sequence amplicons in *Aedes aegypti* genome amplified by using primer set PF1S/ PF2R-bis analysed by agarose gel electrophoresis and viewed under UV showing a single band of 272 base pair in all mosquito specimens. 32 samples that had good quality amplified DNA were used for sequencing and further analysis.

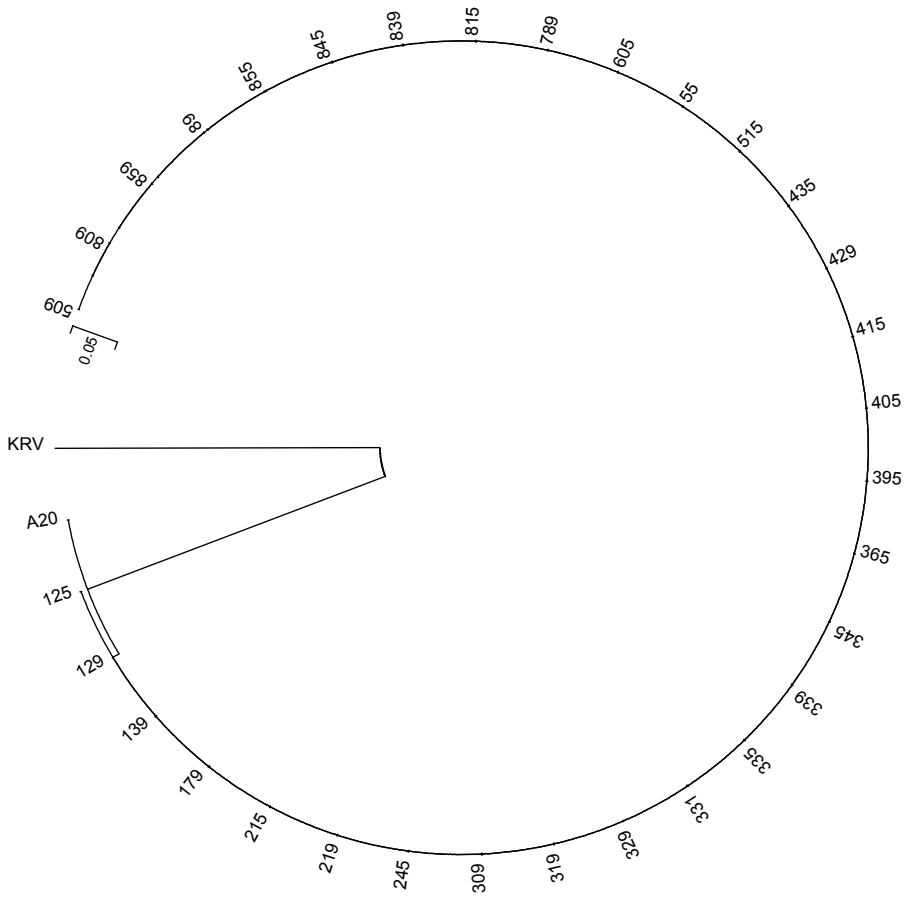

Figure 6.2. NS5 Neighbour joining phylogenetic tree of 32 sequences of field collected mosquitoes from Pakistan and its relation with that of KRV and reference A20 sequences.

6.4 DISCUSSION

The PCR detection of flavivirus like sequences in mosquito genome has already been reported in *Aedes albopictus* (C6/36) and *Aedes aegypti* (A20) derived cell lines (Crochu et al. 2004). This study also reports the same sequences in *Aedes aegypti* collected from Pakistan and our sequence analysis of the PCR products have shown that all products contained the expected sequence of NS5 that was originally identified in the A20 cell line. This cell line was derived in 1969 (Varma and Pudney, 1969) from fertilized eggs of the Liverpool strain of *Ae.aegypti* (originally from West Africa) that was colonized in 1957. KRV was described in 2003 (Sang et al. 2003, Crabtree et al. 2003). This virus has no known vertebrate host (Lutomiah et al. 2007) and is an insect flavivirus of the cell fusing agent (CFA) group within the genus *Flavivirus*. CFA was originally described by Stollar & Thomas in 1975, and sequenced in 1992 by Cammisa-Parks.

The sequences that we identified using NS5 detection primers are most closely related to that of Kamiti River Virus like those reported by Crochu et al. (2004). However the nucleotide identity reported in current study was slightly higher (76.8%) than those of A20 cell line sequences (75%) reported by Crochu et al. (2004). The nucleic acid identity suggests that NS5 sequences are fully conserved (100% identity) among mosquitoes from Pakistan. Many studies on mosquito evolution suggests that the split between *Aedes albopictus, Ae. aegypti and Ae. w-albus* from a common ancestor is roughly between 34-42 million years ago (Simmons and Weller 2001, Meyerhans and Vartanian 1999). Crochu et al. (2004) reported the most recent common ancestor of the two insect only flavivirus CFAV and KRV and the integrated flavivirus sequence known as CSA to be around 3500 years ago (with a range between 350 to 350000) (Corchu et al. 2004). This time line was calculated by assuming the mutation rate for the integrated CSA and flavivirus was the same. RNA viruses mutate at the rate of 10^{-3} to 10^{-5} substitutions/site/year (Drake et al. 1998) whereas eukaryote DNA mutates at the rate of 10^{-9} substitutions/site/year (Kumar and Subramanian 2002). The time of divergence from the most recent common ancestor calculated by Crochu et al. (2004) was on the basis of mutation rate of insect flaviviruses where as the CSA is a part of nuclear genome which make this assumption doubtful. On the other hand an

approximately 2% difference (3/165 nucleotides) was observed in the sequences from Pakistan and that of the A20 cell line which if approximately calculated on the basis of mutation rate of nuclear DNA (10^{-9}) indicates a divergence between these inserted sequences much earlier (approximately 2 Million years ago) than that indicated by Crochu et al. (2004).

In conclusion the insertion of viral genomes reported by Crochu et al. (2004) were also present in mosquitoes collected from Pakistan and these sequences are highly conserved. A study should be carried out to examine *Aedes aegypti* from other parts of the world to determine whether NS5 like sequences are present in all *Aedes aegypti* populations. With this we will get a better evolutionary picture of these sequences and their evolutionary importance should be properly investigated in detail.

Chapter 7

CONCLUSION AND FUTURE WORK

This thesis has been focussed on the dengue vector i.e. *Aedes aegypti* in Pakistan. Previously there have been very few reports of the presence of this mosquito in Pakistan (Barraud 1934, Qutubuddin 1960) and indeed at one point it was thought that it was eradicated from Pakistan as a byproduct of the malaria control program (Suleman 1996). Later this mosquito was thought to be limited to the port city of Karachi (Kamimura et al. 1986, Tariq and Zafar 2000) and the Northern city of Peshawar (Suleman et al. 1996). My study has shown that the mosquito has now extended its range to a number of cities across Pakistan, which means that its spatial distribution is vast. *Aedes aegypti* once eradicated from Peshawar and Lahore has reoccupied these areas and was also collected from Hyderabad, Multan, Faisalabad, Gujranwala, Sheikhupura, Rawalpindi, Taxilla, Hasanabdal, Haripur and Attock for the first time. *Aedes aegypti* is breeding in almost all types of artificial containers, with tyres identified as the key breeding site of *Aedes aegypti* in Pakistan (Chapter 3).

Tyres are not only acting as the preferable breeding point but as shown by the population genetics analysis (Chapter 4) could also be responsible for the passive dispersal of the mosquito in Pakistan. Our analysis suggests that there is a single *Aedes aegypti* population that shows the characteristic pattern of isolation by distance over large spatial scale. A strong negative correlation between genetic distances and road connection suggests a human mediated passive dispersal of this vector mosquito in Pakistan. A negative correlation between observed heterozygosity and geographical distance showed expansion of this mosquito from Karachi. Overall this work suggests that colonization started from Karachi and had moved towards the north aided by human mediated transportation of *Aedes aegypti* in Pakistan (Chapter 4).

The role of tyres in the spread of the dengue virus itself was also investigated by examining the prevalence of the virus in larvae collected from tyres.

Larvae collected not only from tyres but also from other containers were however found to be negative for the presence of dengue virus. Given this low rate of infection in the larvae, we would suggest that at least within our study area at this time, tyres are unlikely to be playing any significant role in the spread of dengue virus in Pakistan. This failure to detect virus in larvae also suggests that the role of vertical transmission in the maintenance of the disease may be none or non-significant (Chapter 5).

We did find flavivirus like sequences in the mosquito genome that were first detected by Crochu et al. (2004) in the A20 cell line of *Aedes aegypti* from West Africa (Varma and Pudney, 1969). The sequences detected in the current study were similar to those of Crochu et al. (2004) with only a few changes. My study therefore shows that these sequences are present not only in African *Aedes aegypti* but also in those from Asia and that these sequences are relatively conserved (Chapter 6).

This study has explored a number of aspects of the biology of the dengue vector in Pakistan. However considerable research work is still required to understand the ecology of *Aedes aegypti* and the biology of its role in disease transmission in Pakistan. Very little is known about the ecology of this mosquito in Pakistan. Previous studies report this mosquito only from Karachi (Kamimura et al. 1986, Tariq and Zafar 2000) and Peshawar (Suleman et al. 1996), and these studies had reported only cemented tanks (Kamimura et al. 1986) and tyres (Suleman et al. 1996) as breeding containers of *Aedes aegypti* in Pakistan. My study has identified a number of different types of containers used by this mosquito for laying eggs in the study area. However a considerable amount of research on the ecology of this mosquito needs to be carried out in Pakistan. In particular I would suggest that research needs to be focussed on the seasonal variation and relative abundance of this mosquito in each city of Pakistan. Seasonal variation should be studied with special reference to the occurrence of dengue disease in the infected areas. This study had identified various types of containers that can be used by *Aedes aegypti* to breed but various population indices like House index (HI), Container index (CI) and Breteau index (BI) should be investigated to determine the abundance of this mosquito in detail in Pakistan. Various vector factors (Preechaporn 2006) including

mosquito density, behaviour, food level, duration of development, size at emergence, flight range, biting activity and survival are still required to be investigated in Pakistan.

The interaction between *Aedes aegypti* and the dengue virus itself also needs to be investigated in more detail in Pakistan. Most critically the vectorial capacity of *Aedes aegypti* in different cities of Pakistan should be studied to determine if the mosquitoes in various cities are all acting equally as vectors or as is more likely are some mosquitoes more efficient then others (Huber et al. 1999). Laboratory based studies are also required to determine the rate of vertical transmission of dengue virus in Pakistani *Aedes aegypti*. This will help in predicting the vertical transmission efficiency of dengue virus in wild mosquitoes. There is a strong need to conduct a full scale epidemiological study of dengue in Pakistan to rule out the presence of any other haemorrhagic fever causing virus like chikungunya, encephalitis and West Nile virus etc. It has been observed that every time the dengue epidemics occurs it is not pandemic so a study should be designed to collect adults as well as larvae from the outbreak area for few consecutive years to determine the prevalence of virus in circulation in adults as well as larvae. In addition during the epidemic every case should be followed and collection should be carried out from the vicinity where dengue cases occur. These studies could help us to better understand the mechanism of dengue transmission and its persistence in Pakistan.

The findings of the current study could be used to formulate, test and apply various control strategies to reduce the vector population in Pakistan. Entomological surveys conducted here have shown that *Aedes aegypti* is breeding in almost all types of artificial container in Pakistan, indoor as well as outdoor. Indoor breeding *Aedes aegypti* are even using drinking water containers to lay their eggs. In such circumstances it becomes difficult to use larvicide or crude oil/petrol due to health hazards. Involving the community could help considerably in decreasing the population of this mosquito. Strategies like “4 o’clock Habit” or “Kill the Mosquito, Knock Out Dengue” adopted by Philippines to eradicate dengue vector in the area should be adopted in Pakistan also. In these strategies, during dengue epidemics every afternoon people were requested to go to their respective

areas at 4 o'clock to clean up and reduce potential mosquito breeding sites (Mahilum et al. 2005). Such strategies involving local people, especially during the dengue epidemic period could be helpful in controlling the disease. This could also help in reducing the use of indoor containers for laying eggs by *Aedes aegypti*. During the inter epidemic periods immature stages may become a reservoir of the virus such that it becomes very important to control the larval population of the vector mosquito also (Lee and Rohani 2005). Large outdoor containers and especially tyres could be prevented from acting as a breeding site by emptying them or using carosine oil. Implementing such strategies would also educate people and continuing them for a number of years will give enough knowledge to the local people that they are likely to start following these guidelines.

The findings of the population genetic analysis could also be used to investigate and implement various strategies to control *Aedes aegypti* in Pakistan. My study implies that there is movement of this mosquito in Pakistan over large spatial scales. This long-range dispersal could be advantageous for using genetically modified *Aedes aegypti* in Pakistan or for infecting the local mosquito population with endosymbiotic bacterial *Wolbachia.* Phuc et al. (2007) developed a transgenic line of *Aedes aegypti* with tetracycline-repressible dominant lethality in both males and females. This lethality could kill the insect at the larval-pupal boundary in the absence of tetracycline supplement. In the presence of tetracycline supplement (30µl/ml) their fitness is similar to wild type mosquitoes but in the absence of tetracycline only 3-4% of the individuals survive from first larval instar to adulthood (Phuc et al. 2007). Compared to sterile insect technology these transgenics have a late lethal phase, and therefore compete for resources as larvae and therefore reduce the survival of their con-specifics. Such transgenics could be used in Pakistan to eliminate the dengue vector in Pakistan but the disadvantage of this method is that it requires a continuous influx of released adults prepared in the lab for a number of consecutive years. They cannot proliferate in wild because of the death of their progeny before sexual maturity.

Another strategy which could be successful in Pakistan without completely eliminating *Aedes aegypti* or disturbing the ecosystem is the introduction of

maternally transmitted endosymbiotic bacterial *Wolbachia* (Hoffmann et al. 2011). The *w*Me1Pop-CLA *Wolbachia* strain not only reduces the life span of *Aedes aegypti* which in itself reduces the potential of dengue transmission but also blocks dengue transmission in *Ae. aegypti* (Walker et al. 2011). Maternal inheritance and cytoplasmic incompatibility provides a powerful mechanism to replace wild *Aedes aegypti* population with the *Wolbachia* infected mosquitoes. Cytoplasmic incompatibility leads to early embryonic death of progeny produced as a result of mating of uninfected females with infected males. On the other hand *Wolbachia*-infected females produce viable offsprings that either mate with infected or uninfected males. This mechanism not only prevents the proliferation of *Aedes aegypti* produced from uninfected females mosquitoes but also increases the number of *Wolbachia* infected mosquitoes (Walker et al. 2011).

Field studies conducted in Australia have shown the successful invasion of *Wolbachia* into two natural *Ae. aegypti* population, reaching near fixation in a few months following the release of *w*Me1 infected *Ae. aegypti* adults. Due to its ability to block dengue transmission the release of *Wolbachia* infected mosquitoes could immediately reduce the occurrence of dengue in Pakistan. However this requires an investigation of the potential of introducing these endosymbiotic bacteria into locally reared *Aedes aegypti* and the development of techniques for the production in large numbers for releasing into the wild. Investigating different aspects of the interaction including its compatability in *Aedes aegypti* from Pakistan and then the blockage of dengue virus could make this control strategy very successful for the control of dengue disease in Pakistan. Secondly, long range dispersal and human mediated movement of these mosquitoes will increase the spatial distribution of *Ae. aegypti* in Pakistan but they will not be able to transmit dengue virus and will therefore reduce the spread of the disease to non-dengue areas of Pakistan.

In summary, in this study I have not only reported the spatial distribution of *Aedes aegypti* in Pakistan but have also identified the types of containers used as breeding point by this mosquito in the study area. Although no dengue virus was detected in mosquitoes collected from Pakistan but by using microsatellite markers and various statistical techniques I had suggested that there is a single population of this mosquito in Pakistan with isolation by distance and that this mosquito had

started its colonization from south in Karachi and had moved towards the north facilitated by passive dispersal. In addition I showed that the cities connected by good road connections are less differentiated, which is also in agreement with the idea of longrange dispersal of this mosquito in Pakistan. The findings of this study could be useful in formulating a better control strategy to control dengue vector in Pakistan ultimately controlling spread of dengue in the coming years.

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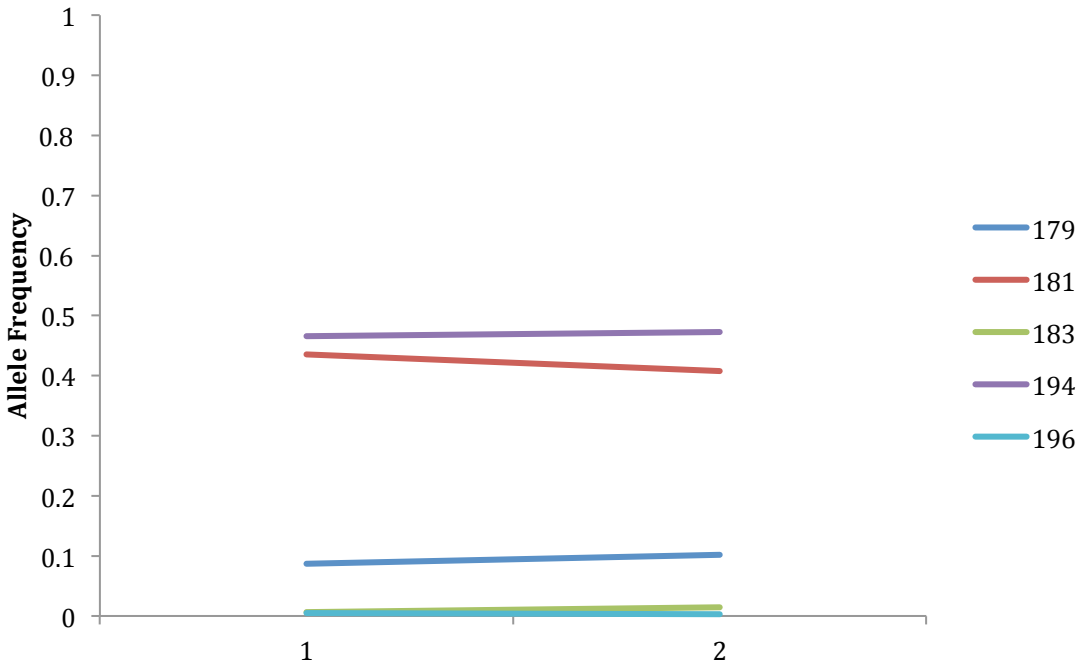
APPENDICES

Location	Sample no	No. of Larvae	Longitude/latitude	
Peshawar	1	6	N34°00.264	E071°34.051
Peshawar	2	5	N34°00.266	E071°34.057
Peshawar	3	9	N34°00.263	E071°34.054
Peshawar	4	4	N34°00.261	E071°34.058
Rawalpindi	5	7	N33°36.744	E073°03.119
Rawalpindi	6	5	N33°36.744	E073°03.113
Rawalpindi	7	9	N33°36.748	E073°03.748
Rawalpindi	8	4	N33°37.595	E073°02.889
Rawalpindi	9	10	N33°37.593	E073°02.888
Rawalpindi	10	6	N33°37.592	E073°02.886
Rawalpindi	11	20	N33°43.866	E072°48.351
Rawalpindi	12	6	N33°43.865	E072°48.353
Rawalpindi	R	1	N33°37.540	E073°01.758
Lahore	13	8	N31°34.507	E074°20.233
Lahore	14	8	N31°34.504	E074°20.234
Lahore	14A	12	N31°34.506	E074°20.235
Lahore	15	10	N31°34.567	E074°20.200
Lahore	16	13	N31°34.569	E074°20.201
Lahore	17	11	N31°31.038	E074°18.102
Lahore	18	8	N31°34.523	E074°18.287
Lahore	19	2	N31°34.501	E074°18.322
Lahore	20	1	N31°33.627	E074°18.521
Sheikhupura	21	9	N31°43.062	E074°00.015
Sheikhupura	22	11	N31°43.063	E074°00.016
Gujranwala	23	26	N32°11.903	E074°10.471
Gujranwala	24	17	N32°11.913	E074°10.510
Faisalabad	25	8	N31°25.909	E073°05.699
Faisalabad	26	17	N31°25.908	E073°05.698
Faisalabad	27	26	N31°25.926	E073°05.688
Faisalabad	28	20	N31°25.926	E073°05.688
Multan	29	25	N31°10.797	E071°29.769
Peshawar	30	24	N34°00.256	E071°34.059
Peshawar	31	29	N34°00.263	E071°34.058
Peshawar	32	16	N34°00.257	E071°34.034
Peshawar	33	13	N34°00.258	E071°34.038
Attock	34	32	N33°34.574	E072°13.704
Attock	35	37	N33°34.621	E072°13.748
Attock	36	1	N33°34.608	E072°13.692
Attock	37	11	N33°33.029	E072°15.658
Attock	38	19	N33°33.033	E072°15.602
Attock	39	11	N33°33.094	E072°15.724

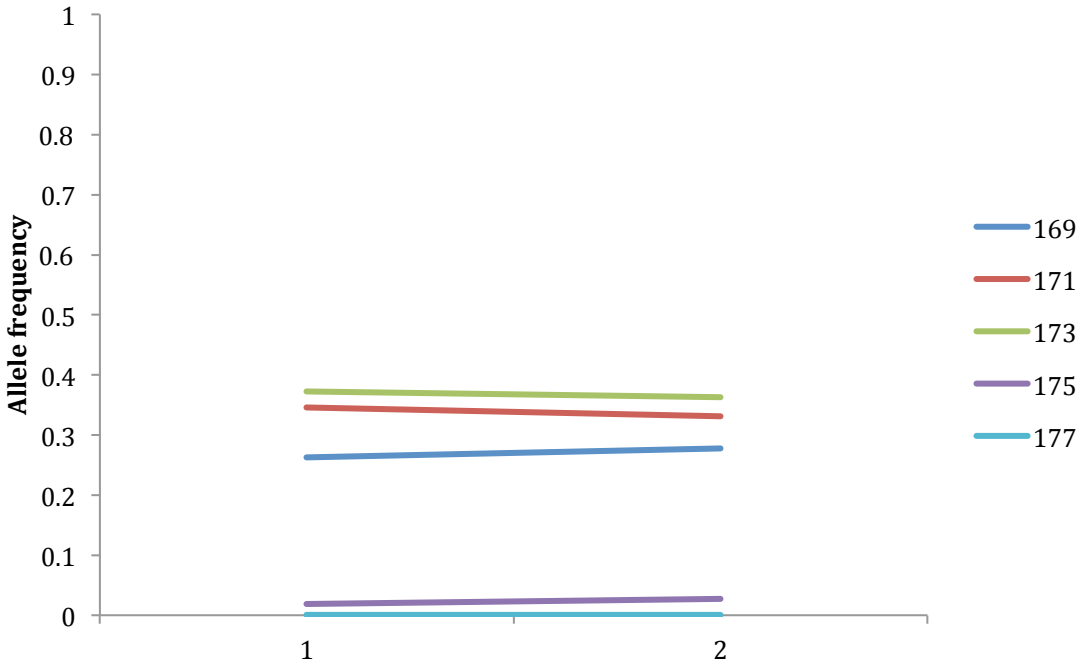
Attock	40	26	N33°33.079	E072°15.647
Karachi	41	36	N24°52.770	E066°59.816
Karachi	42	47	N24°52.780	E066°59.853
Karachi	43	31	N24°52.108	E066°59.522
Karachi	44	18	N24°52.076	E066°58.640
Karachi	45	27	N24°52.876	E067°03.189
Karachi	46	17	N24°52.853	E067°03.182
Karachi	47	6	N24°52.854	E067°03.145
Haripur	48	7	N33°59.066	E072°54.807
Haripur	49	19	N33°59.087	E072°54.805
Haripur	50	37	N33°59.087	E072°54.807
Haripur	51	12	N33°59.084	E072°54.806
Haripur	52	8	N34°00.345	E072°56.300
Haripur	53	1	N34°00.345	E072°56.301
Haripur	54	23	N34°00.339	E072°56.298
Haripur	55	33	N34°00.339	E072°56.298
Haripur	56	6	N34°00.338	E072°56.297
Peshawar	57	27	N33°59.683	E071°32.836
Karachi	83	2	N24°53.630	E067°01.681
Hasanabdal	58	8	N33°48.705	E072°41.511
Hasanabdal	59	8	N33°48.710	E072°41.520
Attock	60	8	N33°34.745	E072°13.353
Attock	61	8	N33°34.745	E072°13.355
Attock	62	8	N33°34.755	E072°13.369
Taxilla	63	8	N33°42.904	E072°48.720
Taxilla	64	8	N33°42.875	E072°48.667
Taxilla	65	8	N33°43.866	E072°48.351
Rawalpindi	66	8	N33°37.842	E073°02.554
Rawalpindi	67	8	N33°37.892	E073°02.594
Rawalpindi	68	8	N33°37.550	E073°01.798
Rawalpindi	69	8	N33°37.540	E073°01.758
Rawalpindi	70	8	N33°37.560	E073°01.728
Rawalpindi	71	8	N33°37.530	E073°01.738
Sheikhupura	72	8	N31°43.093	E073°58.846
Sheikhupura	73	7	N31°43.109	E073°58.894
Lahore	74	8	N31°33.863	E074°18.279
Lahore	75	8	N31°33.071	E074°18.009
Lahore	76	8	N31°33.560	E074°18.296
Lahore	77	8	N31°34.735	E074°18.059
Lahore	78	8	N31°34.745	E074°18.049
Multan	79	8	N30°10.857	E071°29.869
Multan	80	8	N30°10.697	E071°29.704
Hyderabad	81	8	N25°23.070	E068°22.174
Hyderabad	82	8	N25°23.141	E068°22.165
Karachi	84	5	N24°53.440	E067°01.583

Karachi	85	8	N24°54.222	E067°01.026
Karachi	86	8	N24°53.634	E067°01.217
Karachi	87	8	N24°53.446	E067°01.204
Karachi	88	4	N24°56.850	E067°01.359
Karachi	89	5	N24°56.581	E066°59.798
Karachi	90	8	N24°56.854	E067°01.356
Karachi	91	8	N24°56.533	E067°01.217
Karachi	92	8	N24°56.802	E066°59.686
Karachi	93	8	N24°56.437	E066°59.564
Karachi	94	3	N24°49.716	E067°07.774
Karachi	95	2	N24°49.498	E067°09.410
Karachi	96	8	N24°49.314	E067°10.405
Karachi	97	8	N24°55.596	E067°00.063
Karachi	98	8	N24°56.159	E067°00.078
Karachi	99	8	N24°54.846	E067°00.256
Karachi	100	8	N24°53.445	E067°01.538
Karachi	101	8	N24°51.041	E067°12.468
Karachi	102	8	N24°50.970	E067°10.890
Karachi	103	8	N24°52.943	E067°10.854
Karachi	104	8	N24°50.754	E067°09.643
Karachi	105	8	N24°55.556	E067°00.066
Karachi	106	5	N24°51.983	E067°21.256
Karachi	107	8	N24°52.187	E067°12.021
Karachi	108	8	N24°51.665	E067°21.033
Karachi	109	8	N24°53.008	E067°10.689

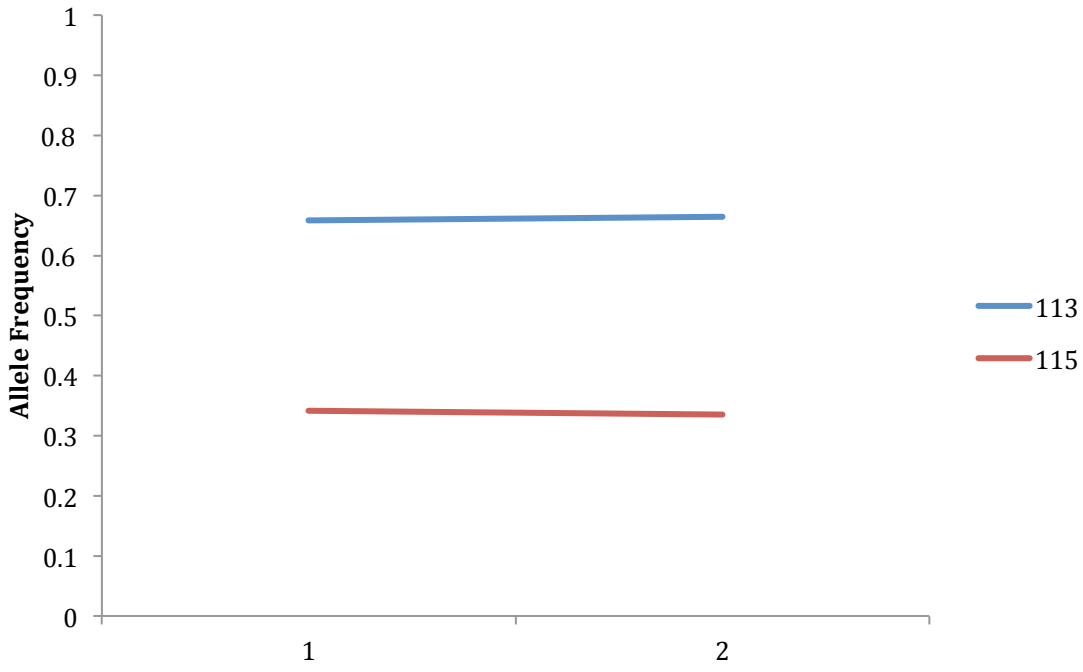
Appendix 1. Geographical coordinates and number of larvae collected from each location.



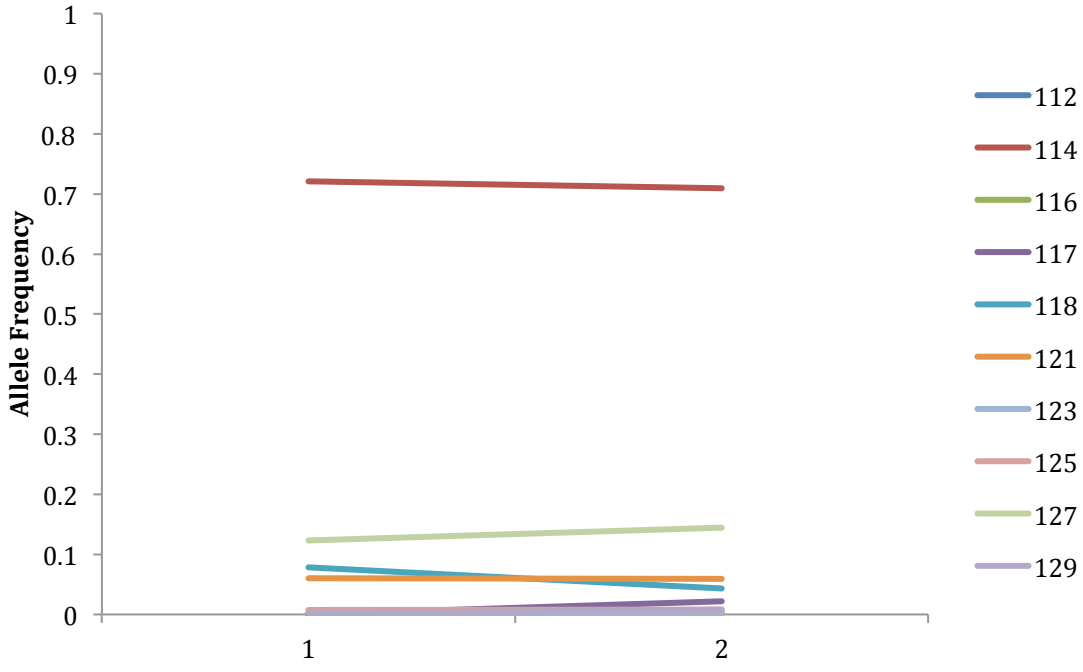
Appendix 2: Change in allele frequency of AC1 in genotypic data from 2009 and 2010.



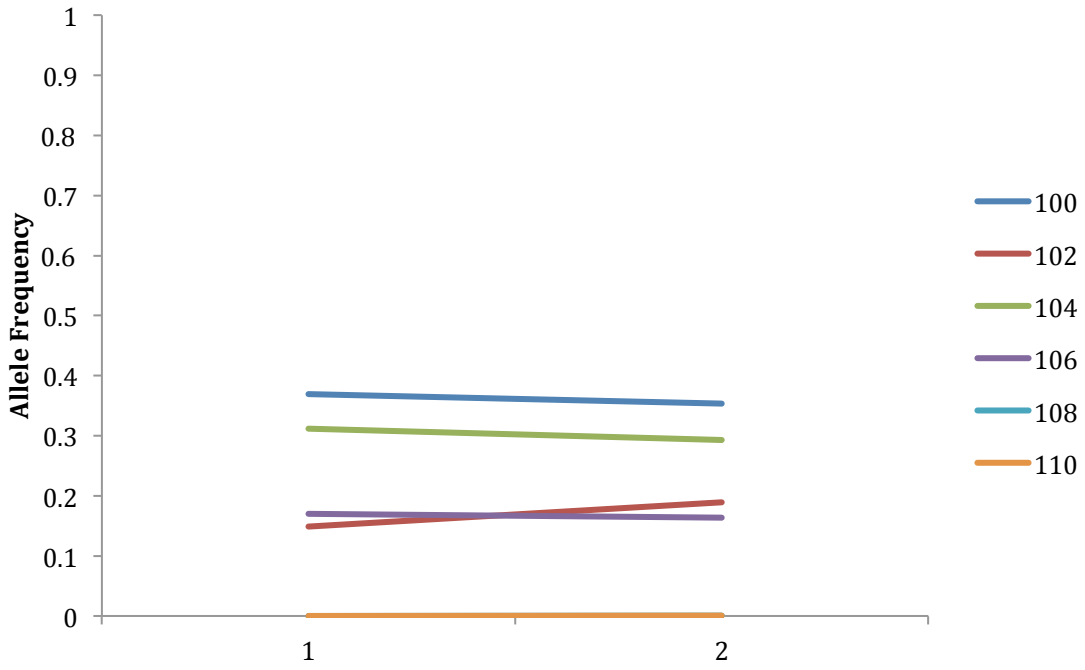
Appendix 3: Change in allele frequency of AC2 in genotypic data from 2009 and 2010.



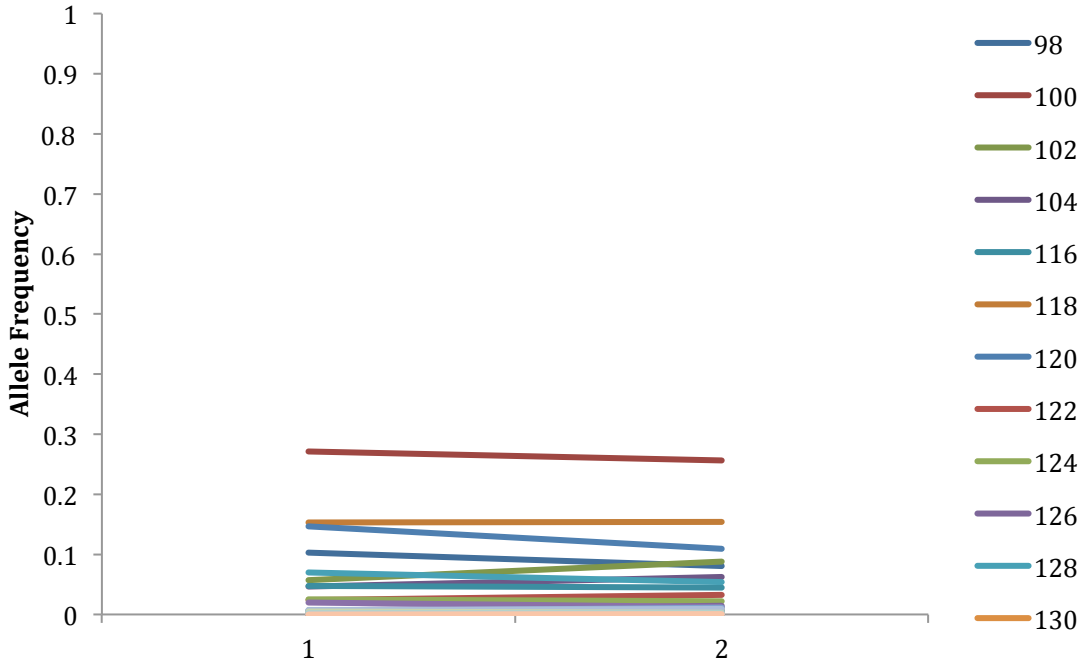
Appendix 4: Change in allele frequency of AC4 in genotypic data from 2009 and 2010.



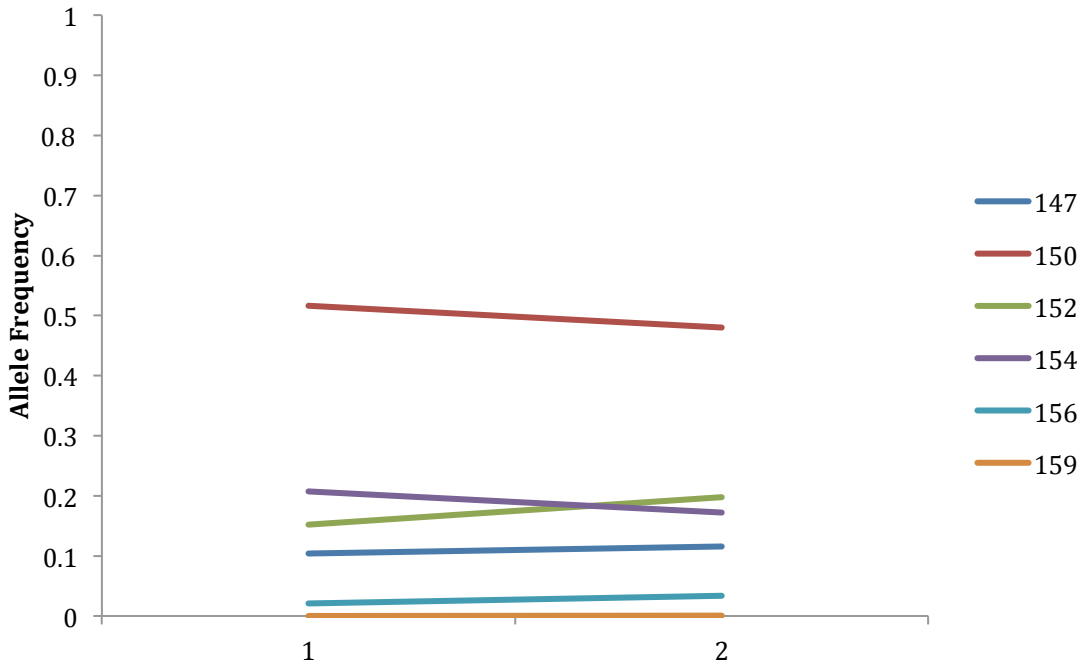
Appendix 5: Change in allele frequency of AC7 in genotypic data from 2009 and 2010.



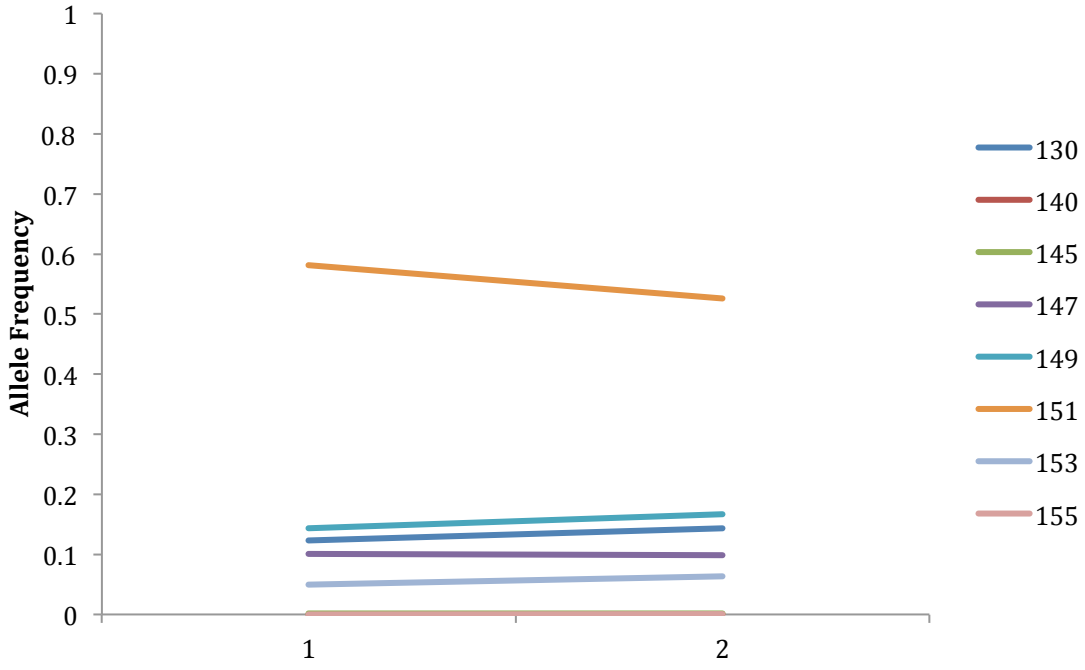
Appendix 6: Change in allele frequency of AG1 in genotypic data from 2009 and 2010.



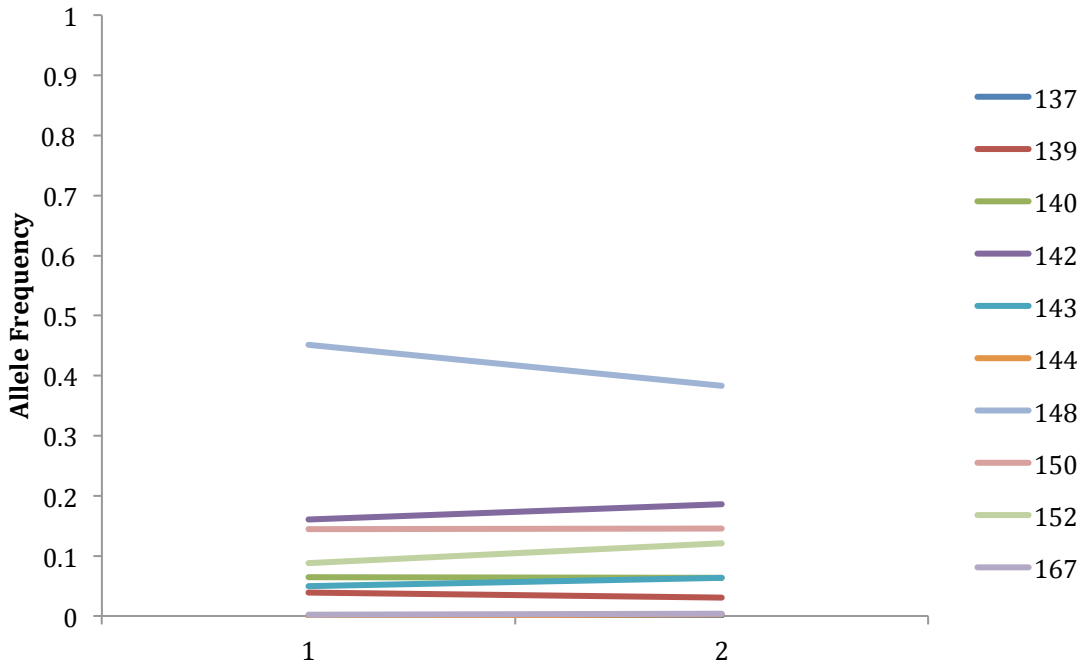
Appendix 7: Change in allele frequency of AG2 in genotypic data from 2009 and 2010.



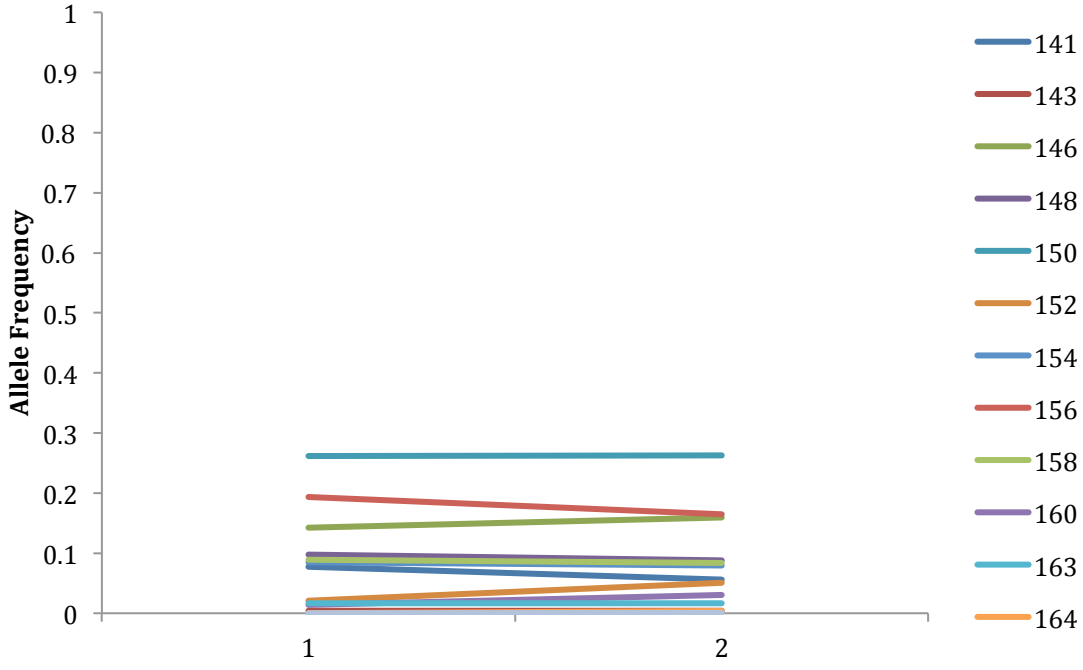
Appendix 8: Change in allele frequency of AG3 in genotypic data from 2009 and 2010.



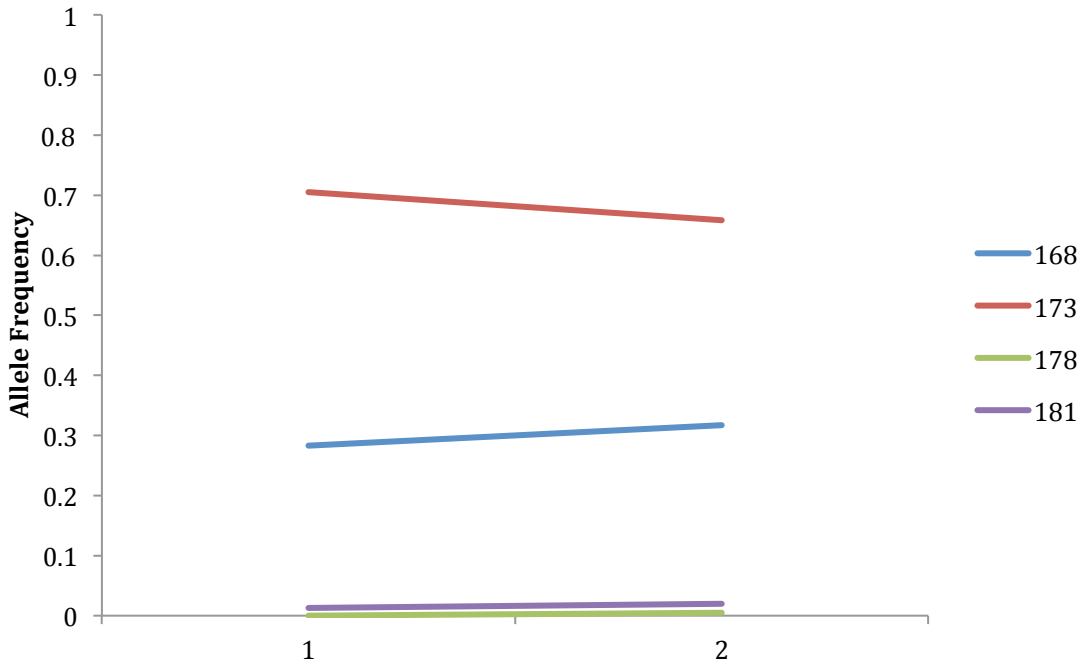
Appendix 9: Change in allele frequency of AG4 in genotypic data from 2009 and 2010.



Appendix 10: Change in allele frequency of AG7 in genotypic data from 2009 and 2010.



Appendix 11: Change in allele frequency of AT1 in genotypic data from 2009 and 2010.



Appendix 12: Change in allele frequency of CT2 in genotypic data from 2009 and 2010.

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CLUSTAL X (1.81) multiple sequence alignment

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839     GAGAAGCAACCGACCATAGCTGGGGAGCCGAAAGGATCCCGGACCATTTGGTATATGTGG
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        ****** *** ** * * ***** * * * * ** ***** ** ***** ***** ******

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245    GCCCGTGAAAATTTCCCGGGTGGCGTGGGAGGTCTCGGCGTAAA
219    GCCCGTGAAAATTTCCCGGGTGGCGTGGGAGGTCTCGGCGTAAA
215    GCCCGTGAAAATTTCCCGGGTGGCGTGGGAGGTCTCGGCGTAAA
179    GCCCGTGAAAATTTCCCGGGTGGCGTGGGAGGTCTCGGCGTAAA
139    GCCCGTGAAAATTTCCCGGGTGGCGTGGGAGGTCTCGGCGTAAA
129    GCCCGTGAAAATTTCCCGGGTGGCGTGGGAGGTCTCGGCGTAAA
125    GCCCGTGAAAATTTCCCGGGTGGCGTGGGAGGTCTCGGCGTAAA
A20    GCCCGTGAAAATCTCCCGGGTGGCGTGGGAGGTCTCGGCGTAAA
KRV    AGCCGGGAAAACTTCCCCGGTGGTGTTGGAGGTCTAGGAGTGAA
         *** *****  **** *****
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Appendix 13: Flavivirus like sequeces detected in mosquito genome.

City	Patch	Sample	AC1		AC2		AC4		AC7		AG1		AG2		AG3		AG4		AG5		AG7		AT1		CT2	
Attock	17	34	194	194	169	171	113	115	114	121	100	100	120	124	150	150	151	151	158	162	148	152	150	156	173	173
Attock	17	34	181	181	169	169	113	115	114	114	100	100	98	120	150	150	149	153	158	158	148	148	150	150	173	173
Attock	17	34	179	194	169	171	113	113	114	114	100	100	120	128	150	150	151	151	158	158	142	152	150	156	173	173
Attock	17	34	179	181	169	169	113	115	114	114	100	100	120	120	150	150	151	153	154	158	148	148	148	150	173	173
Attock	17	34	194	194	169	169	113	115	114	114	100	106	100	120	150	150	151	151	158	162	142	152	150	154	168	173
Attock	17	34	181	181	171	171	113	113	114	114	106	106	100	116	150	150	147	151	162	162	152	152	150	150	173	173
Attock	17	34	181	194	169	173	115	115	114	114	100	100	120	120	150	150	151	151	158	166	148	152	150	156	168	173
Attock	17	34	181	181	169	169	113	115	114	114	100	100	100	100	150	150	147	151	156	156	148	148	150	156	168	173
Attock	17	35	181	181	169	169	113	113	114	114	100	100	100	100	150	150	147	149	162	162	148	148	150	156	173	173
Attock	17	35	181	194	169	169	113	115	114	114	100	100	120	138	150	150	147	151	156	162	148	148	150	156	168	173
Attock	17	35	194	194	169	169	113	115	114	114	100	106	120	164	150	150	130	151	162	162	148	152	150	150	168	173
Attock	17	35	181	181	169	173	113	115	114	114	100	100	100	156	150	150	147	151	158	158	148	148	141	156	173	173
Attock	17	35	194	194	169	171	115	115	114	114	100	106	98	120	150	150	151	151	158	162	142	142	154	154	168	168
Attock	17	35	181	194	171	173	113	113	114	114	100	100	100	100	150	150	151	151	162	162	148	148	148	150	168	173
Attock	17	35	194	194	169	169	113	113	114	114	100	100	100	100	150	150	147	149	162	162	148	148	148	150	173	173
Attock	17	35	181	194	169	169	113	113	114	114	100	100	100	100	150	150	147	151	162	162	148	148	156	156	173	173
Attock	17	36	179	194	169	171	113	113	114	114	100	100	98	120	150	150	147	151	162	162	148	152	150	156	173	173
Attock	17	37	181	194	169	173	115	115	114	127	100	100	116	116	150	150	149	151	162	164	148	148	150	156	173	173
Attock	17	37	181	194	169	173	113	115	114	127	100	100	116	138	150	150	149	151	162	162	142	148	150	156	173	173
Attock	17	37	181	194	169	169	113	113	114	114	100	100	116	138	150	150	147	149	162	162	142	142	150	156	173	173
Attock	17	37	181	194	169	173	115	115	127	127	100	100	118	138	150	150	147	149	162	164	142	148	150	156	173	173
Attock	17	37	181	194	169	169	113	113	114	127	100	100	116	138	150	150	149	151	162	164	148	148	150	156	173	173
Attock	17	37	179	194	173	173	115	115	114	127	100	106	98	98	150	150	147	151	162	162	148	152	148	156	168	173
Attock	17	37	181	194	169	173	113	115	127	127	100	100	116	116	150	150	149	151	162	162	142	148	150	156	173	173
Attock	17	37	181	194	169	169	115	115	114	114	100	100	116	118	150	150	147	149	162	162	142	148	150	156	173	173
Attock	18	38	181	194	169	173	113	113	114	114	100	100	98	98	150	150	147	151	164	164	148	148	141	150	168	173
Attock	18	38	181	194	169	173	113	115	114	127	100	100	98	98	150	150	151	151	158	162	142	148	148	150	168	173
Attock	18	38	181	194	171	173	113	115	114	127	100	100	98	100	150	150	151	151	156	162	142	152	150	150	168	173
Attock	18	38	181	194	171	173	115	115	114	127	100	100	98	98	150	150	151	151	158	158	148	148	148	148	168	173
Attock	18	38	181	194	173	173	113	115	114	127	100	100	98	100	150	150	151	151	158	162	148	148	148	148	173	173
Attock	18	38	181	181	171	173	115	115	114	127	100	100	98	98	150	150	151	151	158	162	142	148	148	150	168	173
Attock	18	38	194	194	171	173	113	115	114	127	100	100	98	100	150	150	151	151	156	158	148	152	148	150	173	173
Attock	18	38	194	194	171	173	113	115	114	127	100	100	98	100	150	150	151	151	156	158	148	152	148	150	168	173
Attock	18	39	179	194	169	171	113	115	114	114	100	100	98	116	150	150	151	151	158	162	148	152	141	148	173	173
Attock	18	39	179	181	173	173	113	115	114	114	100	106	118	120	150	150	149	151	162	162	148	152	141	156	173	173
Attock	18	39	194	194	171	171	113	113	114	127	100	104	98	116	147	150	151	151	156	162	148	152	150	150	173	173
Attock	18	39	181	181	169	173	113	115	114	127	100	106	118	120	150	150	149	149	162	164	139	148	156	158	173	173

Attock	18	39	194	194	171	173	113	115	114	114	100	106	98	98	150	150	147	147	158	162	142	148	148	158	173	173
Attock	18	39	179	194	171	171	113	115	114	127	100	104	98	118	147	150	151	151	158	162	148	152	148	150	168	173
Attock	18	39	179	194	169	171	113	113	114	127	100	104	98	98	147	150	149	151	162	162	148	152	150	150	168	173
Attock	18	39	179	194	171	171	113	113	114	127	100	104	100	118	147	150	151	151	162	162	148	152	150	156	168	173
Attock	18	40	179	181	173	173	115	115	114	114	106	106	116	118	150	150	147	151	162	162	148	152	141	156	168	173
Attock	18	40	181	194	173	173	113	115	114	114	104	106	98	116	147	150	151	151	162	162	148	152	156	156	168	173
Attock	18	40	179	181	169	173	113	115	114	114	100	100	98	98	150	150	130	151	164	166	142	148	150	156	168	173
Attock	18	40	194	194	173	173	113	113	114	127	106	106	98	116	150	150	149	151	162	164	139	148	150	158	168	173
Attock	18	40	181	194	169	169	113	113	114	127	100	106	118	118	150	150	151	151	158	162	148	148	148	150	168	173
Attock	18	40	194	194	173	173	113	115	114	114	100	104	98	116	147	150	151	151	162	162	148	152	156	156	168	173
Attock	18	40	179	194	169	173	113	115	114	114	100	100	100	118	150	150	149	151	164	164	139	139	156	158	168	173
Attock	18	40	194	194	173	173	113	113	114	114	100	106	98	100	150	150	149	151	158	158	148	152	148	156	173	173
Attock	18	40	181	194	171	173	113	115	114	127	104	106	98	100	147	150	149	151	158	166	148	152	150	150	173	173
Attock	18	40	179	194	169	171	113	113	114	114	106	106	98	118	150	150	147	151	162	162	148	152	156	156	168	168
Attock	17	38	181	194	169	173	115	115	112	114	100	100	98	98	150	150	151	151	158	158	139	148	148	150	173	173
Attock	17	35	194	194	171	171	113	115	114	114	100	100	98	120	150	150	151	151	162	162	142	142	154	154	173	173
Attock	17	35	194	194	169	173	113	115	114	114	100	100	98	124	150	150	151	151	162	162	148	148	150	156	173	173
Attock	17	34	194	194	171	173	113	115	114	114	100	100	120	120	150	150	147	151	158	162	148	152	150	150	173	173
Attock	17	34	181	181	169	169	113	115	114	114	100	100	120	120	150	150	149	153	162	162	148	148	150	150	173	173
Attock	25	60	181	194	169	171	113	113	114	114	100	100	98	100	150	150	147	147	162	162	148	152	148	156	173	173
Attock	25	60	181	194	169	169	113	113	114	114	100	100	100	128	150	150	147	147	162	162	142	152	148	156	173	173
Attock	25	60	181	194	169	173	113	113	114	114	100	106	100	120	150	150	151	151	156	162	142	148	146	156	173	173
Attock	25	60	194	194	169	173	113	113	114	114	100	106	100	120	150	150	151	151	156	162	142	148	146	156	173	173
Attock	25	60	181	194	169	171	113	113	114	114	100	100	98	100	150	150	147	147	158	162	148	152	148	156	173	173
Attock	25	60	194	194	169	173	113	113	114	114	100	106	100	120	150	150	151	151	156	162	148	148	156	156	173	173
Attock	25	60	181	194	169	171	113	113	114	114	100	100	98	100	150	150	147	147	158	162	152	152	150	156	173	173
Attock	25	60	181	194	169	169	113	113	114	114	100	100	98	100	150	150	147	147	162	162	152	152	156	156	173	173
Attock	25	61	181	194	169	173	113	115	114	114	100	106	100	116	150	150	151	151	156	162	148	148	156	156	173	173
Attock	25	61	194	194	169	171	113	115	114	114	100	100	98	116	150	150	151	153	162	162	148	148	148	156	168	173
Attock	25	61	194	194	169	171	113	115	114	114	100	100	116	120	150	150	147	151	162	166	142	148	148	150	168	173
Attock	25	61	194	194	169	169	115	115	114	114	100	100	100	120	150	150	149	151	158	162	148	152	148	150	173	173
Attock	25	61	194	194	169	169	113	113	114	114	100	100	98	116	150	150	147	151	162	162	152	152	148	148	168	173
Attock	25	61	181	194	169	171	113	113	114	114	100	100	100	100	150	150	147	147	156	162	148	152	148	156	173	173
Attock	25	61	181	194	171	171	113	115	114	114	100	100	116	156	150	150	147	151	162	162	142	148	148	148	173	173
Attock	25	61	181	194	169	171	113	115	114	114	100	100	98	116	150	150	147	151	162	162	152	152	148	148	173	173
Attock	25	62	194	194	169	169	115	115	114	114	104	104	100	120	147	150	149	151	158	162	148	152	148	150	173	173
Attock	25	62	194	194	169	171	115	115	114	114	104	106	100	100	147	150	149	151	158	162	148	152	148	150	173	173
Attock	25	62	194	194	169	171	115	115	114	114	104	106	100	120	147	150	151	151	156	162	148	148	154	156	173	173
Attock	25	62	194	194	169	171	115	115	114	114	100	106	98	100	150	150	149	151	158	162	148	152	148	150	173	173
Attock	25	62	194	194	169	171	115	115	114	114	100	106	100	100	150	150	149	151	158	162	148	152	150	154	173	173
Attock	25	62	194	194	169	169	115	115	114	114	100	100	98	120	150	150	151	151	156	162	148	148	154	156	173	173
Attock	25	62	194	194	169	169	115	115	114	114	100	104	100	100	147	150	149	151	158	162	148	152	150	154	173	173

Attock	25	62	194	194	169	171	115	115	114	114	100	106	100	100	150	150	149	151	158	162	148	152	148	150	173	173
Faisalabad	13	25	179	181	173	173	113	115	114	118	104	106	98	100	150	154	151	151	154	162	148	152	148	156	168	168
Faisalabad	13	25	179	181	171	173	113	115	114	114	104	104	118	128	154	154	130	130	166	166	140	148	146	150	168	173
Faisalabad	13	25	194	194	171	171	113	115	114	121	100	106	100	104	150	150	151	151	156	162	148	148	150	154	168	173
Faisalabad	13	25	181	194	173	173	113	115	114	114	102	104	102	104	152	154	130	151	162	162	148	148	146	150	168	168
Faisalabad	13	25	179	194	169	171	113	113	114	114	100	100	98	104	150	150	130	151	162	162	139	142	150	152	168	173
Faisalabad	13	25	181	181	169	169	115	115	114	118	102	104	104	118	152	154	151	153	158	162	139	148	146	150	168	168
Faisalabad	13	25	181	194	173	173	113	113	114	118	104	106	118	128	150	154	151	151	154	162	148	152	156	156	168	173
Faisalabad	13	25	179	194	169	173	113	115	114	114	100	102	98	116	150	152	151	151	162	162	142	148	141	156	168	173
Faisalabad	13	26	181	194	169	173	113	115	118	121	104	106	100	104	150	154	130	151	162	162	139	148	148	158	173	173
Faisalabad	13	26	181	181	169	173	115	115	118	121	104	106	100	102	150	154	130	130	162	162	139	142	146	148	168	173
Faisalabad	13	26	181	194	171	173	113	113	114	114	106	106	120	120	150	156	151	151	154	162	148	148	146	158	173	173
Faisalabad	13	26	179	179	173	173	113	113	114	114	102	104	98	102	152	154	149	151	156	156	142	148	150	156	173	173
Faisalabad	13	26	181	181	171	173	113	115	121	127	100	106	104	104	150	156	149	151	158	162	139	142	150	158	173	173
Faisalabad	13	26	181	181	169	171	115	115	114	118	100	104	100	104	150	154	130	151	162	162	148	148	154	158	173	173
Faisalabad	13	26	181	181	173	175	113	113	114	114	100	100	100	104	150	150	151	153	162	162	142	148	146	154	173	173
Faisalabad	13	26	181	194	171	171	113	115	114	118	100	104	100	104	147	150	130	149	158	162	148	148	148	158	173	173
Faisalabad	14	27	181	181	169	171	113	113	114	121	106	106	118	120	150	150	130	151	156	162	142	148	152	154	173	173
Faisalabad	14	27	181	181	169	171	113	113	114	114	104	106	104	120	150	154	153	153	158	162	142	148	146	150	168	173
Faisalabad	14	27	181	194	171	173	113	115	118	127	102	104	104	118	152	154	130	147	162	162	148	152	146	146	173	173
Faisalabad	14	27	181	181	173	173	113	115	114	118	100	106	104	128	150	150	130	130	162	162	148	152	150	158	173	173
Faisalabad	14	27	181	194	171	173	113	113	114	114	104	104	100	118	154	154	149	151	154	154	148	148	148	150	173	173
Faisalabad	14	27	194	194	169	173	113	115	114	127	104	104	98	120	154	154	130	147	156	158	139	148	146	146	168	173
Faisalabad	14	27	181	181	171	171	113	113	114	127	102	104	118	120	152	154	151	151	162	166	142	142	152	156	168	173
Faisalabad	14	27	181	181	171	173	113	113	114	127	100	106	116	128	150	150	130	130	156	162	139	150	154	156	173	173
Faisalabad	15	28	181	181	171	173	113	115	114	127	100	106	104	128	150	150	130	130	162	162	148	152	150	158	168	168
Faisalabad	15	28	181	181	171	171	113	113	114	114	102	104	98	122	152	154	130	151	156	156	148	148	146	163	173	173
Faisalabad	15	28	179	194	169	171	113	115	114	127	100	104	98	104	150	154	130	151	162	162	148	152	148	150	168	173
Faisalabad	15	28	179	181	173	173	113	113	114	118	100	104	118	118	150	154	151	151	154	162	142	142	150	152	168	173
Faisalabad	15	28	179	194	169	171	113	115	114	127	100	104	118	126	150	154	130	130	162	162	139	148	148	150	173	173
Faisalabad	15	28	181	181	169	173	113	115	114	121	104	106	104	118	150	154	130	147	158	158	148	148	141	146	168	173
Faisalabad	15	28	181	194	169	173	113	113	114	123	104	106	118	128	150	154	149	153	158	162	148	150	148	150	168	173
Faisalabad	15	28	194	194	173	173	113	115	114	114	104	104	100	118	147	154	151	153	162	162	139	148	146	154	168	168
Faisalabad	15	28	194	194	173	173	115	115	114	114	100	104	100	128	150	154	151	153	162	162	139	148	146	154	168	168
Faisalabad	15	28	179	181	171	173	113	113	114	127	100	106	104	128	150	150	130	130	162	162	148	152	150	158	173	173
Faisalabad	14	27	181	181	171	173	113	113	114	127	100	106	104	118	150	150	130	130	162	162	139	148	150	158	168	173
Faisalabad	14	27	179	194	169	173	113	113	114	114	100	102	122	124	150	152	149	149	162	162	142	148	143	160	168	168
Faisalabad	13	26	181	194	169	171	113	115	114	114	100	104	100	102	147	150	130	130	158	162	139	142	146	148	168	173
Gujranwala	12	23	181	181	169	173	113	115	114	114	102	106	118	120	152	152	151	151	162	162	142	148	150	158	173	173
Gujranwala	12	23	181	181	173	175	113	115	114	114	100	104	120	120	150	154	149	153	158	158	140	148	152	163	168	173
Gujranwala	12	23	179	183	171	171	113	113	114	114	106	106	100	160	150	150	151	151	158	162	148	150	150	160	173	173
Gujranwala	12	23	179	181	171	171	113	113	114	114	106	106	118	118	150	150	151	151	162	162	148	150	150	150	173	173

Gujranwala	12	23	181	194	169	169	113	113	114	114	104	104	100	160	147	147	151	151	162	162	150	152	150	160	168	173
Gujranwala	12	23	181	181	169	173	113	115	114	114	102	106	100	118	150	152	151	151	162	162	148	150	150	158	173	173
Gujranwala	12	23	181	194	169	169	113	113	114	127	104	106	120	124	150	154	149	151	158	158	142	152	150	154	168	173
Gujranwala	12	23	181	194	169	171	113	115	114	114	106	106	100	118	150	150	149	151	162	162	148	150	148	150	173	173
Gujranwala	12	24	181	181	169	173	113	113	114	114	102	104	100	118	152	154	151	151	158	162	148	150	150	154	173	173
Gujranwala	12	24	179	194	169	171	113	115	114	114	104	106	100	124	150	154	149	149	158	162	148	152	154	154	173	173
Gujranwala	12	24	181	181	169	175	113	113	118	127	106	106	118	120	150	156	130	147	162	162	148	148	148	154	173	173
Gujranwala	12	24	179	181	169	171	113	115	114	114	100	106	120	120	150	150	151	151	162	162	148	152	148	150	173	173
Gujranwala	12	24	181	181	169	169	113	115	114	114	104	106	120	120	147	150	151	151	156	162	140	152	152	156	173	173
Gujranwala	12	24	179	194	169	169	113	115	114	114	106	106	100	118	150	150	151	151	158	158	148	148	158	158	173	173
Gujranwala	12	24	179	181	171	173	113	115	114	114	104	106	98	128	147	150	151	151	158	162	142	150	150	156	173	173
Gujranwala	12	24	181	194	171	173	113	113	114	129	104	106	120	120	147	150	130	149	162	162	148	150	141	150	168	173
Gujranwala	12	24	179	179	169	169	113	113	114	114	102	106	160	160	150	152	149	151	162	162	140	148	150	158	173	173
Gujranwala	12	23	179	181	173	173	113	113	114	114	104	104	98	120	147	154	151	151	158	162	142	148	141	156	173	173
Gujranwala	12	23	181	194	169	169	113	113	114	127	104	106	100	120	150	154	151	151	158	162	148	150	141	150	168	173
Gujranwala	12	22	181	181	169	169	113	115	114	125	100	100	118	118	150	150	151	151	156	156	148	150	150	156	173	173
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Rawalpindi	4	7	181	181	171	173	113	113	114	127	100	106	98	98	150	150	151	151	156	156	142	148	150	156	168	173
Rawalpindi	4	7	181	181	171	173	113	113	114	114	100	104	98	118	147	150	151	151	156	156	142	148	150	156	168	173
Rawalpindi	4	7	181	194	171	173	113	115	114	114	100	104	98	118	147	150	151	151	156	156	142	148	150	156	168	173
Rawalpindi	4	7	181	181	169	173	113	113	114	121	100	106	98	116	150	150	147	151	162	162	148	148	148	156	173	173

Rawalpindi	4	7	181	181	171	173	113	113	114	114	100	104	118	118	147	150	151	151	156	156	142	140	150	156	168	173
Rawalpindi	4	7	181	181	171	173	113	113	114	114	100	104	98	98	147	150	151	151	156	162	142	150	146	150	168	173
Rawalpindi	4	7	181	181	169	169	113	113	114	114	104	104	100	116	147	154	147	149	156	158	148	148	150	158	173	173
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Taxilla	26	65	179	181	171	173	113	115	114	121	100	100	100	118	150	150	151	151	158	162	148	150	156	158	173	173
Taxilla	26	65	181	194	169	171	113	115	114	114	104	104	100	118	147	154	147	151	156	156	148	152	148	158	173	173
Taxilla	26	65	181	181	169	171	115	115	114	114	106	106	100	120	150	150	149	151	162	162	142	142	148	150	173	173
Taxilla	26	65	179	194	173	173	113	113	114	114	104	106	126	148	147	150	149	151	158	162	148	152	146	152	173	173
Taxilla	26	65	179	194	171	173	113	113	114	114	100	104	100	122	147	150	149	151	156	162	148	148	146	148	173	173
Taxilla	26	65	181	181	169	171	113	113	114	114	106	106	100	120	150	150	151	151	162	162	142	148	141	148	173	173
Taxilla	26	65	179	194	171	171	113	113	114	114	104	106	98	100	147	150	151	151	158	162	148	150	156	158	173	173
Taxilla	26	65	181	194	169	171	113	113	114	121	104	106	100	100	147	150	147	151	158	158	148	150	154	154	173	173

Appendix 13: Genotypic data of 850 mosquitoes collected from Pakistan.