

# **Chikungunya virus outbreak in Dhaka, Bangladesh in 2017 - A seroprevalence study**



Inspiring Excellence

**A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL  
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF  
SCIENCE IN MICROBIOLOGY**

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# Declaration

I, hereby certify that the thesis work entitled “**Chikungunya virus outbreak in Dhaka, Bangladesh in 2017 - a seroprevalence study**” submitted to the Department of Mathematics and Natural Science, BRAC University in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology is a record of work carried out by me under joint supervision and able guidance of my supervisors Mahbubul Hasan Siddiquee, Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University and Dr. Fahmida Khanam, Assistant Professor of Virology, Department of Parasitology, NIPSOM. It is further declared that the research work presented here is original and the contents of this report in full or parts have not been submitted to any other university and institution for any degree or diploma. Any references to work done by any other person or institution or any material obtained from other sources have been duly cited and referenced.

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**Dedicated To**  
**My Greatest Strength**

**‘My Beloved Family’**

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# LIST OF ABBREVIATIONS

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|          |  |
|----------|--|
| BSL-2    | Biosafety level 2                                |
| CHIK     | Chikungunya                                      |
| CHIKV    | Chikungunya virus                                |
| CHIKF    | Chikungunya fever                                |
| CDC      | Centers for Disease Control and Prevention       |
| E1       | Envelope gene 1                                  |
| ECSA     | East Central South African                       |
| ELISA    | Enzyme-linked immunosorbent assay                |
| FRNT     | Focus reduction neutralization test              |
| HI       | Hemagglutinin inhibition                         |
| HRP      | Horseradish peroxidase                           |
| IFA      | Immunofluorescence assay                         |
| IFN      | Interferon                                       |
| IgG      | Immunoglobulin G                                 |
| IL       | Interlukin                                       |
| IOL      | Indian Ocean lineage                             |
| ORF      | Open reading frame                               |
| NK cells | Natural Killer cells                             |
| NSP      | Non Structural protein                           |
| PFU      | Plaque forming unit                              |
| PCR      | Polymerase chain reaction                        |
| PRNT     | Plaque reduction neutralization test             |
| RNA      | Ribonucleic acid                                 |
| RT-LAMP  | Real-time loop-mediated isothermal amplification |
| RT-PCR   | Reverse transcriptase-polymerase chain reaction  |
| SFV      | Semliki Forest virus                             |
| TMB      | Tetramethylbenzidine                             |

## Abstract

Chikungunya is a viral disease transmitted by mainly *Aedes aegypti* and *Aedes albopictus* mosquitoes. In 2017, Chikungunya virus was introduced into the Dhaka city of Bangladesh and triggered a massive outbreak which affected millions of lives and forced upon significant damages in socioeconomic factors. Since the disease appeared quite recently in this region and this is the first time such a widespread transmission occurred, there is a lack of adequate data to assess the viral burden in the population and the effectiveness of laboratory procedures that can be implemented in resource limited countries like Bangladesh. Hence, this study was conducted to evaluate the rapid techniques available for the serological diagnosis of Chikungunya. For this research, a population based cross-sectional study was carried out to correlate the clinical cases with laboratory confirmation by two main serological techniques called ICT and ELISA. Immunochromatographic (ICT) test was performed on a total of 1201 collected convalescent blood samples and among them Enzyme linked immunosorbent assay was performed on a total of 81 individuals who were selected through proper sampling technique for the detection of IgM or IgG antibodies against CHIKV which confirms the infection. The sensitivity and specificity of the ICT test was determined as 83% and 100% respectively by taking ELISA as the reference method. The seroprevalence of the convalescent samples drawn by the rapid test is 75.2%. Gender distribution of Convalescent seropositive Chikungunya cases showed a higher infection rate in male than female with seroprevalence rates of 79.7% and 68.5% respectively. The data of age distribution revealed that seroprevalence rate is much higher in older people than younger people. In addition, statistical analyses confirmed the association of age and gender with seropositivity determining them as possible risk factors. Furthermore, it was detected that fever, joint pain and rash were significantly related with CHIKV seropositivity. Overall, considering all these findings Chikungunya infection must be given considerable weight and proper diagnostic procedure and management strategies should be taken to control the situation.

# *Chapter 1*

## *Introduction*

# Introduction

## 1.1 Background of the study

Emerging and constantly evolving arthropod-borne viruses (arboviruses) represent a significant warning to human health worldwide. Chikungunya virus (CHIKV) infection caused by Chikungunya virus is an emerging, febrile, arthropod-borne viral disease associated with significant impact and potential substantial effects on different areas of public health. Chikungunya virus is a member of the genus *Alphavirus* belonging to the family *Togaviridae* which is accountable for the unexpected rise in crippling febrile arthralgia in the past decade (Pastorino et al., 2004; Lumsden et al., 1955; Powers et al., 2000). Until recently, Chikungunya fever did not evoke the fear associated with other arboviruses, such as dengue and West Nile viruses and attracted only minor interest in the medical community. However, the recent resurgence of Chikungunya fever has drawn global attention due to its explosive onset, rapid spread, high morbidity, and myriad clinical manifestations. (Simon et al., 2008). Therefore it has become a disease of global concern due to its tremendous social and economic impact in different parts of the world.

Chikungunya virus (CHIKV) is an arthropod-borne virus that is mainly transmitted by *Aedes* mosquitoes. Chikungunya is a Makonde word meaning “the one which bends up” referring to the stooped posture of the affected patient acquired due to severe pain in the joints, a characteristic feature of the disease (Robinson, 1955). Historically, Chikungunya was not considered a life-threatening infection but recent epidemiological evidence suggests a case fatality rate of around 0.1 % (Pialoux et al., 2007). Even though the mortality rate remains in question, high attack rates are often seen throughout different epidemics (Mohan et al., 2010)

## 1.2 Epidemiology

### 1.2.1 Chikungunya Emergence, Distribution and Spread

CHIKV was first isolated from the serum of a febrile patient during an outbreak that occurred in the Makonde Plateau, southern province of Tanzania in 1952–1953 (Robinson, 1955; Ross,

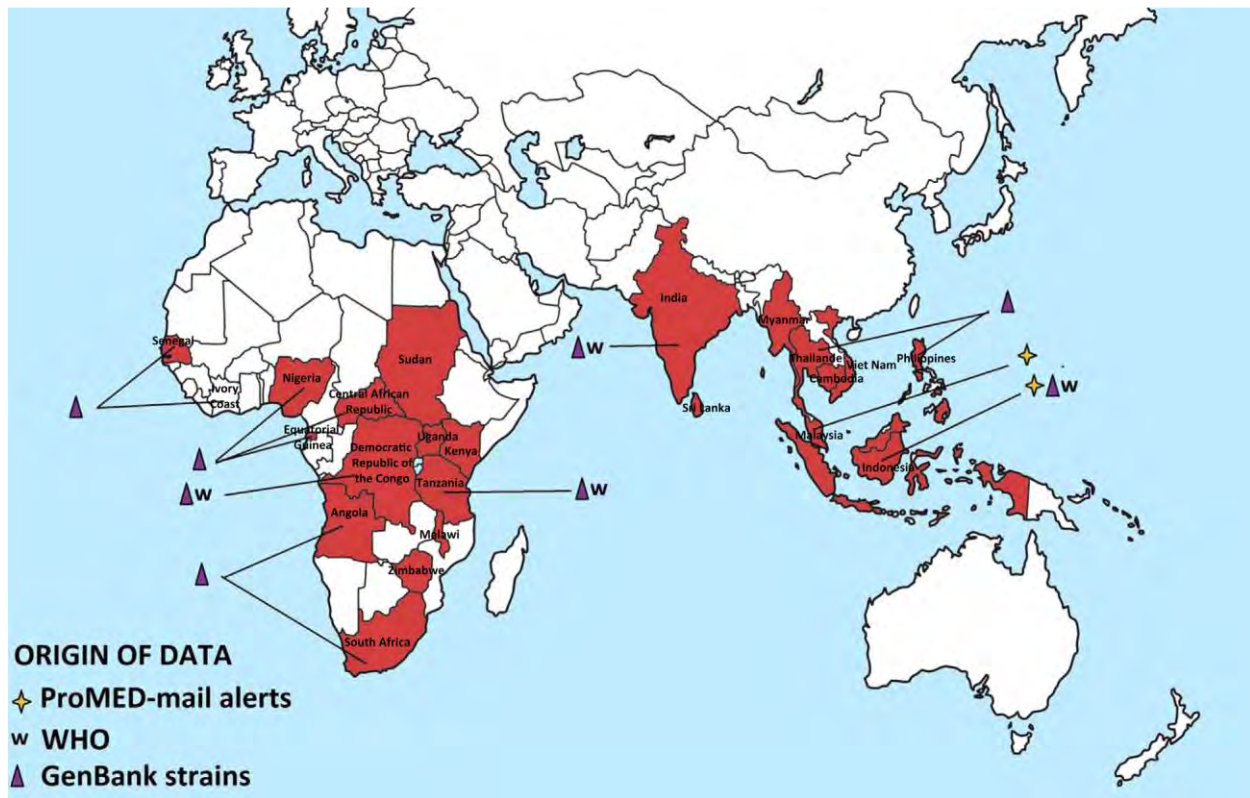
1956). After that a combination of increased global travel and trade, wide distribution of the mosquito vectors, and lack of herd immunity contributes to the introduction and rapid spread of CHIKV in naive populations (Kuan et al., 2016). Since the first isolation of CHIKV in Tanzania, it has been endemic in many parts of Africa and Asia. However, within the last decade it has reemerged as a major threat to human health globally, causing massive outbreaks in endemic areas, as well as in new regions (Weaver & Forrester, 2015). Both sporadic cases and major epidemics of CHIKV disease have been reported in Africa (Muyembe-Tamfum et al., 2003), India (Myers et al., 1965), South-East Asia and the Western Pacific (Mackenzie et al., 2001; Laras et al., 2005).

After a period of quiescence, numerous African countries faced the re-emergence of CHIKV infection at the end of 1990s, with outbreaks in Senegal in 1996 and 1997 (Thonnon et al., 1999), the Democratic Republic of Congo in 2000 (Pastorino et al., 2004). Cases were reported in Sudan in 2005 (Gould et al., 2008) , Tanzania in 2007 and 2008 (Hertz et al., 2012) and outbreaks occurred in Gabon in 2007 and 2010 (Karon et al., 2012), Cameroon in 2006 and again in Republic of Congo in 2011 (Kelvin, 2011). Furthermore, a number of studies indicates CHIKV circulation in Kenya and Cameroon during inter-epidemic periods (Sutherland et al., 2011; Kuniholm et al., 2006; Mease et al., 2011). The first documented Asian outbreak took place in 1958 in Bangkok, Thailand. Since then, many outbreaks have been recorded from Cambodia, Vietnam, Laos, Myanmar, Malaysia and Indonesia before the virus virtually disappeared following the 1973 outbreak in India. (Mathew et al., 1973; Laras et al., 2005; Lam et al., 2001; Jupp et al., 1988).

A large outbreak in Kenya in 2004 initiated a reappearance of the virus leading to widespread infection in the Indian Ocean islands of the Comoros, Seychelles, Mauritius and the French islands of Mayotte and La Réunion. The epidemiology of the virus changed, with the major vector on La Réunion identified as *Aedes albopictus* (Ligon, 2006). At the time, this was the largest documented outbreak, with over 266,000 cases estimated to have occurred (Staples et al., 2009). The rapid geographic spread of the virus led to a large Chikungunya disease epidemic in India during 2006-2007 (Mavalankar et al. 2007). Countries in Europe, Asia and North America documented imported cases associated with travelers returning from India and the Indian Ocean



islands (Powers et al., 2007; Lanciotti et al., 2007; Parola et al., 2006; Lee et al., 2006). In 2007 the first chikungunya epidemic in a temperate country was recorded in the region of Emilia-Romagna in north-eastern Italy (Rezza et al., 2007). In September 2010, two autochthonous cases were documented in the southern French city of Fréjus where the *Aedes albopictus* vector is present (Institut de Veille Sanitaire, 2011). In the Americas, autochthonous CHIKV transmission was first reported in the Caribbean on the island of St. Martin in December 2013 (Leparc-Goffart et al., 2014; Cauchemez et al., 2014).



**Figure 1.1: Geographic distribution of CHIKV cases**

Although Bangladesh faced periodic epidemics of Dengue time to time from 1964 including the large outbreak in 2000 (Sharmin et al., 2015), Chikungunya infection was uncommon here. The first outbreak of Chikungunya fever was detected in northern Rajshahi and Chapainawabganj districts bordering India in 2008 (icddr, 2009). In late October 2011, an outbreak of fever and severe joint pain was reported by a local health official in Dohar Sub-district in Dhaka District

where the responsible species was identified as *Ae. Albopictus* (Khatun et al., 2015). Recently in 2017, a major outbreak occurred in Dhaka city caused by Chikungunya virus which seems to start in May of 2017 after the heavy rainfall in April where the magnitude surpassed the previous outbreaks (The daily star, 2017).

### 1.2.2 Genetic Origins

On the basis of the phylogenetic analysis of the open reading frame of several CHIKV strains, the virus has been divided into three lineages: West African (WAf), East/Central/South African (ECSA) and Asian (Powers et al., 2000). The current CHIKV strains derived from a common ancestor that existed around 500 years ago. The divergence between the ECSA and the Asian clades occurred during the end of the nineteenth and the beginning of the twentieth centuries. The recent Indian Ocean monophyletic lineage (IOL) originated from the ECSA group at the beginning of the twentieth century (Volk et al., 2010). Over the last decade, the ECSA lineage became prevalent worldwide causing outbreaks in Europe, Africa, Indian Ocean, and south Asia (Burt et al., 2012).

### 1.2.3 Arthropod vectors

The principal mosquito vectors responsible for CHIKV infection are *Ae. aegypti* and *Ae. albopictus*. Until recently, *Ae. aegypti* was considered as the primary vector for CHIKV transmission but in 2006, *Ae. albopictus* was surprisingly identified as a second major vector of the virus both in places where *Ae. aegypti* is considered to be rare (e.g., in Reunion island) and also in places where both mosquito species are prevalent (e.g., in Madagascar, India and Gabon). This emergence of *Ae. albopictus* as a major vector of CHIKV was largely attributable to a single mutation in the E1 protein of CHIKV which facilitated enhanced virus uptake, replication and transmission by the vector (Tsetsarkin et al., 2011b).

The species *Ae. albopictus* flourishes in a wider range of water-filled breeding sites than *Ae. aegypti*, including bamboo stumps, coconut husks, cocoa pods, , tree holes and rock pools, in addition to artificial containers such as saucers beneath plant pots and vehicle tires. This diversity of habitats explains the abundance of *Ae. albopictus* in rural as well as peri-urban

areas. *Ae. aegypti* is more closely associated with human habitation and uses indoor breeding sites, including flower vases, water storage vessels and concrete water tanks in bathrooms, as well as the same artificial outdoor habitats as *Ae. albopictus*. Adult mosquitoes rest in cool and shady areas and bite humans during the daytime (WHO, 2017).

#### **1.2.4 Vertebrate reservoirs and transmission cycles**

Chikungunya fever epidemics show cyclical, secular and seasonal trends. These epidemics are characterized by explosive outbreaks interspersed by periods of disappearance ranging from several years to a few decades. Several mechanisms including a complex interaction between various factors such as the susceptibility of humans and the mosquito vectors to the virus; conditions facilitating mosquito breeding resulting in a high vector density, ability of the vector to efficiently transmit the virus are all thought to play a role (Mohan, 2006; Simon et al., 2008; Pialoux et al., 2007). International travel has facilitated the introduction of the virus from endemic areas to other areas resulting in outbreaks of the illness and imported cases of Chikungunya fever in different regions (Simon et al., 2008).

Human beings serve as reservoir hosts for the virus during epidemic periods whereas during inter-epidemic periods, several other reservoir hosts have been incriminated such as monkeys, rodents and birds (Inoue et al., 2003; Wolfe et al., 2001). The virus is transmitted from human to human by the bites of infected female mosquitoes. Two distinct transmission cycles have been described for CHIKV. It is circulated in a sylvatic cycle in Africa where the virus is transmitted between forest or savannah associated mosquitoes and non-human primates and possibly rodents with occasional spillover of the virus into nearby human populations living in or close to the sylvatic environment (Wolfe et al., 2001). Under these latter circumstances the virus may then encounter mosquitoes primarily associated with urban environments (i.e., domestic or peridomestic mosquitoes) thus initiating an urban human–mosquito–human virus transmission cycle such as those seen in Asia, the Indian Ocean, Africa and more recently, Europe (Rezza et al., 2007). The mosquito species involved in sylvatic cycles vary geographically and with ecological conditions; however, the major species involved are *Ae. fuscifer*, *Ae. taylori*, *Ae. luteocephalus*, *Ae. africanus* and *Ae. neoafricanus* (McIntosh et al., 1977). *Ae. aegypti* and *Ae.*

*albopictus* act as the main species in urban human-mosquito-human transmission cycle (Myers et al., 1965).

### 1.3 Chikungunya virus in brief

#### 1.3.1 Virus Structure, Classification and Genomic organization

The Chikungunya virus (CHIKV) has a single-stranded, positive sense RNA genome of approximately 11.8 kb in length (Lumsden, 1955). It is a member virus of the Semliki Forest virus antigenic complex. The complex consists of CHIKV (Africa, Asia), O'nyong-nyong virus (Africa), Ross River virus, Barmah Forest virus (Australia), and Mayaro virus (South America), which are antigenically closely related and all of them cause similar clinical manifestations (Tesh, 1982). The virion has an icosahedral capsid enclosed by a lipid envelope and a diameter of 60–70 nm. It is sensitive to desiccation and to temperatures  $>58$  °C.

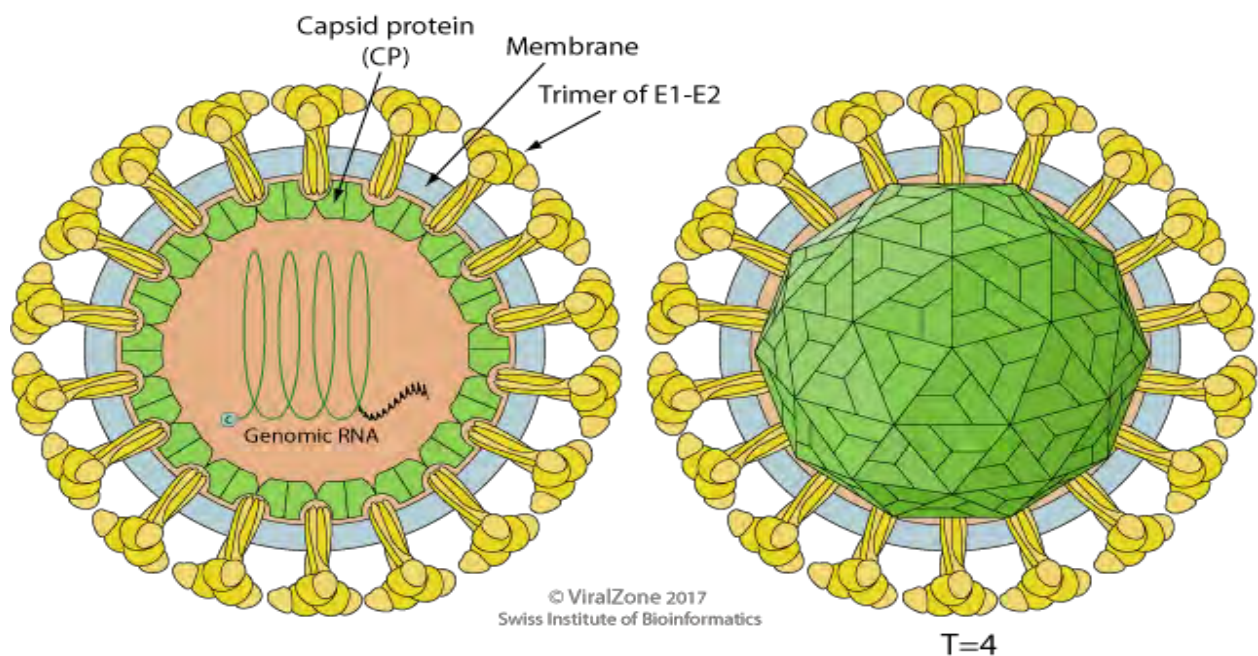
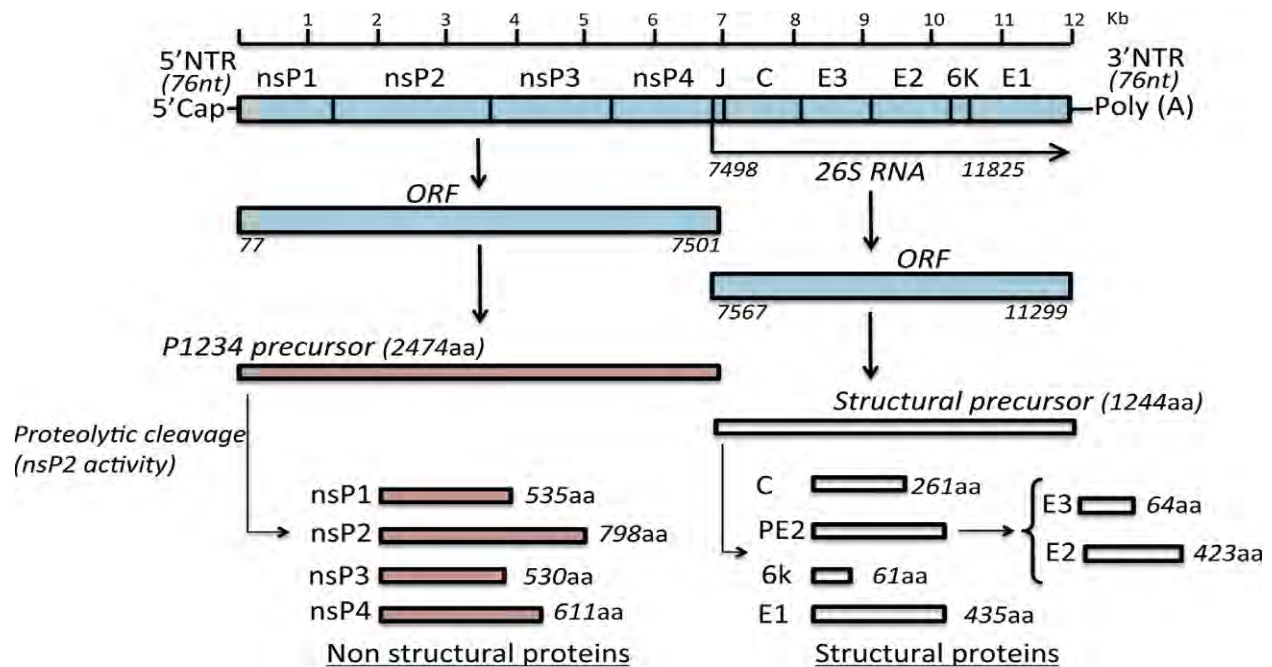


Figure 1.2: Structure of Chikungunya virus

The genomic organization is arranged as: **5'-nsP1–nsP2–nsP3–nsP4–junction region–C–E3–E2–6k–E1–poly (A)-3'** with two open reading frames (ORFs). The 5' ORF is translated from genomic RNA and encodes four non-structural proteins (nsP1, 2, 3 and 4) (Jose et al., 2009). The 3' ORF is translated from a subgenomic 26S RNA and encodes a polyprotein that is processed as the capsid protein (C), two surface envelope glycoproteins (E1 and E2) and two small peptides designated E3 and 6k (King et al., 2012; Simizu et al., 1984; Voss et al., 2010).



**Figure 1.3: Organization of the CHIKV genome and gene products.**

The glycoproteins E1 and E2 are embedded in a heterodimeric form in the viral envelope and are responsible for virus attachment and membrane fusion. Virus fusion with the cell membrane is mediated by the E1 glycoprotein, a class II fusion protein, in a process dependent on low-pH. Acidic conditions induce a conformational change in the virus envelope proteins, dissociation of the E2–E1 heterodimers and formation of E1 homotrimers. The E1 trimer is inserted into the target membrane via its hydrophobic fusion peptide and refolds to form a hairpin-like structure.

Cholesterol is required for both cell membrane fusion and budding during alphavirus infection (Solignat et al., 2009).

### **1.3.2 Replication cycle**

Replication of CHIKV occurs in the cytoplasm, both in vertebrate and insect cells, in close association with the Golgi apparatus. The nonstructural proteins (nsP1–4) and their cleavage intermediates are involved in RNA replication. The five structural proteins (C, E3, E2, 6k, E1) and their cleavage intermediates are required for viral encapsidation and budding (Fros et al., 2010; Leung et al., 2011). Virus enters the cell at the plasma membrane, mostly by endocytosis, via a pH-dependent mechanism which results in fusion pore formation and release of the nucleocapsid into the cytosol. It begins with attachment where E2 is primarily responsible for interactions with cellular receptors and fusion of virus particles in the membrane of the host cell. The fusion peptide is located at the tip of the E1 molecule in domain II, close to amino acid 226.

Following virus entry, two rounds of translation occur. Positive-sense genomic RNA acts directly as mRNA and is partially translated from 5' end to produce non-structural proteins. These proteins are responsible for replication and formation of a complementary negative strand, the template for further positive-strand synthesis. Subgenomic mRNA (26S) replication occurs through the synthesis of full-length negative intermediate RNA, which is regulated by nsP4 and p123 precursor in early infection, and later by mature nsPs. Translation of the 26S sub-genomic RNA results in production of 5 structural proteins (C, E3, E2, 6k, E1). Assembly occurs at the cell surface, and the envelope is acquired as the virus buds from the cell (Solignat et al., 2009).

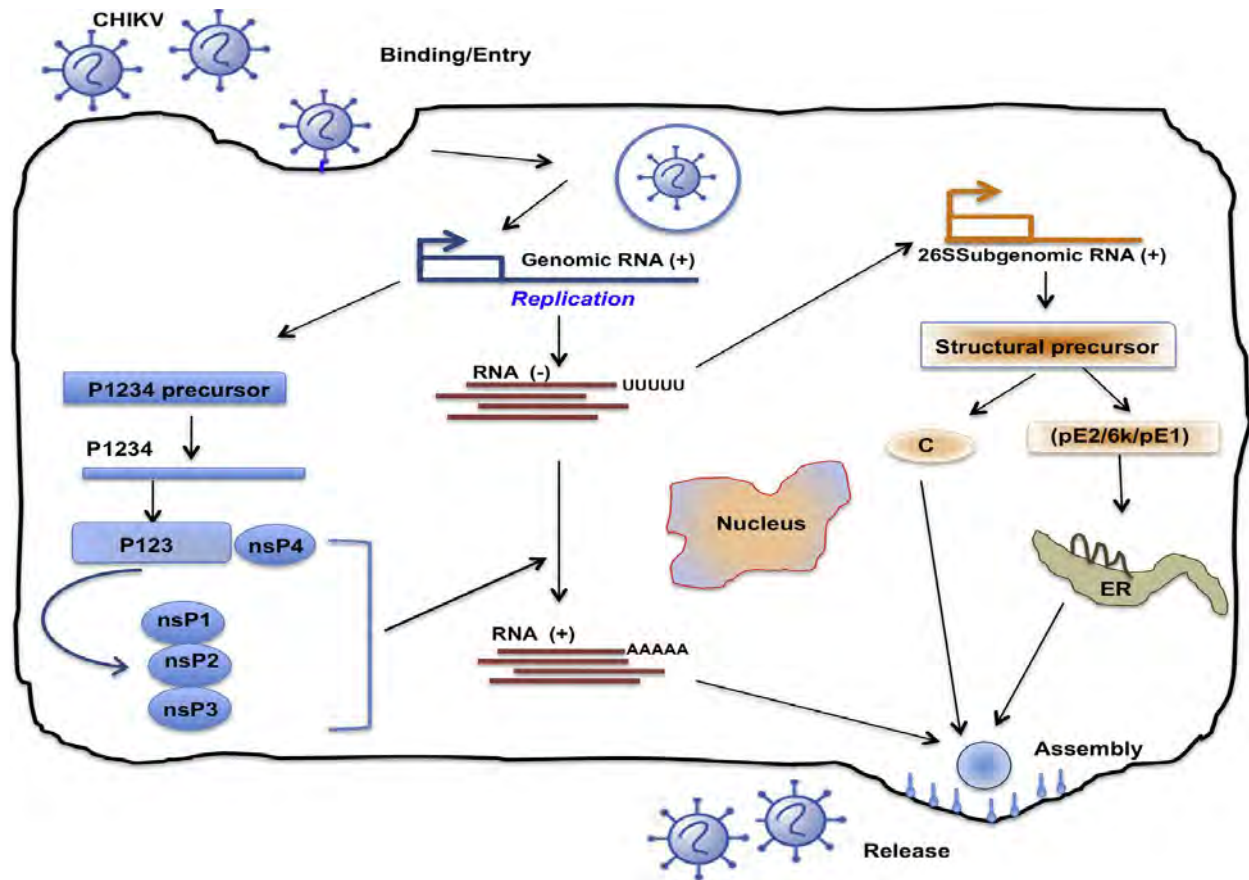


Figure 1.4: Life cycle of CHIKV in infected cells.

## 1.4 Clinical manifestations

CHIK infections are usually initiated by bites of infected female mosquitoes which are followed by several characteristic clinical features. Based on their clinical presentation, Chikungunya cases are often categorized into two primary groups - Acute stage or the ‘viral stage’ (days 1–4), associated with rapid decrease of viraemia, followed by rapid improvement of clinical presentation and late stage of illness, also known as convalescent stage (days 5–14) that was associated with no detectable viraemia and a slow clinical improvement (Thiberville et al., 2013).

Clinical disease manifestations emerge after an incubation period that lasts an average of 4 to 12 days. The first symptom is usually a high fever, followed hours later by myalgia and generalized arthralgia and arthritis, which are often incapacitating and debilitating, the most significant feature of this disease. After a few days, fever may abate and recrudesce, giving rise to a “saddleback” fever curve. These are often associated with chills, headache, malaise, vomiting, headache and back pain. Most cases of chikungunya fever are self limiting and the symptoms usually resolve within 7 days (Mahendradas, 2009).

Certain patients, however, experience persistent joint pain for weeks or months and, occasionally, years after the initial onset of illness. Joint pain is mostly polyarticular, bilateral and symmetrical. This polyarthropathy frequently involves the peripheral joints of the hand, wrist, and ankles and the larger joints such as the knee and shoulder. Disabling acute tenosynovitis is also frequently present and Periarticular swelling is frequently observed (Simon et al., 2008; Pialoux et al., 2007; Borgherini et al., 2007).

Cervical or generalized lymphadenopathy may be present. Mucocutaneous manifestations, such as morbilliform eruptions, scaling, macular erythema, intertrigo, hypermelanosis, xerosis, excoriated papules, urticaria, and petechial spots have been described in patients with chikungunya fever. A maculopapular rash and facial swelling are present in approximately 40 to 50% of patients (Thiberville et al., 2013). In children, vesicobullous eruptions with intense subsequent flaking may occur, along with petechiae and gingivorrhagia. Ocular manifestations can also occur and generally achieve satisfactory resolution in six to eight weeks (Pialoux et al., 2007).

Neurological complications such as meningoencephalitis have been reported in rare cases during the first Indian outbreak and the French Reunion Island outbreak. Other neurological manifestations reported thus far include neuropathy, myelitis, entrapment neuropathy, altered mental functions, seizures, focal neurological deficit myeloneuropathy, and acute flaccid paralysis (Pialoux et al., 2007). Severe systemic disease such as multiple organ involvements, sudden sensory neural hearing loss, hypokalemic periodic paralysis, liver failure, respiratory failure, and renal failure are extremely rare but have been reported (Mahendaras et al., 2008).



Chikungunya and dengue infections can overlap in clinical presentation, but low platelet count in dengue can be used to differentiate between the two conditions (Lee et al., 2012).

## **1.5 Immunopathogenesis**

### **1.5.1 Cellular response**

All the components of immune system both cell-mediated and humoral immunity are involved in response to Chikungunya infection. Following inoculation with chikungunya virus through a mosquito bite, the virus directly enters the subcutaneous capillaries, with some viruses infecting susceptible cells in the skin, such as macrophages or fibroblasts and endothelial cells. Local viral replication seems to be minor and limited in time, with the locally produced virus probably being transported to secondary lymphoid organs close to the site of inoculation. (Talarmin et al., 2007). Antiviral cytokines such as, TYPE-1 IFN (IFN-alpha & IFN-beta) are released to counteract the viral growth by apoptotic lysis of the virus infected cells. Type-1 IFN also activates NK cells which directly kills virus infected cells by linking with the ligand on infected cell to the activating receptor- NKG2C expressed on their surface. Despite robust innate response, viral replication continues and multiplying viruses within macrophages will disseminate through circulation to reach secondary lymphoid organs like lymph nodes and lymphadenopathy develops. Replicating viruses also disseminates to a wide range of internal organs like- liver, spleen, brain, joint synovium and muscles. During this period, clinical manifestations of chikungunya first appears with a very high viraemic load of  $10^7$  pfu/ml on average after 5-6 days of clinical onset of symptoms (Schwartz & Albert, 2010).

Due to massive viral replication, certain chemokines like CCL2 and CXCL10 are released and there is infiltration of macrophage into the infected organs. Pro inflammatory cytokines like IL-1, IL-6 & TNF-alpha are released from macrophages which potentiates inflammation within joint synovium and produces fever by directly acting on temperature regulating centre of hypothalamus. IL-8 is also released, causing chemotaxis of Neutrophil into the infected organs which further accentuates the tissue injury (Ng et al., 2009). Here viruses hijack the synovial macrophage, persist within it and inflammatory response continues which ultimately causes the

development of tenosynovitis, responsible for severe disabling arthralgia. Macrophages also present viral antigens to CD4+ helper T cell and switches Th0 to Th1 by releasing IL-12. IFN-gamma is released from Th1, ultimately potentiates inflammation by activated macrophages. IL-2 is also released, causing activation of cytotoxic T cell(CD8+) which ultimately destroys the virus infected endothelial cells of skin micro capillaries. Ultimately skin rashes will develop due to leakage of RBC from damaged vessels. CD8+ cytotoxic T cells ultimately infiltrates into the joint synovium and also responsible for tissue injury. (Lum et al., 2015).

### **1.5.2 Humoral response**

Following initial infection, a rapid humoral response occurs with huge production of neutralizing Anti Chickungunya antibody. The immunoglobulin M (IgM) can be detected very early, usually from the third to fourth day after the onset of clinical symptoms and antibody levels are highest 3 to 5 weeks after the onset of illness (Litzba et al., 2008). Due to rapid seroconversion, Immunoglobulin G (IgG) appears only after two or three days of appearance of IgM where IgG subclass 3 is the predominant. Persistence of IgM antibodies was found to be varying in different studies and it did not persist normally more than three to four months period, at detectable levels (Chia et al. 2010). However, in some studies it is reported to persist for 12 to 18 months. This unusual persistence of IgM may be attributed to continual viral replication within synovial macrophage. IgG antibodies are found to be detectable in convalescence and remains so for years (Chia et al. 2010).

### **1.6 Laboratory Diagnosis**

The clinical presentation of CHIKV infection during the acute stages can be quite similar to other arthritogenic alphaviruses and other tropical arboviruses such as dengue and Zika. Therefore definitive diagnosis is required to differentiate the CHIKV infection from other similar clinical diseases. The testing algorithm to diagnose CHIKV infections is based on the characteristics of CHIKV infection and the timing of specimen collection. The gold standard for

the diagnosis of Chikungunya fever is viral culture based on inoculation of mosquito cell cultures, mosquitoes, mammalian cell cultures, or mice. (Simon et al., 2008; Pialoux et al., 2007; Powers et al., 2007). However, viral culture is seldom done in routine clinical practice as these facilities are not widely available in poor and developing countries. Reverse Transcriptase Polymerase Chain reaction (RT-PCR) act as the most sensitive method for the detection of CHIKV. Real-time loop-mediated isothermal amplification (RT-LAMP) have also been found to be useful molecular tool for the rapid diagnosis of Chikungunya in the acute stage (WHO, 2009; Pastorino et al., 2004). Serodiagnostic methods for the detection of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies against Chikungunya virus are frequently performed during the first few weeks or months as the virus starts to disappear after four days of clinical onset due to the presentation of antibodies against the virus in the immune system. Among them Enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA) and hemagglutination inhibition (HI) are simple, rapid, and sensitive techniques widely used for the diagnosis of CHIKV infection. However, these methods represent a screening tool and often require confirmation by a second-line assays. Serum virus neutralization assay is more specific than ELISA , IFA and inhibition tests and is considered a confirmatory test. Neutralization assay is employed to determine the titer of virus neutralizing antibodies against CHIKV in patients' sera. (WHO, 2009).

## 1.7 Literature review

Blacksell et al., (2011) conducted a study where two serodiagnostic assays for detecting Chikungunya infection - IgM antibody rapid immunochromatographic test (ICT) device and Chikungunya IgM antibody enzyme-linked immunosorbent assay (ELISA) manufactured by Standard Diagnostics (SD; Standard Diagnostics, South Korea) were being evaluated. For the evaluation, diagnostic accuracy indices of sensitivity, specificity, negative predictive values (NPV), and positive predictive values (PPV) with exact 95% confidence intervals (CI) were calculated for a total of 292 patients. The sensitivity and specificity of the SD IgM ELISA for convalescent samples were found 84% and 91% respectively which gives the indication that this test device is quite reliable for detecting convalescent Chikungunya infections. The diagnostic accuracy of ICT test device was not determined for convalescent cases which requires attention.

According to Johnson et al. (2015), Nine commercial CHIKV IgM detection assays are available for the Laboratory Diagnosis of convalescent Chikungunya Virus Infections which includes Chik IgM ELISA kit of SD Diagnostics, Korea. They were evaluated at 3 reference laboratories namely CDC, the Public Health Agency of Canada National Microbiology Laboratory (NML), and the Caribbean Public Health Agency (CARPHA) in Trinidad-Tobago to provide guidance to public health diagnostic laboratories on their performance parameters. The Euroimmun and Inbios CHIKV MAC-ELISA kits had the highest accuracy (99% and 100%, respectively) and reproducibility and the Euroimmun IIFT also had high performance (96% accuracy). However, their diagnostic accuracies in different clinical settings were not clearly defined and needs further assessment.

Wasonga et al., (2015) developed an in-house ELISA technique and compared it with CDC ELISA and focus reduction neutralization test (FRNT). The in-house ELISA had a sensitivity of 97.6% and specificity of 81.3% compared to the CDC ELISA and a sensitivity of 91.1% and specificity of 96.7% compared to FRNT. The study also demonstrated age distribution of seropositive cases where CHIK cases were detected in all age groups, with a higher positive ratio being observed in children of 14 year and below, and in adults of 55 year and above.

By contrast, Sergon et al (2004) determined that during the CHIK outbreak in Lamu Island, Kenya in 2004, which was the first documented outbreak in the coastal region, all age groups were equally infected, indicating that the population at that time were immunologically naive and all individuals were equally susceptible to CHIKV infection.

De Salazar et al., (2017) demonstrated the importance of evaluating commercial kits for the detection of CHIKV infections. In this study, three commercially-available assay test kits—two enzyme immunoassays (EIA) of Euroimmun and one immunofluorescence antibody technique (IFA) of InBios — that detect IgG antibodies against CHIKV were evaluated. Results showed sensitivity and specificity values ranging from 92.8% – 100% and 81.8% – 90.9%, respectively, with a significant number of false-positives ranging from 12.5% – 22%.

Another study was conducted by Kosasih et al., (2012) where evaluation of two IgM rapid immunochromatographic tests during circulation of Asian lineage Chikungunya virus was performed. The sensitivity and specificity of the OnSite Chikungunya IgM Rapid Test-Cassette and the SD Bioline CHIK IgM rapid test were evaluated in comparison to a capture ELISA. The sensitivity of the OnSite test was 20.5% while its specificity was 100%. The sensitivity of the SD Bioline test was 50.8% while its specificity was 89.2%. The sensitivity of the SD Bioline test increased with increasing CHIK IgM titers and with days of onset in samples collected before day 21 of illness. Increasing the reading time from the manufacturer's suggested time of 10 to 20 minutes significantly increased the sensitivity of the SD Bioline test to 68.2%, but did not significantly change its specificity.

For determining the association between clinical manifestation and seropositivity Macpherson et al., (2016) showed that joint pain was by far the most statistically significant clinical symptom distinguishing between positive and negative CHIKV cases. Of 441 cases with joint pain, 395 (90%) tested positive for CHIKV, whereas only 31 of 49 (63%) cases without joint pain tested positive. Thus, the proportion of cases who presented with joint pain and tested positive for CHIKV was 42% greater than the proportion of cases without joint pain. Moreover it was also found in this study that only joint pain and fever alone were insufficient to reliably classify

Chikungunya cases. According to them, the decision rule to “Diagnose as CHIKV positive patients who present with joint pain along with any combination of the fever, body pain, or rash,” would have provided 85% agreement with the serological tests.

A cross sectional seroprevalence study was performed by Azami and his colleagues (2013), which was carried out in Malaysian cohort participants. They showed that there were no significant differences of the seroprevalence between age groups ( $p=0.078$ ). They also found in their study that gender, locality, ethnicity and state of residency were statistically significant univariate predictors of CHIK seroprevalence. Males were more likely to be CHIK seropositive compared to females with an odds ratio of 2.112 (95% CI: 1.223- 3.647).

In a study conducted by Moro et al. (2010), after an outbreak of Chikungunya infection in Emilia-Romagna Region of North-eastern Italy the prevalence of antibodies against Chikungunya virus was found 10.2% (33 cases out of 325) by ELISA method. The prevalence of antibody to CHIK virus significantly increased with age and was higher in men than women where in the univariate analysis, the prevalence of infection was higher in older age groups, and in males.

Sissoko et al., (2008) conducted a household-based cross sectional serosurvey in Mayotte in November and December 2006 using complex multistage cluster sampling. The overall weighted seroprevalence rate for anti-CHIKV antibodies in the study population was 37.2% (95% CI 33.9–40.5) where a total of 1154 individuals were analyzed. The weighted prevalence of specific IgM and IgG antibodies was 18.1% and 37.2% respectively. Significant differences in seroprevalence were observed according to gender (40.6% for men and 33.8% for women,  $p = 0.03$ ), but not for age, although subjects in the 2–14 and 45–54 year age groups appeared to be less infected.

A community based cohort study for ages 2-14 years and a cross-sectional survey for ages  $\geq 15$  years was conducted by Kuan et al., (2016) in Managua, Nicaragua after the first

Chikungunya epidemic of 2014-2015. Routine annual serum samples of a total of 3,362 cohort participants and a total of 848 age-stratified samples for the cross-sectional survey were analyzed. In the cohort study, 204 children were seropositive for CHIKV, for a seroprevalence of 6.1% where 13.1% of the 848 participants were seropositive for CHIKV in the cross-sectional study. Comparing the two populations, the seroprevalence in children aged 2–14 was 7.0 percentage points lower than those  $\geq 15$  ( $p < 0.001$ ).

Demanou et al., (2010) performed a seroprevalence study one year after a Chikungunya outbreak in the rural area of western Cameroon in 2006. They reported that 54 subjects (51.4%) had detectable IgM anti-CHIKV in their sera among a total of 105 persons. Amongst these, 52 showed both anti-CHIKV IgM and IgG and 2 (1.9%) had IgM anti-CHIKV in the absence of IgG. Isolated anti-CHIKV IgG positives were detected in 41 (39%) cases. Moreover, IgM seropositivity to CHIKV was similar between females (52%) and males and anti-CHIKV IgM antibody prevalence did not vary significantly across different age strata ranges from 5 to  $\geq 60$  years.

Two serosurveys were implemented by Gerardin et al., (2008) after the two-wave seventeen-month-long outbreak in La Réunion Island in 2005–2006. The first survey was a rapid survey where stored sera of pregnant woman was used and the second survey population-based survey conducted among a random sample of the community. 18.2% of the pregnant women were tested positive for CHIKV specific antibodies (13.8% for both IgM and IgG, 4.3% for IgM, 0.1% for IgG only) which provided a congruent estimate with the 16.5% attack rate calculated from the surveillance-system. In survey 2, the seroprevalence in community was estimated to 38.2% (95% CI, 35.9 to 40.6%). They also assessed the association between clinical features and serology where people who declared a Chikungunya with sudden fever and incapacitating arthralgia represented 88.5% of all people who declared the disease and 87.0% of all positive serology (sensitivity and PPV for both signs: 88.5% and 87.0%, respectively).

In another study conducted on Pregnant Women living in an urban Area in Benin of West Africa by Sambri et al., (2015) where 36.1% seropositive cases were determined among pregnant women.

Gay et al. (2016) conducted a serosurvey in the population of Saint Martin after seven months of the outbreak in the region where the chikungunya virus was detected of Asian lineage. A total of 203 individuals was tested and screened for the presence of Both IgM and IgG anti-CHIKV specific antibodies using in-house ELISA. The seroprevalence detected was 16.9% with 95% confidence interval.

Another household based study was conducted by Sergon and his colleagues (2007) after the occurrence of a Chikungunya outbreak in Grande Comore Island from January through May 2005 where 5,202 cases were reported. A total of 481 households were surveyed with 331 (69%) of the selected survey participants consenting to blood collection. Among the 331 serum specimens tested, IgM or IgG antibodies to CHIKV were detected in 209 (63%). IgM antibodies were detected in 198 (60%) of the sera and IgG antibodies were found in 89 (27%). Only 11 seropositive sera had IgG, but no IgM antibodies. It was also found that seropositivity was 68% (139 of 204) among females compared with 55% (70 of 126) among males which indicated that females were more likely to be infected than males. The highest seropositivity was in the (45–54) year age group (75%). However, there was no statistical difference in antibody prevalence when comparing young ( $\leq 15$  years of age) and older ( $>15$  years old) participants.

Gudo et al., (2015) investigated Serological Evidence of Chikungunya Virus among Acute Febrile Patients in Southern Mozambique. In this study Paired blood samples were obtained from 209 patients, of which 26.4% (55/208) were presented anti-CHIKV IgG antibodies in the convalescent sample. Seroconversion or a four-fold titer rise was confirmed in 9 (4.3%) patients. The median age of study participants was 26 years (IQR: 21–33 years) and 57.5% (224/391) were female. In regard to convalescent sample, the average number of days from disease onset was 25 days.

A study was conducted by Kawle et al., (2017) to determine Seroprevalence and clinical manifestations of chikungunya virus infection in rural areas of Chandrapur district of Maharashtra state of India. Here, seroprevalence of CHIKV infection was found to be



46% in 482 infected enrolled participants during the epidemic period. Among risk factors, ageing and female gender was strongly associated with a raised seroprevalence of CHIKV infection along with symptoms such as rashes, small joints pain and neck stiffness. The participants consisted of 41% male and 59% female aged between 5 and 92 year.

In an attempt to investigate the Chikungunya outbreak that occurred in rural area of Bangladesh in 2011 Khatun and her team (2015) conducted a house-to-house serosurvey. A total of 1105 suspected blood samples were collected from 897 households and tested for the presence of anti Chikungunya IgM antibodies. 80% (196/245) of them had IgM antibodies against Chikungunya virus. They also found out that Patients tested within 1 week of illness onset were unlikely to have IgM antibodies, while 93% of suspected cases tested between 30 and 60 days post illness onset had IgM antibodies against Chikungunya. The attack rate was estimated as 29% (1105/3840).

# 1.8 Aims and Objectives

Recently Dhaka city of Bangladesh confronted an outbreak on Chikungunya in 2017 where severe damages have been imposed on the social and economic lives of people. Although several minor cases have been reported in Bangladesh previously in the year of 2008 and 2011, this was first time when the scale is so immense where such a wide distribution occurred. It almost paralyzed the whole city where the sufferings of people surpassed all boundaries. Chikungunya infection is quite recent in this country and very few reports are available to elucidate the infection of Bangladeshi population. There is a lack of sufficient data to properly understand the magnitude and assess the different perspectives of this disease. In addition, CHIKV infection is clinically similar to other arboviral infections such as dengue virus and Zika virus which makes the diagnosis of this disease quite challenging. Unlike developed countries, Bangladesh do not contain the diagnostic setup and financial system to apply the expensive and sensitive procedure required for the diagnosis of Chikungunya. Hence, this study aims to evaluate the immunochromatographic test (ICT) which is a rapid test to detect the presence of anti Chikungunya IgM or IgG antibody by taking the ELISA as the reference method and to see whether it can be established as a reliable technique for initial mass screening of convalescent Chikungunya patients in the context of developing countries like Bangladesh where resource facilities are poor and identify possible risk factors associated with the infection.

## General Objective

Comparative analysis of serodiagnostic procedures on convalescent Chikungunya virus infections of Dhaka city, Bangladesh

## Specific Objectives

1. To find out seropositive Chikungunya cases using ICT and ELISA
2. To evaluate immunochromatographic (ICT) test by taking ELISA as the standard method through sensitivity and specificity test.
3. To detect age and gender distribution of convalescent Chikungunya cases
4. To determine possible risk factors of Chikungunya infection associated with seropositivity for ensuring better prognosis and management of CHIK patients

*Chapter 2*

*Materials and*

*Methods*

# Materials and methods

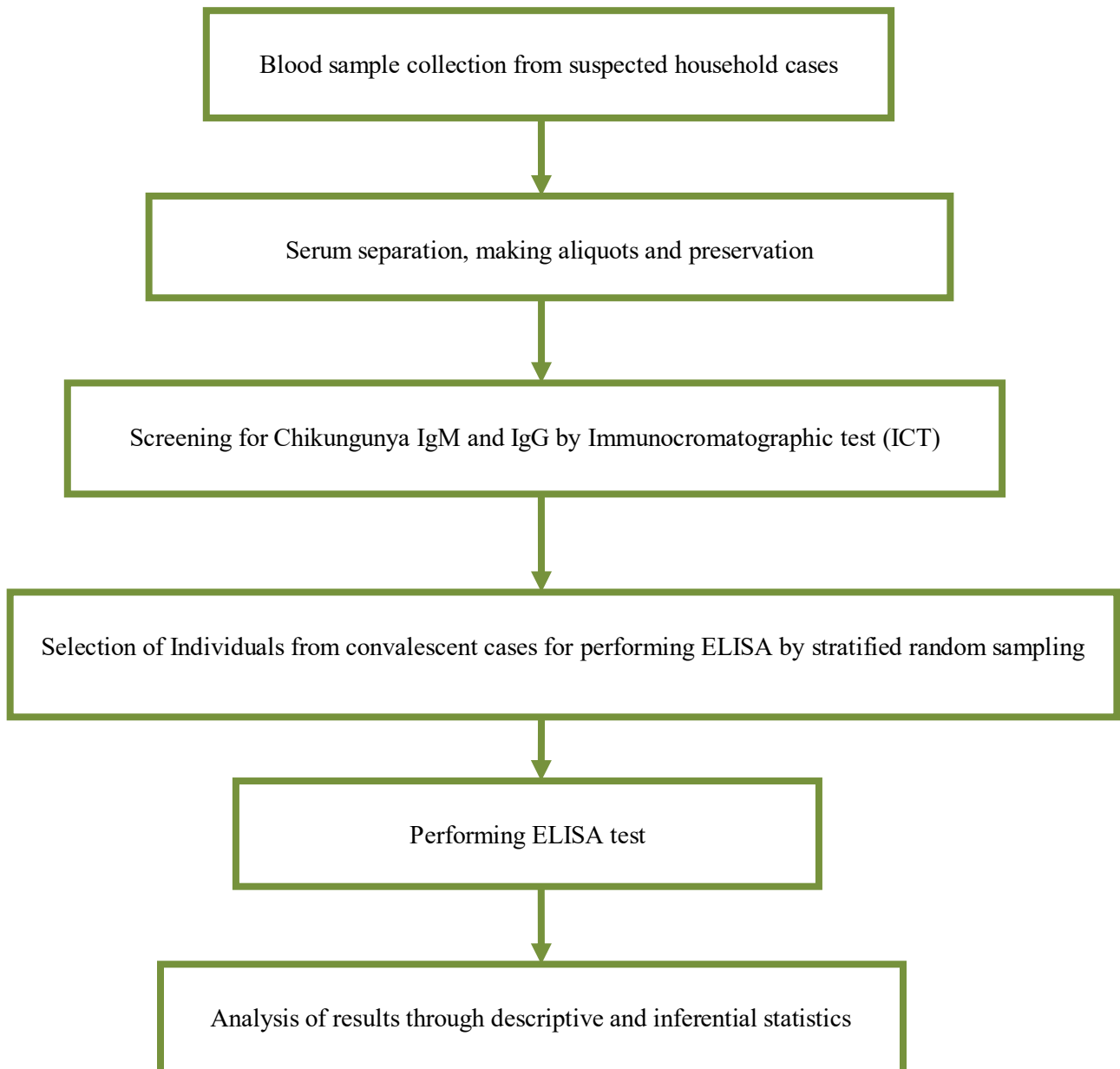
## 2.1 Place of study

This study was conducted at the laboratory facility of National Institute of Preventive and Social Medicine.

## 2.2 Period of Study

This study was carried out between August 2017 and January 2018.

## 2.3 Flow Diagram of the Study Design



## **2.4 Study design**

Household- based cross sectional study was conducted among individuals living in hundred different probabilistically selected spacial locations in Dhaka city, Bangladesh. Serum samples were obtained from individuals of different age groups in selected locations, and tested for anti Chikungunya antibodies (IgM and IgG) against chikungunya viruses.

### **2.4.1 Study setting**

Dhaka is the capital and largest city of Bangladesh. It lies along the east bank of the Buriganga river in the heart of the Bengali delta and has an average elevation of 4m above sea level. It is one of the world's most populated cities with a population of 18.89 million people, a total area of 306.38 kilometer square and a population density of 46,997 per square kilometer square. Tropical vegetation and moist soils characterize the land, which is flat and close to sea level. This leaves Dhaka susceptible to flooding during the monsoon seasons owing to heavy rainfall and cyclones. The city has a distinct monsoon season, with an annual average temperature of 26 °C and monthly means varying between 19 °C in January and 29 °C in May. Approximately 87% of the annual average rainfall of 2,123 millimeters occurs between May and October.

### **2.4.2 Selection of Study Population**

To conduct a household survey for the detection of the magnitude of Chikungunya outbreak at Dhaka city in 2017 and its epidemiological perspectives 92 wards and 8 peri-urban areas of Dhaka North and South City Corporation was divided into 100 different cluster locations. 30 households were selected from each cluster for the collection of suspected Chikungunya blood samples where the number is determined using the probability proportional to population count. For each location, starting coordinates were generated in locations where the first positive larvae of *Aedes* mosquito was found by the entomological team. Selected locations were visited in random order by 10 epidemiological study teams of NIPSOM in 10 different spots. The samples were collected from all age groups who are being suspected to have Chikungunya according to proper clinical history and case definition from July 19 to July 28, 2017 during the peak of the

epidemic. A structured questionnaire was followed for data collection. From these blood samples collected by the epidemiological team, the convalescent cases (duration of fever >7 days) were selected according to the history of fever for Immunochromatographic test (ICT) to check for the presence of IgM and IgG antibodies. Among them specific individuals were chosen for the ELISA technique. A total of 1487 Blood samples were collected.

**Population size, N = 1487**

### **2.4.3 Study participants**

People living in the selected households were eligible to participate in the study if they had ongoing fever at the time of the study or if they were recovered from fever not more than three months ago along with other clinical manifestations characteristic of Chikungunya infection such as debilitating arthralgia or rash. All age groups were included in the study. Exclusion criteria included pregnant women, individuals with psychiatric disease, individuals with a readily identifiable focus of infection, such as otitis media, sinusitis, purulent pharyngitis, cellulites, urinary tract infection, dental abscess, septic arthritis, pneumonia or pelvic inflammatory disease and inability to give consent.

#### **Case definitions**

Probable chikungunya is defined as a patient meeting both the clinical (a characteristic triad of fever, rash and joint manifestations) and epidemiological criteria (residing or having visited epidemic areas). Confirmed acute chikungunya was defined as a positive PCR result.

Convalescent cases can be confirmed by the presence of anti-chikungunya IgM or IgG antibodies in blood serum.

## 2.4.4 Sample size determination

### a. Sample size determination for Immunochromatographic (ICT) test

Individuals with duration of fever >7 days from the onset of illness at the time of blood collection were considered as convalescent cases. Immunochromatographic (ICT) test was performed for these convalescent cases. A total of 1215 blood samples were identified as convalescent cases according to the definition.

**Sample size of ICT test = 1215**

### b. Sample size determination for Enzyme linked Immunosorbent Assay (ELISA) test

The following formula was applied for the determination of sample size for the ELISA test –

$$n = \frac{z^2 \times P \times (1-P)}{d^2}$$

Where, n= sample size

z = normal deviate for two-tailed alternative hypothesis at a level of significance

P = prevalence of event of interest for the study

d = precision or degree of accuracy or margin of error

Therefore, for 5% level of significance at 95% confidence interval the value of z = 1.96

$$\text{Prevalence, P of convalescent Chik IgM} = \frac{835}{1203} \times 10 = 70\%$$

No of positive cases of Chik IgM by ICT = 835

No of total convalescent cases for whom the ICT test was performed = 1201

d = 0.1

By inputting all the values in the previous formula the sample size for ELISA test is determined.

**Sample size for ELISA test = 81**

The prevalence value was determined from the Immunocromatographic test (ICT).

### **2.4.5 Sampling technique**

Specific individuals were selected for Enzyme linked Immunosorbent Assay (ELISA) test among convalescent cases through stratified random sampling technique. For gender based distribution the total population was divided into two strata - male and female.

For the determination of male and female stratum size following formula was used -

$$n_h = N_h / N \times n$$

where  $n_h$  = stratum sample size

$N$  = population size

$n$  = sample size

$$\text{Therefore, Male stratum sample size} = \frac{725}{1215} \times 81 = \mathbf{48}$$

$$\text{and Female stratum sample size} = \frac{490}{1215} \times 81 = \mathbf{33}$$

For age group distribution the total population was divided into five different age group strata. They are- (0-14 years), (15-29 years), (30-44 years), (45-59 years) and  $\geq 60$  years. Similar formula is utilized for the determination of sample size in each age group stratum.

Therefore, sample size for (0-14years) age group = **6**

Sample size for (15-29 years) age group = **24**

Sample size for (30-44 years) age group = **28**

Sample size for (45-59 years) age group = **17**

Sample size for ( $\geq 60$  years) age group = **6**

The 81 individuals were selected from a random number table to avoid biasness and ensure homogeneity.



## 2.4.6 Sample processing

### a. Collection, transport and Processing of blood specimens

Blood samples were collected, processed and preserved by the following method-

1. 3-5 ml blood sample was collected from antecubital vein of each suspected individual and labeled in the heparinized green screw capped vacutainer tube from febrile patients by taking all aseptic precautions. Duplicate label was put in the data collection form.
2. The blood containing tube is placed in the tube rack in upright position in the sample collection ice box and transferred to the laboratory as early as possible along with one copy of data collection form.
3. After arrival in the lab, the blood samples was checked for any hemolysis and other rejection criteria. Hemolyzed and improperly labeled samples was discarded.
4. The collected blood samples were divided into two aliquots. One aliquot of whole blood was kept at  $-70^{\circ}\text{C}$  in an eppendorf tube after proper labeling for further study. Serum is separated from other aliquot and kept at  $-20^{\circ}\text{C}$  freezer for ICT and ELISA test.

### b. Separation of serum from Whole Blood

Following the proper steps for Blood collection, serum was separated from the whole blood by the following steps

2. The whole blood in the vacutainer tube was directly transferred into a separate sterile eppendorf tube by using pipette and the tube was labeled with the sample ID.
3. Since Blood is collected at heparinized tubes, separation of clotting factors was not necessary.
5. Then the samples were centrifuged for 10 minutes at 1500g to get a supernatant which contains pure serum.
6. The clear serum was again collected in an appropriately labeled eppendorf tube with the sample ID.
7. Samples are refrigerated at  $4^{\circ}\text{C}$  immediately after collection. If it is not analyzed within 7 days, samples should be frozen at  $-80^{\circ}\text{C}$ . The same procedure was followed for every collected blood sample.



**Figure 2.1: Separation of serum of convalescent Chikungunya cases**

## **2.4.7 Laboratory procedures to be followed in achieving stated objectives:**

### **2.4.7.1 Immunochromatographic test (ICT)**

STANDARD Q Chikungunya IgM/IgG test kits of SD biosensor Inc., Korea was used for performing the immunochromatographic test (ICT). A number of trial and error method was applied among different rapid test kits such as OnSite Chikungunya IgM Combo rapid test and this kit was selected since results provided by other kits were not satisfactory.

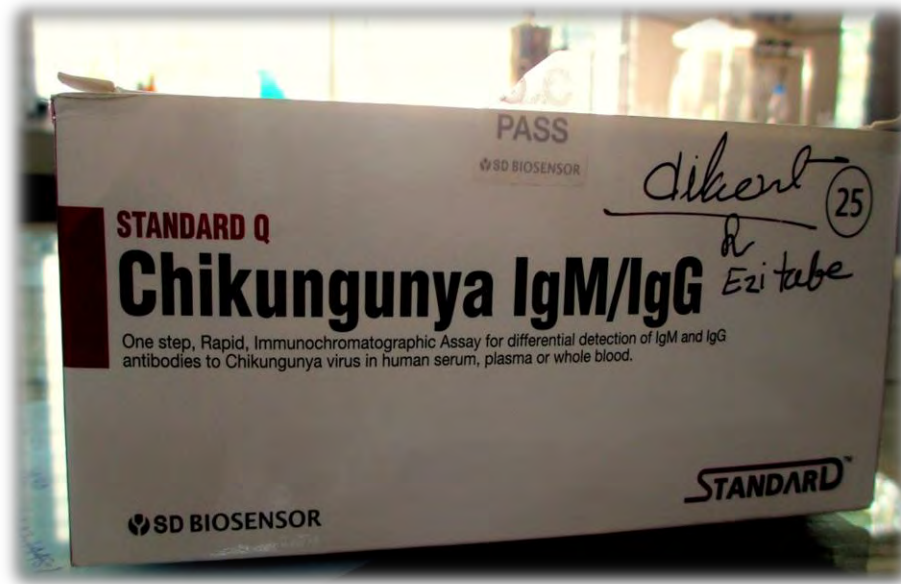


Figure 2.2: STANDARD Q Chikungunya IgM/IgG test kit

### a. Summary of the test kit

STANDARD Q Chikungunya IgM/IgG test is an immunochromatographic assay for the detection of IgM or IgG antibodies against Chikungunya virus in human serum, plasma or whole blood samples. This test kit is for *in vitro* use only and can be intended to use for initial screening. It is a qualitative test which only confirms the presence of anti Chikungunya IgM or IgG antibody in the suspected blood sample. This test kit is approved by European Commission and Food and drug administration (FDA).

### b. Test principle

STANDARD Q Chikungunya IgM/IgG Test Kit has “M”, “G” test lines for the detection of Anti Chikungunya IgM and IgG respectively and “C” control line. Monoclonal anti-human IgM and Monoclonal anti-human IgG are immobilized at two individual test lines respectively (M, G line) on the nitrocellulose membrane. The IgM line in the result window is closer to the sample well and followed by IgG line. Inactivated Chikungunya virus in the antigen pad and monoclonal anti-Chikungunya E1-gold in the conjugate pad is released when assay diluents are added and react with anti-Chikungunya IgM or IgG in the patient sample. If human anti-Chikungunya IgM or IgG exist in patient serum, the individual test line appear as visible bands respectively forming

the complex with anti-human IgM or IgG, human IgM or IgG, inactivated Chikungunya virus, and anti-Chikungunya E1-gold, which indicates a positive test results. The violet line at the control region should always appear if the assay is performed correctly.

### **c. Test Procedure**

#### **[Preparation before test]**

1. The instructions were read carefully for using the test strips
2. The expiry date at the back of the foil pouch was checked. The kit should not be used, if the expiry date has passed.
3. The foil pouch was opened, and the test device and silica gel pack in the foil pouch were checked.

#### **[Test Procedure]**

1. Using a SD Ezi tube, 10µl of serum was loaded to the black line of the SD Ezi tube+.
2. The collected serum sample was added to the sample well of the test device.
3. 3 drops (90µl) of assay diluent was added into the assay diluent well of the test device.
4. The test result was read after 15 minutes, the test can be read up to 30 minutes.

### **d. Warnings and Precautions**

1. The test kit should not be re-used.
2. The test kit should not be used if the pouch is damaged or the seal is broken.
3. The assay diluents of another lot should not be used.
5. Personal protective equipment, such as gloves and lab coats should be worn when handling kit reagents. Hands should be washed thoroughly after the tests are done.
6. Spills should be cleaned thoroughly using an appropriate disinfectant.
8. Established precautions against microbiological hazards should be followed throughout testing procedures.
9. All specimens and materials used to perform the test should be disposed as bio-hazard waste. Laboratory chemical and bio-hazard wastes must be handled and discarded in accordance with all local, state, and national regulations.

10. Silica gel in foil pouch is to absorb moisture and keep humidity from affecting products. If the moisture indicating silica gel beads change from yellow to green, the test device in the pouch should be discarded.

#### **e. Limitations of the test**

1. The test procedure, precautions and interpretation of results for this test must be followed strictly when testing.
2. This test detects the presence of anti-Chikungunya IgMs or IgGs in the specimen and should not be used as the sole criteria for the diagnosis of Chikungunya virus infection.
3. Test results must be considered with other clinical data available to the physician.
4. For more accuracy of immune status, additional follow-up testing using other laboratory methods is recommended.
5. Neither the quantitative value nor the rate of anti-Chikungunya IgM or IgG concentration can be determined by this qualitative test.
6. Failure to follow the test procedure and interpretation of test results may adversely affect test performance and/or produce invalid results.

#### **2.4.7.2 Enzyme linked Immunosorbent Assay (ELISA) test**

After the completion of ELISA test Enzyme linked Immunosorbent Assay (ELISA) test was performed for selected individuals through a sapling technique. STANDARD E Chikungunya IgM ELISA test kit of SD Biosensor Inc. was employed for performing the ELISA test.

#### **a. Summary of the test kit**

STANDARD E Chikungunya IgM ELISA is an enzyme linked immunosorbent assay for the detection of IgM antibodies against Chikungunya virus in human serum, plasma samples. This is *in vitro* diagnostic test kit for laboratory use. It is a qualitative test which confirms the presence

of anti Chikungunya IgM antibodies in the suspected blood samples. This diagnostic kit was already evaluated with 100% sensitivity and 97.3% specificity according to the manufacturer.



**Figure 2.3: STANDARD E Chikungunya IgM ELISA kit**

## **b. Test Principle**

STANDARD E Chikungunya IgM ELISA contains a microplate, which has been pre-coated with monoclonal anti-Human IgMs on well. During the first incubation with the microplate, IgMs in the patient sample are bound to the monoclonal anti-Human IgMs on well and then bind to chikungunya antigen combined with monoclonal anti-Chikungunya-HRP (horseradish peroxidase), forming “Double Sandwich”. After this incubation, all unbound materials are removed by aspiration and washing. The residual enzyme activity found in the wells will thus directly proportional to the anti-Chikungunya IgM concentration in patient sample and evidenced by incubating the solid phase with a substrate solution (TMB: tetramethylbenzidine) in a substrate buffer. Color development is indicative of the presence of anti-Chikungunya IgMs in the patient sample and the level of anti-Chikungunya IgM is directly proportional to the color

intensity of the sample. Colorimetric reading is performed by using a spectrometer or ELISA plate reader at 450nm. Colorless wells appear if anti-Chikungunya IgM is negative.

### c. Preparation of Reagents

1. **Diluted Wash buffer Preparation:** The 20X concentrated wash buffer was diluted 1 to 19 using distilled or deionized water before use.

2. **Reconstituted Chikungunya antigen Preparation:** 1ml of the distilled or deionized water was added per one vial of Chikungunya antigen which was previously lyophilized before use. After the lyophilized Chikungunya antigen has been reconstituted, it was aliquoted in small vials or tubes and stored at below  $-20^{\circ}\text{C}$ .

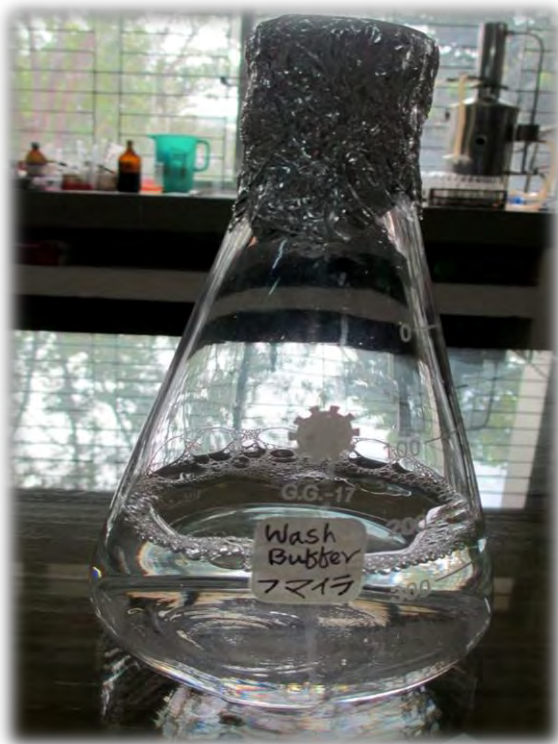


Figure 2.4.1: Diluted Wash Buffer



Figure 2.4.2: Reconstituted Chikungunya antigen

**Table 2.1: Reagent specification of STANDARD E Chikungunya IgM ELISA**

| Reagent                           | Storage              | Stability |
|-----------------------------------|----------------------|-----------|
| Diluted wash buffer               | Room Temp. (15-25°C) | 1 week    |
| Reconstituted Chikungunya antigen | Below -20°C          | 1 month   |

#### **d. Test Procedure**

All reagents was equilibrated at room temperature (15-25°C/59-77°F) and gently shaken before testing.

##### **1. Dilution of sample**

1) Using suitable test tubes or a microplate, the samples were diluted at 1:99 ratio with sample buffer and mixed well.

2) The mixture was incubated for at least 10 minutes at room temperature (15-25°C/59-77°F).

##### **2. Sample incubation**

1) 100µl of the **UNDILUTED** positive control was transferred for 2 wells and 100µl of the **UNDILUTED** negative control for 3 wells, and 100µl of diluted patient samples into each well.

2) The plate was covered with an adhesive plate sealer.

3) The plate was incubated for 37± 1°C/ 96.8-100.4°F for 1 hour.

##### **3. Antigen dilution in working enzyme conjugate**

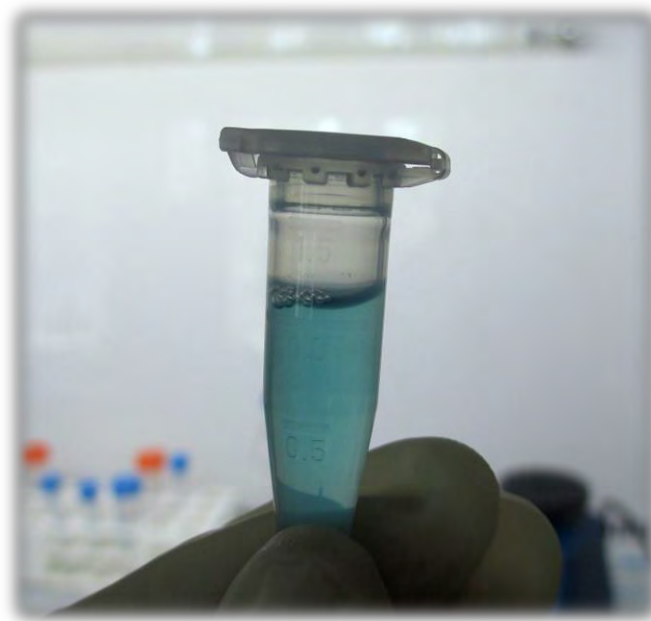
1) 101X enzyme conjugate and diluted conjugate diluent (e.g., 101X enzyme conjugate 100µl : conjugate diluent 10ml) were mixed at 1:100 ratio to make the working conjugate (Refer to the Table 1).

2) Dissolved antigen and working conjugate are mixed at 1:10.1 ratio after vortexing, and incubated for 1 hour at room temperature.



**Table 2.2: Conjugate and Antigen mixture preparation**

| Number of strips | Conjugate Diluent | 101x Enzyme conjugate | Reconstituted Chikungunya antigen |
|------------------|-------------------|-----------------------|-----------------------------------|
| 1                | 1ml               | 10 $\mu$ l            | 0.1ml                             |
| 2                | 2ml               | 20 $\mu$ l            | 0.2ml                             |
| 3                | 3ml               | 30 $\mu$ l            | 0.3ml                             |
| 4                | 4ml               | 40 $\mu$ l            | 0.4ml                             |
| 5                | 5ml               | 50 $\mu$ l            | 0.5ml                             |
| 6                | 6ml               | 60 $\mu$ l            | 0.6ml                             |
| 7                | 7ml               | 70 $\mu$ l            | 0.7ml                             |
| 8                | 8ml               | 80 $\mu$ l            | 0.8ml                             |
| 9                | 9ml               | 90 $\mu$ l            | 0.9ml                             |
| 10               | 10ml              | 100 $\mu$ l           | 1.0ml                             |
| 11               | 11ml              | 110 $\mu$ l           | 1.1ml                             |
| 12               | 12ml              | 120 $\mu$ l           | 1.2ml                             |



**Figure 2.5: Enzyme-Conjugate and Ag mixture preparation**

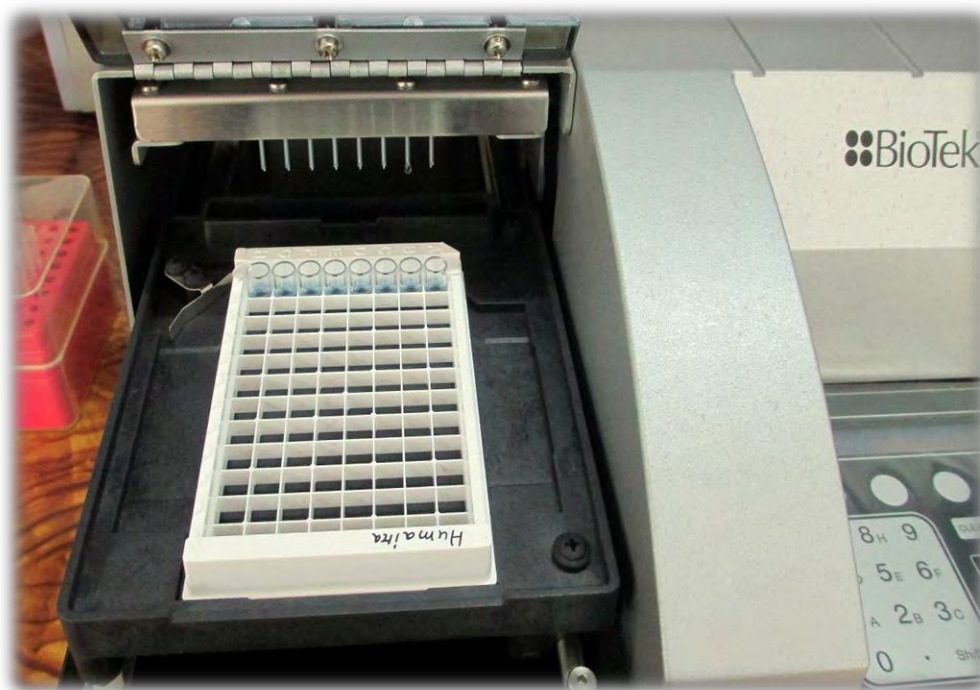
#### 4. Washing procedure

1) The wells were washed five times with 350 $\mu$ l of diluted wash buffer and aspirate all liquid were aspirated from the wells. Automated plate washer of Biotek, USA was utilized for this step.

2) The wash buffer was left in each well for 4-5 seconds per washing cycle and then the wells were emptied.

3) After washing, all liquid were thoroughly disposed from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

**Precaution:** Residual liquid in the reagent wells after washing can interfere with the substrate and lead to false low extinction values. Insufficient washing (e.g., less wash cycles, too small wash buffer volumes, or too short residence times) can lead to false high extinction values.



**Figure 2.6: Automatic washer in operation**

#### 5. Enzyme conjugate mixture incubation

1) Hundred  $\mu$ l of enzyme conjugate mixture was added into each of the wells.

2) The plate was covered with an adhesive plate sealer.

3) The plate was incubated at  $37 \pm 1^\circ\text{C}$  /  $96.8\text{-}100.4^\circ\text{F}$  for 30 minutes.

## **6. Secondary washing**

1) The wells were washed five times with 350µl of diluted wash buffer following the same washing procedure.

## **7. TMB substrate incubation**

1) 100µl of TMB substrate was added into each of the wells.

2) They were incubated for 15 minutes at room temperature (15-25°C/59-77°F) in the dark.

## **8. Stopping the reaction**

1) 100µl of stop solution was added into each of the wells in the same order and at approximately same speed as the TMB substrate.

2) They were mixed by gentle shaking.

## **9. Measurement**

1) The absorbance values of the wells were read at 450nm in an ELISA plate reader (with reference wavelength between 620nm and 650nm) right after from the end of assay, within 30 minutes. ELISA plate reader of Biotek, USA was utilized for plate reading. Gen 5 2.08 software was utilized for obtaining OD values of individual test samples.

## **g. Limitation of the test**

1. The test procedure, precautions and interpretation of results sections for this test kit must be followed closely when testing.

2. Testing could be performed on patients with clinical symptoms on when exposure is suspected.

3. Samples:

1) Heat-inactivation samples (30 minutes at 56°C/133°F) do not impair the test.

2) Anticoagulants such as heparin, EDTA or sodium citrate do not affect the test.

3) Hemolyzed or contaminated samples may give erroneous results. Blood corpuscle in samples may also give nonspecific reaction.

4. Failure to add specimen in the procedure could cause false negative result. Repeat testing should be considered where there is clinical suspicion of infection.
5. Test results must be considered with other clinical data available to the physician.
6. For more accuracy of immune status, additional follow-up testing using other laboratory methods is recommended.

### **Statistical analysis**

Statistical analyses are performed based on the primary data collected by the Epidemiology department of National Institute of Preventive and Social Medicine. All the statistical analyses was done in SPSS version 16. Descriptive statistics showed the relevant data about seroprevalence, age and gender distribution among the seropositive convalescent Chikungunya blood samples. Pearson Chi-square test was performed to detect the association between gender and age with seropositivity. In addition binary logistic regression analysis was carried out to find out the relation between age and seroprevalence. A p value of  $<0.05$  at 95% confidence interval was considered statistically significant.

# *Chapter 3*

## *Results*

# Results

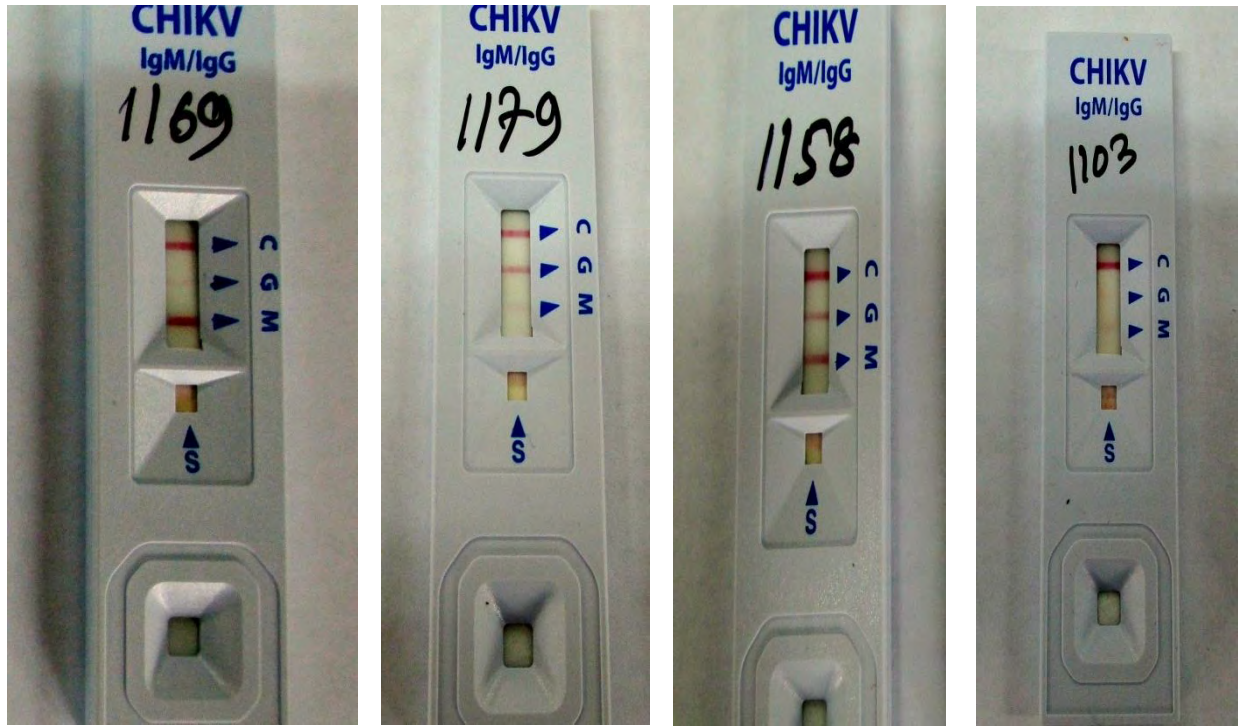
## 3.1 Result of Immunochromatographic (ICT) test

After the collection and processing of total 1487 Blood samples from selected households, they were divided into two disease criteria - Acute and Convalescent cases. Serum was separated and ICT test was performed for convalescent cases per criteria and the test results were recorded. The positive result of ICT test appear as characteristic colored bands beside specific test lines within the result window of the test strip. Absence of colored bands on the test lines indicated negative result. (Figure 1)

### Interpretation of the test results

1. **Negative result:** Only band (“C” Control line) within the result window indicates a negative result.
2. **IgM positive result:** Two colored bands (“C” Control line and “M” Test line) within the result window indicate Chikungunya IgM positive.
3. **IgG positive result:** Two colored bands (“C” Control line and “G” Test line) within the result window indicate Chikungunya IgG positive.
4. **IgM & IgG positive result:** Three colored bands (“C” Control line, “M” Test line, and “G” Test line) within the result window indicate Chikungunya IgM & IgG positive.
5. **Invalid result:** If the control band (“C” Control line) is not visible within the result window, the result is considered invalid. The directions may not have been followed correctly or the test may have deteriorated.

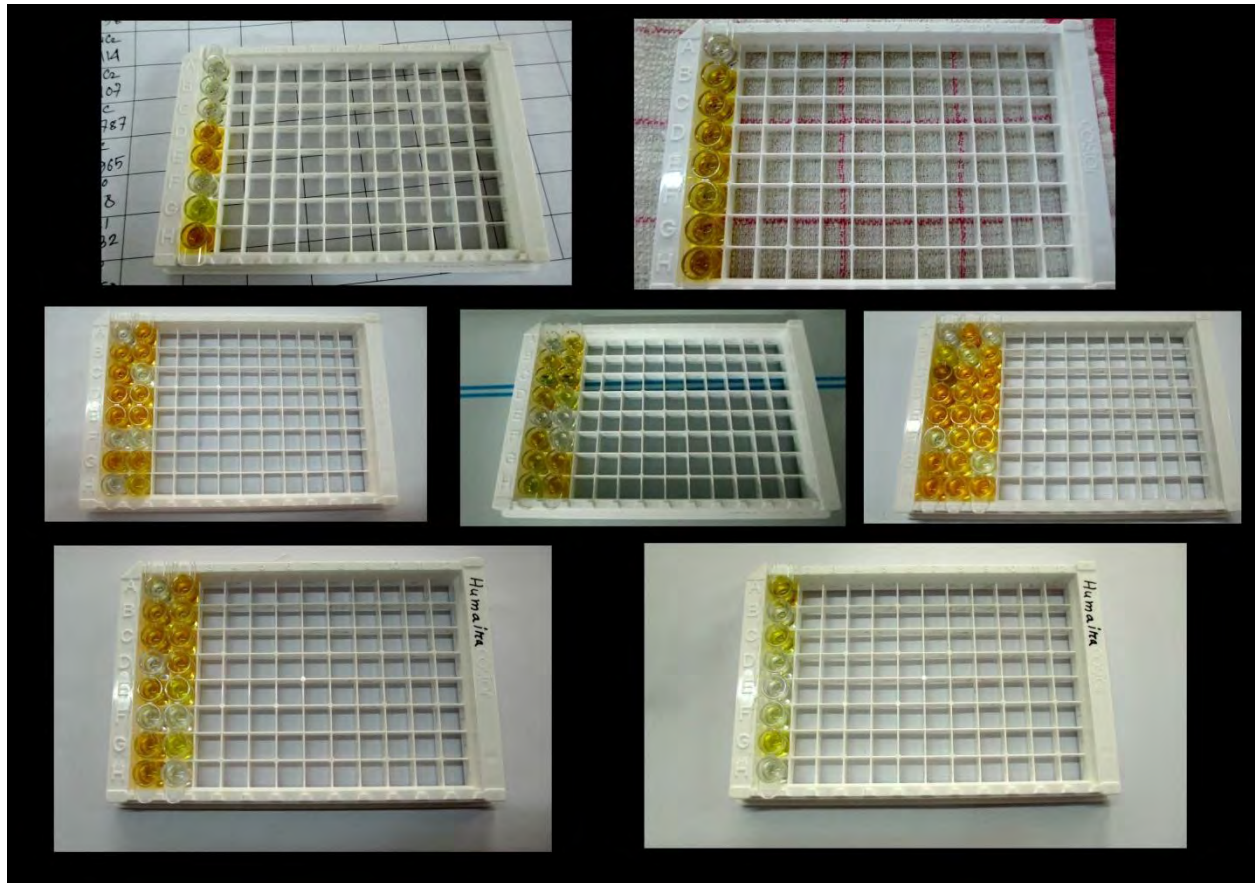
The conclusion of the test results were drawn for each sample according to this interpretation.



**Figure 3.1: Typical banding profile of the rapid diagnostic Immunochromatographic (ICT) test for the detection of anti Chikungunya IgM or IgG antibodies in the patient samples.** The red colored band along their specific lines indicates a positive result where appearance of only band on the control line indicates a negative result. From the left the first picture shows two red colored bands on the "C" control line and "M" test line which confirms the presence of IgM antibody. In the second picture, two red colored bands appeared on the "C" control line and "G" test line which confirms the presence of IgG antibody. The third picture shows three colored bands on the "C" control line, "M" test line and "G" test line within the result window indicating the confirmation of the presence of both IgM and IgG antibodies. Finally the fourth picture shows only one colored band on the "C" control line which indicates the absence of either antibody and a negative result.

### 3.2 Result of Enzyme linked Immunosorbent Assay (ELISA) test

After the completion of Immunochromatographic test for all convalescent blood samples, Enzyme linked Immunosorbent Assay (ELISA) test was performed for specific individuals who were selected through stratified random sampling technique and the test results were recorded. The absorbance values of each sample on specific wells were taken in an ELISA plate reader at 450nm wavelength.



**Figure 3.2: Reaction of color development in wells of microtitre plates where individual samples were loaded in the Enzyme linked Immunosorbent Assay (ELISA) test. A change of yellow coloration from blue after adding the stop solution is indicative of positive result which confirms the presence of anti-Chikungunya IgM in the sample. the level of anti-Chikungunya IgM is directly proportional to the color intensity of the sample. Absence of color development indicates a negative result.**



## Interpretation of test results

1. The mean absorbance of the triplicates of the negative controls were calculated and 0.400 was added with the value. This is the Cut-off value.

\*Cut-off value = Mean absorbance of Negative control + 0.400

$$\begin{aligned}\text{Mean absorbance of Negative control} &= \frac{0.098+0.109+0.098}{3} \\ &= 0.102\end{aligned}$$

$$\text{Cut-off Value} = 0.400+0.102 = \mathbf{0.502}$$

2. An index value can be calculated by dividing the sample absorbance by the Cut-off value (calculated in step 1)

$$\text{Index Value} = \frac{\text{Absorbance of Sample}}{\text{Cut-off Value}}$$

Example: Sample 1 Absorbance = 0.092

Sample 2 Absorbance = 0.678

$$\text{Sample 1 Index Value} = 0.092/0.502 = 0.183 \text{ (NEG)}$$

$$\text{Sample 2 Index Value} = 0.678/0.502 = 1.351 \text{ (POS)}$$

**Table 1: Interpretation of Index values found in the ELISA test**

| Index value                  | Result    | Interpretation   |
|------------------------------|-----------|--|
| Index value < 0.8            | Negative  | No detectable chikungunya IgM antibody. The result does not rule out chikungunya infection. An additional sample should be tested if early infection is suspected. Other chikungunya assays should be performed to rule out acute infection. |
| $0.8 \leq$ Index value < 1.1 | Equivocal | The sample should be re-tested. If the samples remain equivocal, an alternative method or another sample should be tested.   |
| Index value $\geq$ 1.1       | Positive  | Presence of detectable IgM antibodies. Other chikungunya assays should be performed to confirm chikungunya infection.  |

The conclusion of the test results were drawn for each sample according to these interpretations.

### 3.3 Evaluation of ICT test

The Immunochromatographic test is a rapid serological diagnostic test to identify Chikungunya infection. However evaluation of this technique was performed to determine its reliability as a test that can be utilized for mass screening. For the determination of the performance characteristics of ICT test, ELISA test is employed as the reference test since it is considered as the gold standard for any kind of serological diagnosis.

#### a. Sensitivity and specificity of ICT test

**Sensitivity** is the ability of a test to correctly identify those with the disease (true positive rate), whereas **specificity** is the ability of the test to correctly identify those without the disease (true negative rate).

For the detection of sensitivity and specificity of ICT the following truth table can be constructed

**Table 2 : Truth table for detecting Sensitivity, specificity, PPV and NPV of ICT test**

|              |          | ELISA test    |               | Total Result |
|--------------|----------|---------------|---------------|--------------|
|              |          | True Positive | True Negative |              |
| ICT          | Positive | 50            | 0             | 50           |
|              | Negative | 10            | 21            | 31           |
| Total Result |          | 60            | 21            | 81           |

The following formula can be used for determining the sensitivity of a test.

$$\text{Sensitivity: } A/(A+C) \times 100$$

where A = true positive

C = false negative

$$\text{Therefore sensitivity of ICT test} = 50/(50+10) \times 100$$

$$= 83\%$$

The following formula can be used for determining the specificity of a test.

$$\text{Specificity: } D/(D+B) \times 100$$

where B = false positive

D = true negative

**Therefore specificity of ICT test =  $21/(21+0) \times 10$**

$$= 100\%$$

### **b. Positive predictive value and Negative predictive value of ICT test**

**Positive predictive value (PPV)** is the probability that subjects with a **positive** screening test truly have the disease. **Negative predictive value (NPV)** is the probability that subjects with a negative screening test truly do not have the disease. *Positive and negative predictive values are influenced by the prevalence of disease in the population that is being tested.* If the test is performed in a high prevalence setting, it is more likely that persons who test positive truly have disease than if the test is performed in a population with low prevalence.

The following formula can be used for determining PPV

$$\text{Positive Predictive Value: } A/(A+B) \times 100$$

**Therefore PPV of ICT test =  $50/(50+0) \times 100$**

$$= 100\%$$

The following formula can be used for determining NPV

$$\text{Negative Predictive Value: } D/(D+C) \times 100$$

**Therefore NPV of ICT test =  $21/(21+10) \times 100$**

$$= 67.7\%$$

Table 3: Evaluation of ICT test

| ICT test    |             |      |       |
|-------------|-------------|------|-------|
| Sensitivity | Specificity | PPV  | NPV   |
| 83%         | 100%        | 100% | 67.7% |

**Table 4 : Comparison of ICT and ELISA in terms of time**

| Type of test | Time       |
|--------------|------------|
| ELISA        | 3 hours    |
| ICT          | 20 minutes |

**Table 5: Comparison of ICT and ELISA in terms of cost**

| Type of test | Cost      |
|--------------|-----------|
| ELISA        | Expensive |
| ICT          | Cheap     |

**Table 6: Comparison of ICT and ELISA in terms of methodology**

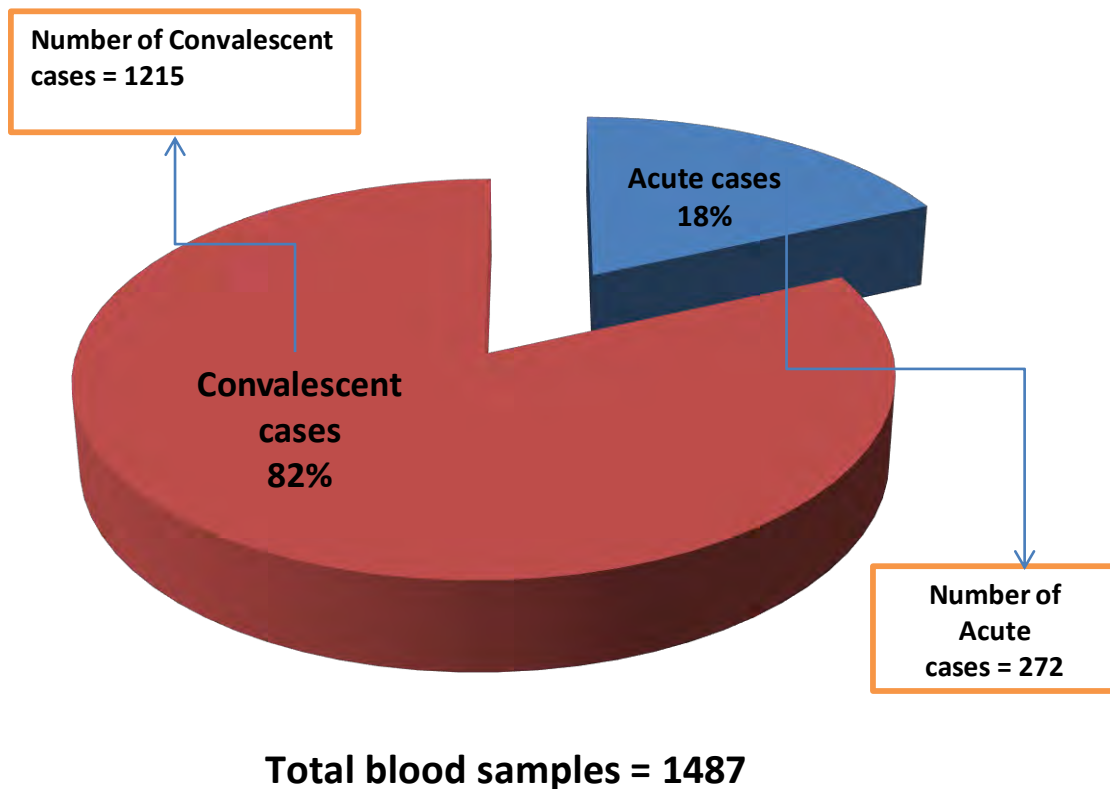
| Type of test | Methodology |
|--------------|-------------|
| ELISA        | Complex     |
| ICT          | Simple      |

The sensitivity and specificity of the rapid test was evaluated as 83% and 100% respectively. It gives the clear indication that the rapid test will not declare any healthy person as diseased but it will miss some positive cases (17%). Therefore we can verdict that ICT test is an efficient diagnostic procedure which provides significantly fast, cost effective and easy system to identify Chikungunya infection by confirming the presence of IgM or IgG antibodies and enables supportive definitive diagnosis of Chikungunya. However the sensitivity of the test is not satisfactory enough to use it solely as a confirmatory test.

### 3.4 Statistical Presentation of Laboratory findings

A total of 1487 blood samples were collected from households of hundred different cluster locations for individuals who falls into the case definition and inclusion criteria and gave consent to participate in the study.

#### Acute and convalescent Chikungunya cases



**Figure 3.3: Distribution of collected blood samples of Chikungunya cases**

Among the total 1487 collected blood sample of suspected cases, the number of Convalescent cases are 1215 and number of acute cases are 272. Therefore, convalescent cases constitute much bigger portion of the total samples. Convalescent cases are individuals whose onset of fever is >7 days during the period of blood collection and acute cases are defined as cases whose onset of fever is <7 days at the time of blood collection.

### 3.4.1 Statistical presentation of ICT test performed on Convalescent cases

The convalescent blood samples according to the test definition criteria were chosen for this study. ICT test was performed for a total of 1201 patient samples where the minor portion was omitted due to sample rejection criteria such as hemolyzation of blood samples or improperly labeled samples. The following bar and column charts shows interpreted results and age and gender distribution of seropositive cases for convalescent cases.

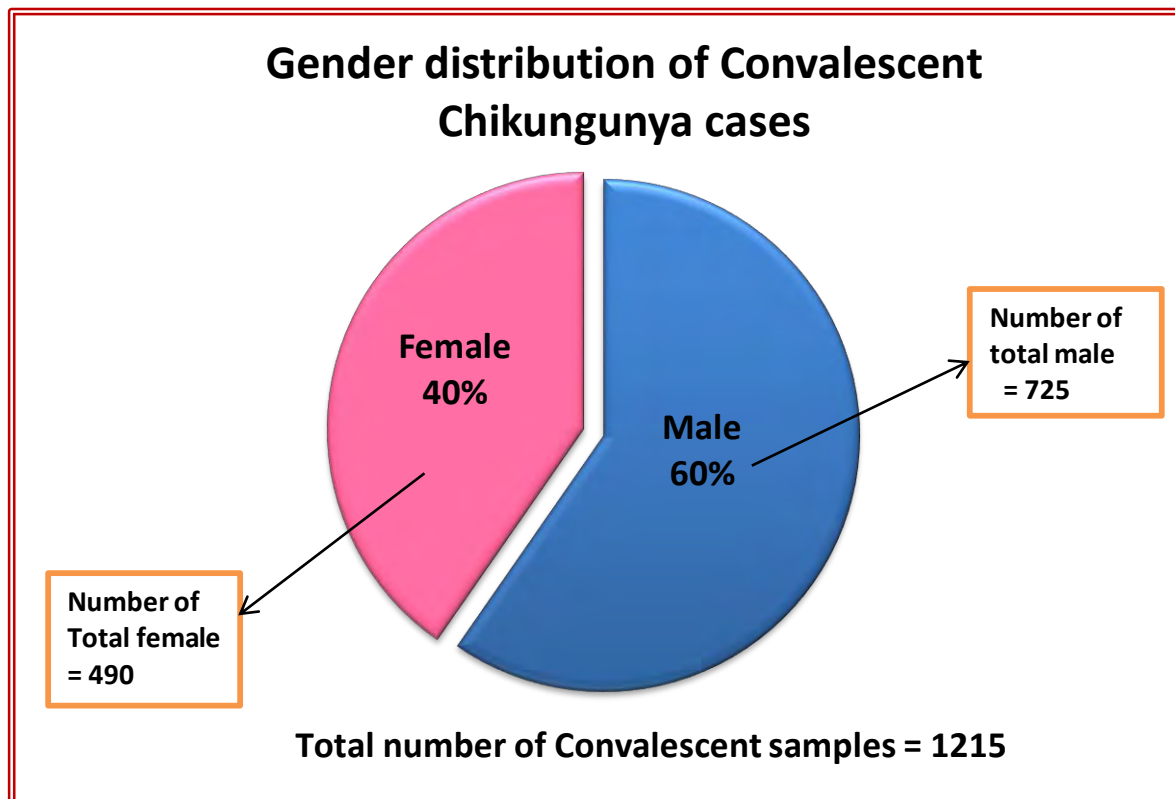
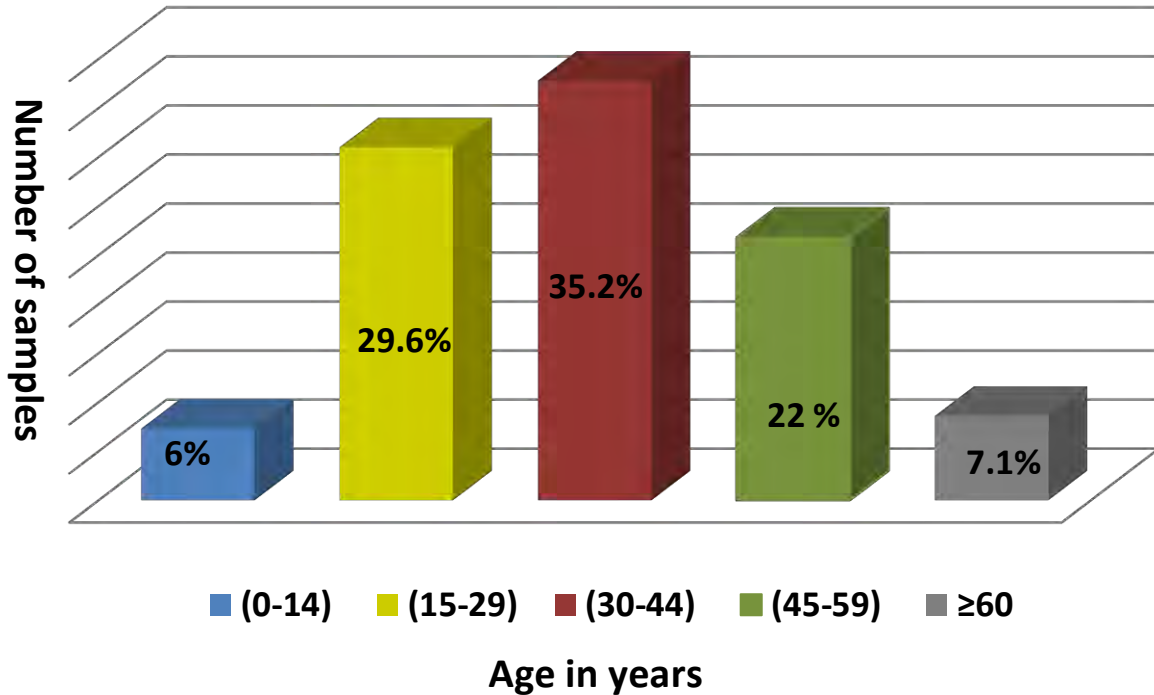


Figure 3.4: Gender distribution of convalescent Chikungunya cases.

Considering the convalescent Chikungunya cases, the number of total male is 725 and the number of total female is 490. Therefore the percentage of male participants was bigger (60%) than the percentage of female participants (40%). It can be concluded that more male persons participated in the study than female.

### Age distribution of Convalescent Chikungunya cases

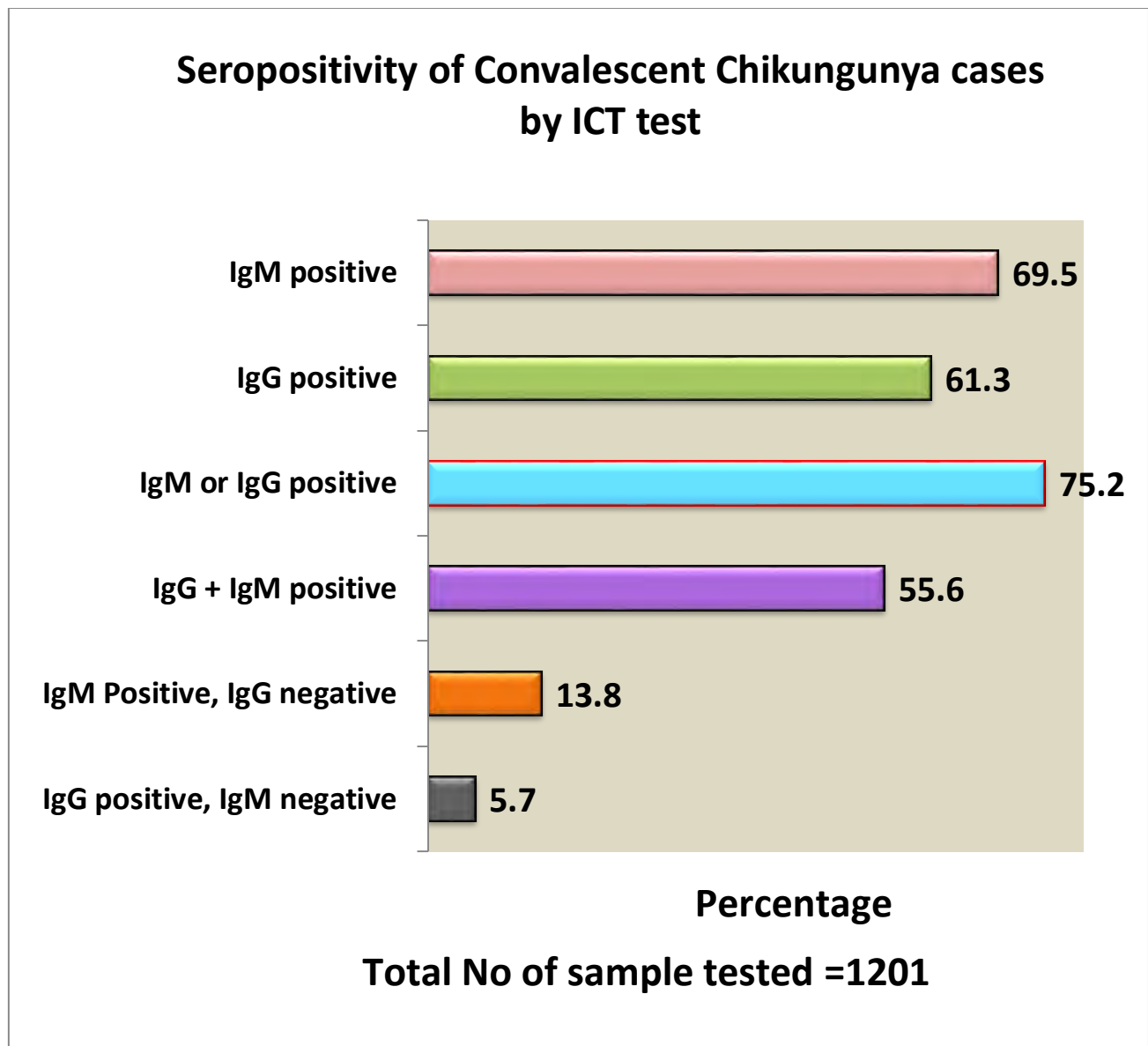


Total number of convalescent samples = 1215

Figure 3.5: Age distribution of Convalescent Chikungunya cases.

Total numbers of convalescent samples were divided into five age groups which are (0-14) years, (15-29) years, (30-44) years, (45-59) years and  $\geq 60$  years. This column chart represents the age distribution of convalescent cases. Participants were distributed across all ages, however most of the suspected blood samples were collected from the (30-44) years age group (35.2%) and a lesser portion was collected from children (6%) and elderly people (7.1%). The median age was calculated as 36 years.



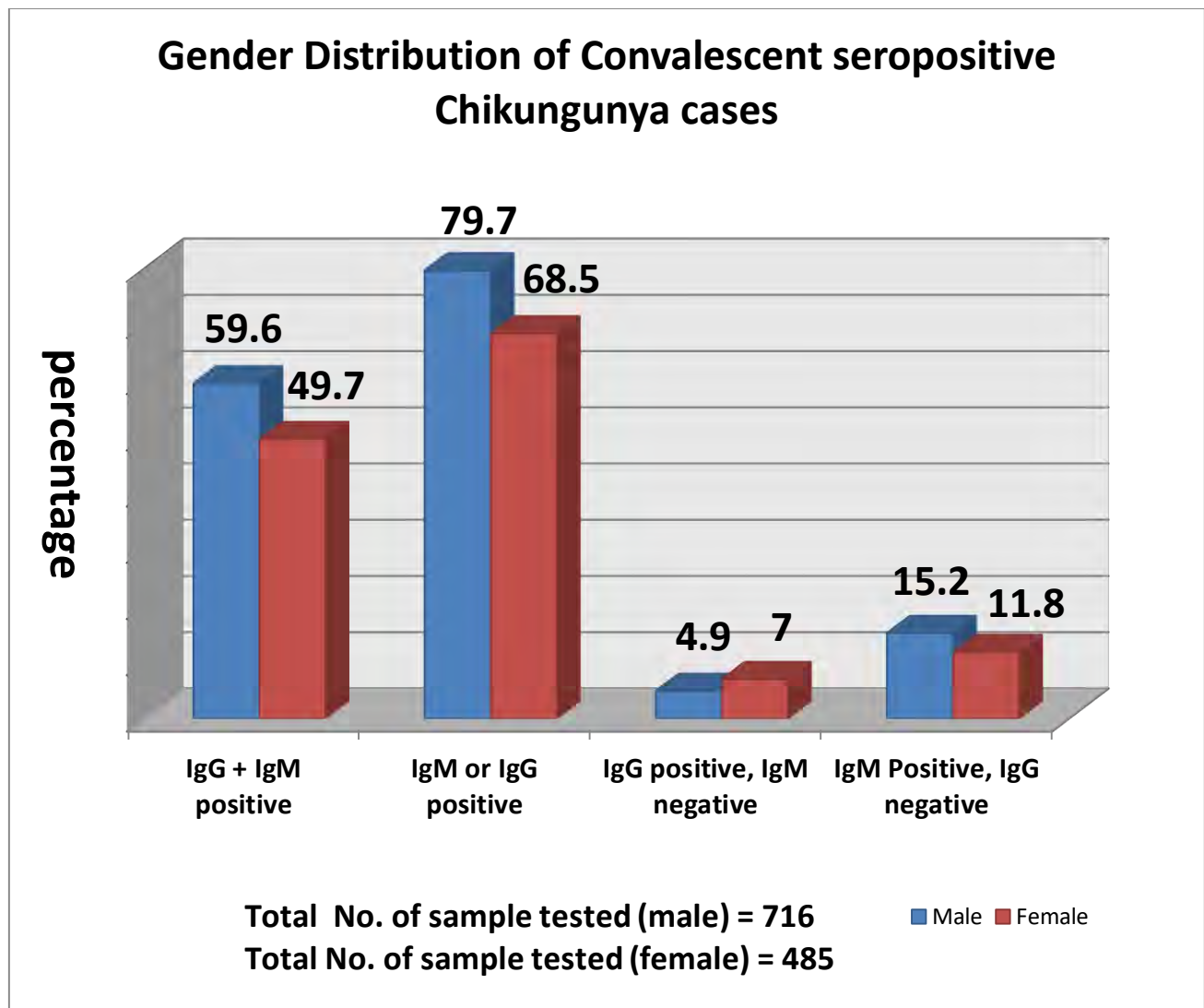


**Figure 3.6: Seropositivity of Chikungunya Convalescent cases by ICT test.**

This bar chart represents the seropositivity of Chikungunya convalescent cases according to ICT test. The percentage of people having active Chikungunya infection is 13.8% who had only IgM antibody in their blood. The seroprevalence of Convalescent Chikungunya cases is 75% which is determined by the presence of at least one antibody (IgM or IgG) in the blood sample. The percentage of people having past infection is 61.3% who had only IgG in their blood. The percentage of people protected from secondary infection is 55.6% who had both IgM and IgG in their blood.

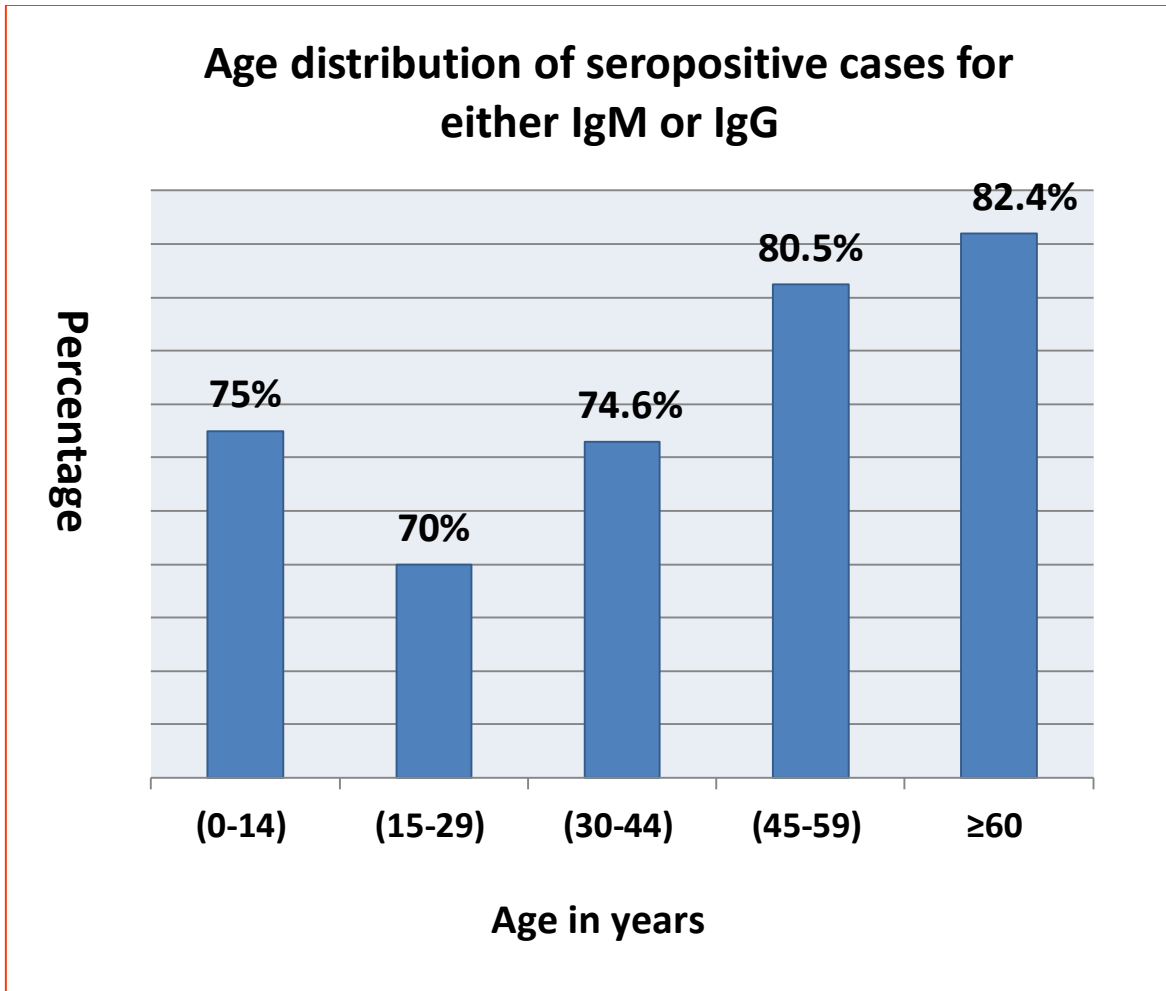
Table 7: Interpretation of Convalescent Seropositive cases by ICT test

| Type of Antibody           | No of positive cases | Percentage | Interpretation                    |
|----------------------------|----------------------|------------|-----------------------------------|
| IgM                        | 835                  | 69.5       | IgM seroprevalence                |
| IgG                        | 737                  | 61.3       | IgG seroprevalence                |
| IgM or IgG                 | 903                  | 75.2       | <b>Overall seroprevalence</b>     |
| IgG + IgM                  | 668                  | 55.6       | Protected for secondary infection |
| IgM Positive, IgG negative | 166                  | 13.8       | Active infection                  |
| IgG positive, IgM negative | 69                   | 5.7        | Past infection                    |



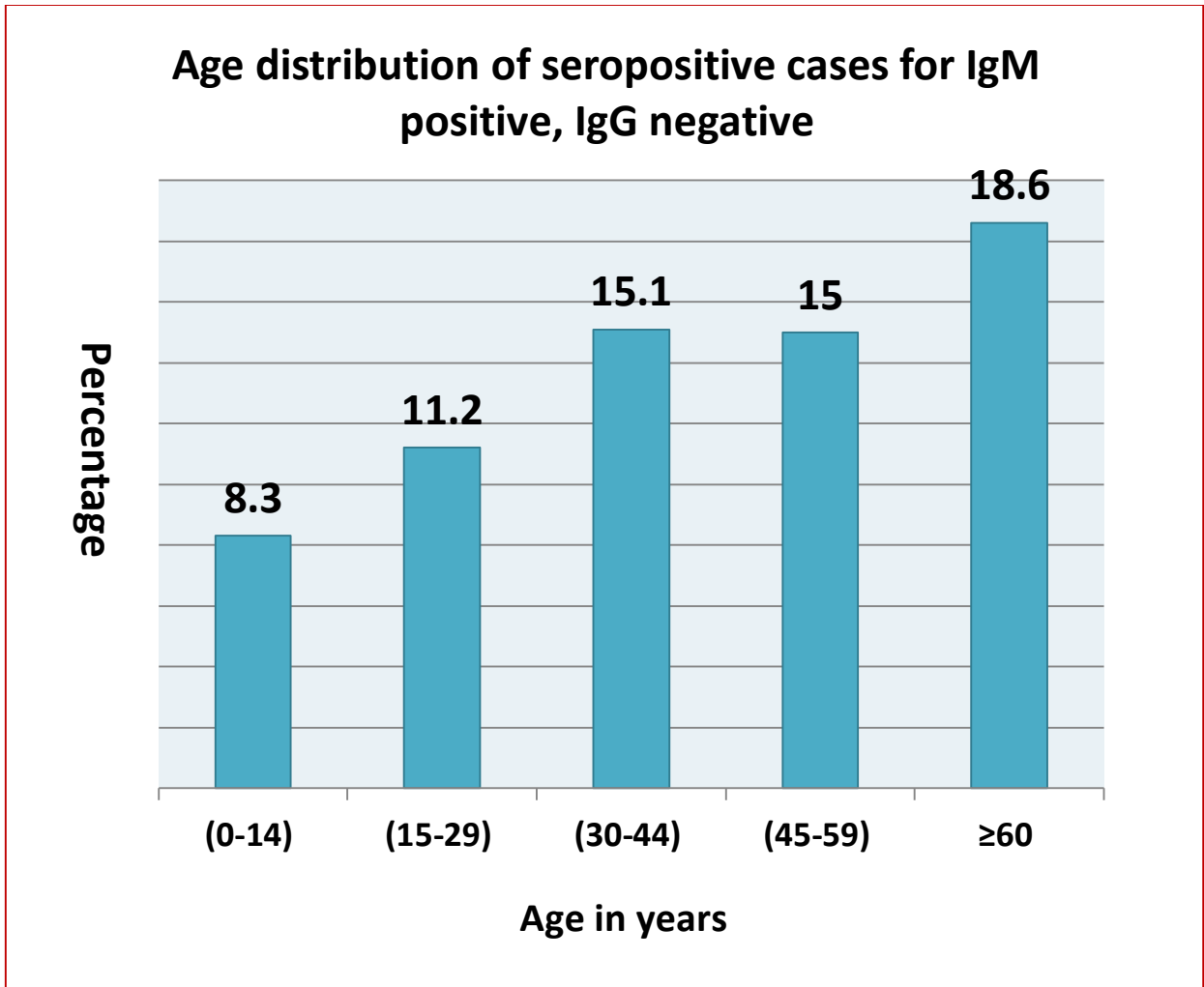
**Figure 3.7: Gender distribution of Convalescent Seropositive Chikungunya cases.**

This side-by-side column chart represents the gender distribution of seropositive convalescent Chikungunya cases. The seroprevalence rate for male and female are 79.7% and 68.5% respectively. Therefore, it is apparent that the seroprevalence rate of male is greater than female. The rate of people having active infection is also high in male (15.2%). However more female persons are having past infection (7%) than male persons (4.9%).



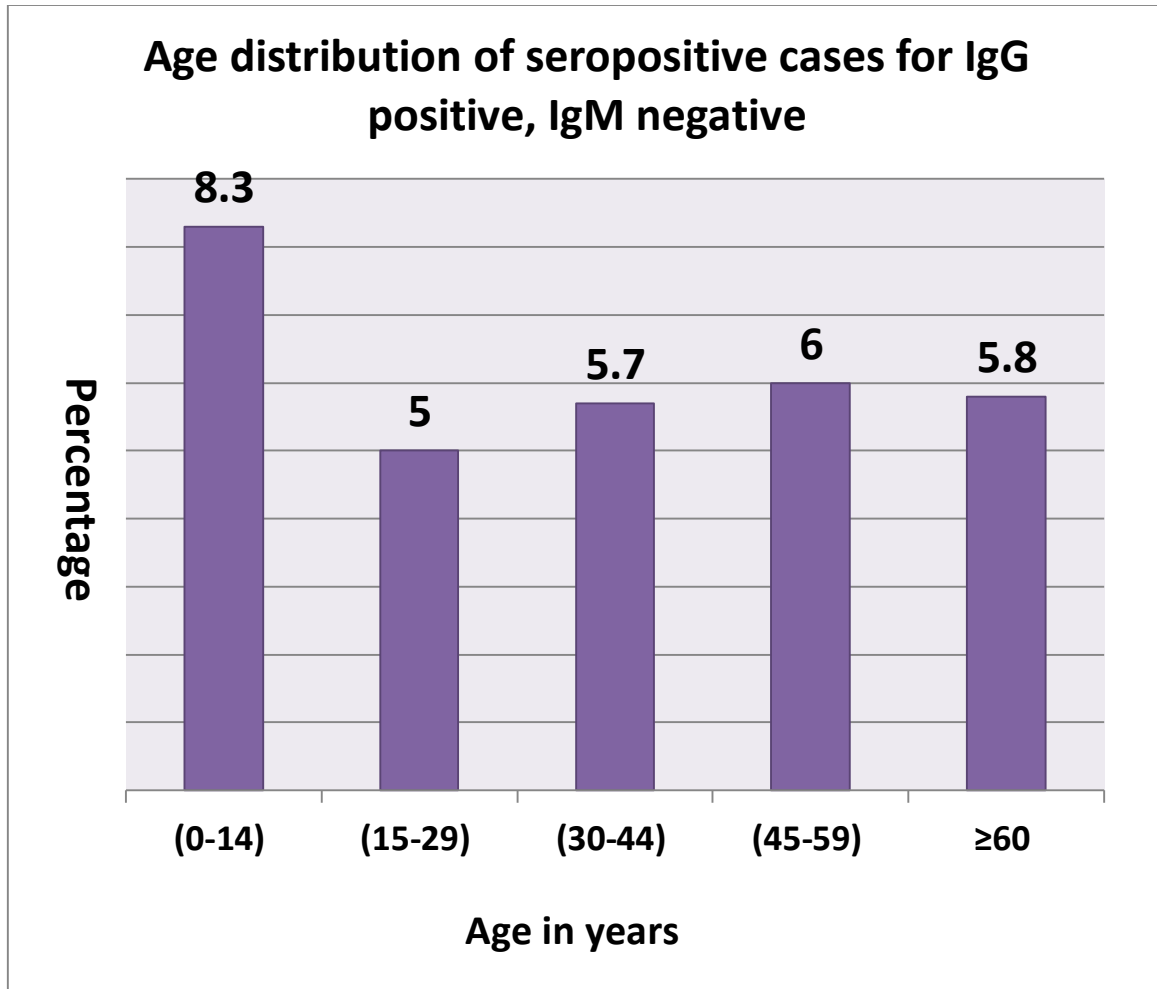
**Figure 3.8: Age distribution of seropositive cases for either IgM or IgG.**

This column chart represents the age distribution of seropositive cases who have at least IgM or IgG in their blood. Here it is apparent that the age group that has maximum presentation of either antibody (IgM or IgG) is  $\geq 60$  years (82.4%) and the age group with least seropositive cases is (15-29) years (70%). Therefore, it can be said that, the seroprevalence rate is much higher in older people than younger age groups.



**Figure 3.9: Age distribution of seropositive cases for IgM positive, IgG negative.**

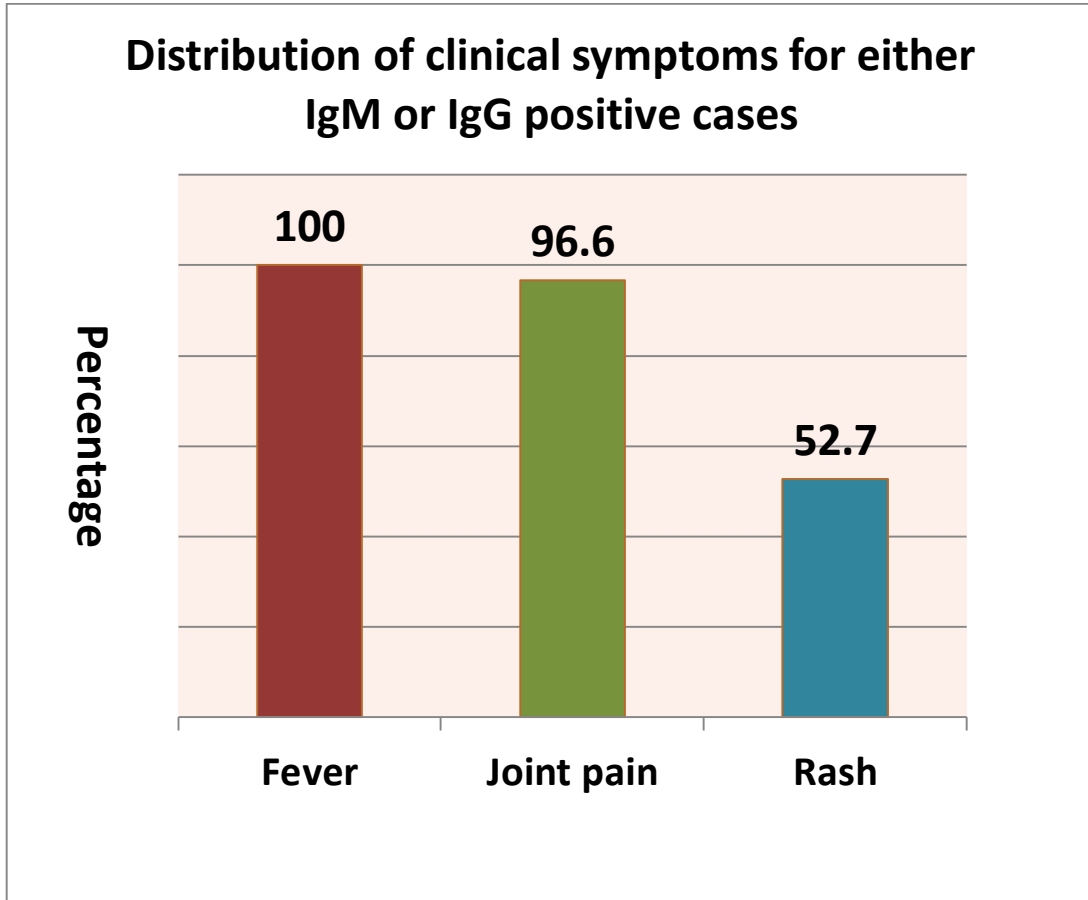
This column chart represents the age distribution of seropositive cases who have only IgM antibody in their blood. Here it is apparent that the age group that has maximum presentation of only IgM antibody is  $\geq 60$  years. Therefore, older people also had more active infection (18.6%) than the other age groups.



**Figure 3.10: Age distribution of seropositive cases for IgG positive, IgM negative**

This column chart represents the age distribution of seropositive cases who have only IgG antibody in their blood. Here it is apparent that the age group that has maximum presentation of only IgG antibody is (0-14) years. Therefore, younger age group (0-14 years) had more past infection (8.3%) than other age groups.

## Clinical manifestations of Seropositive cases of Convalescent samples

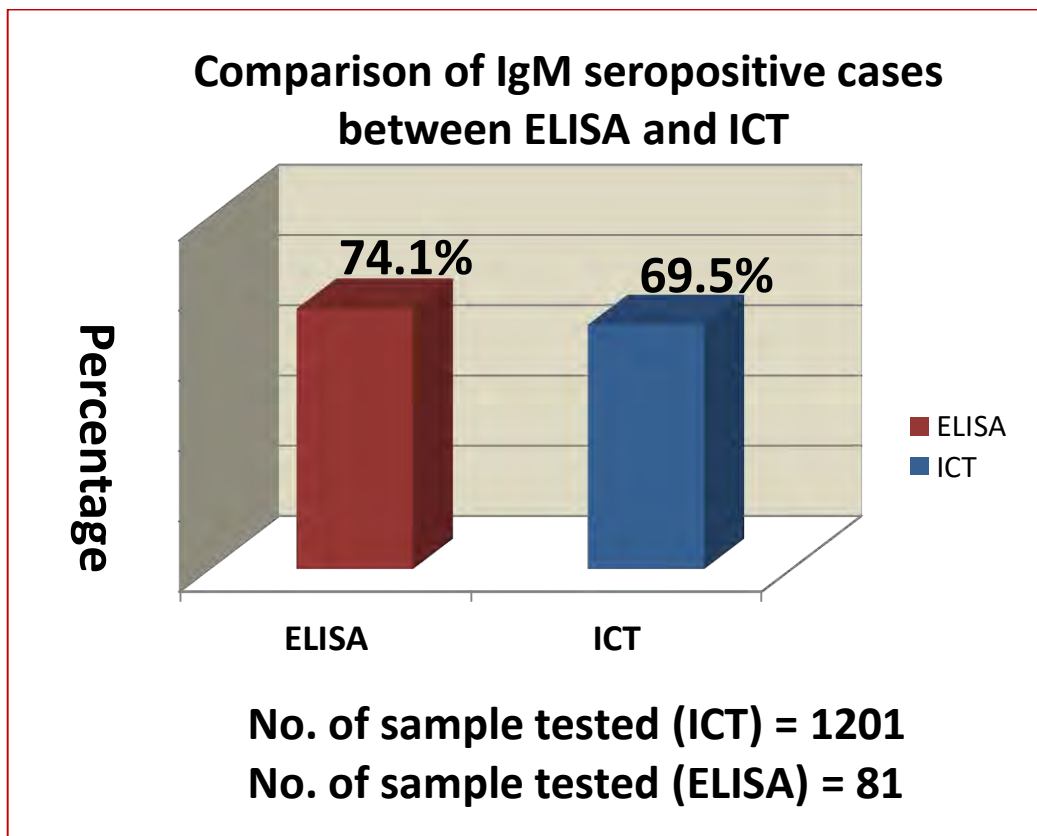


**Figure 3.11: Distribution of Clinical symptoms for either IgM or IgG seropositive cases**

This column chart represents the distribution of clinical symptoms for either IgM or IgG seropositive cases in Convalescent patient samples. Here, It is observed that all the individuals with IgM antibody in their blood (n= 803) had fever, about 96.6% had joint pain and 52.7% had rash along with fever.

### 3.4.2 Statistical presentation for the comparison of ELISA and ICT test

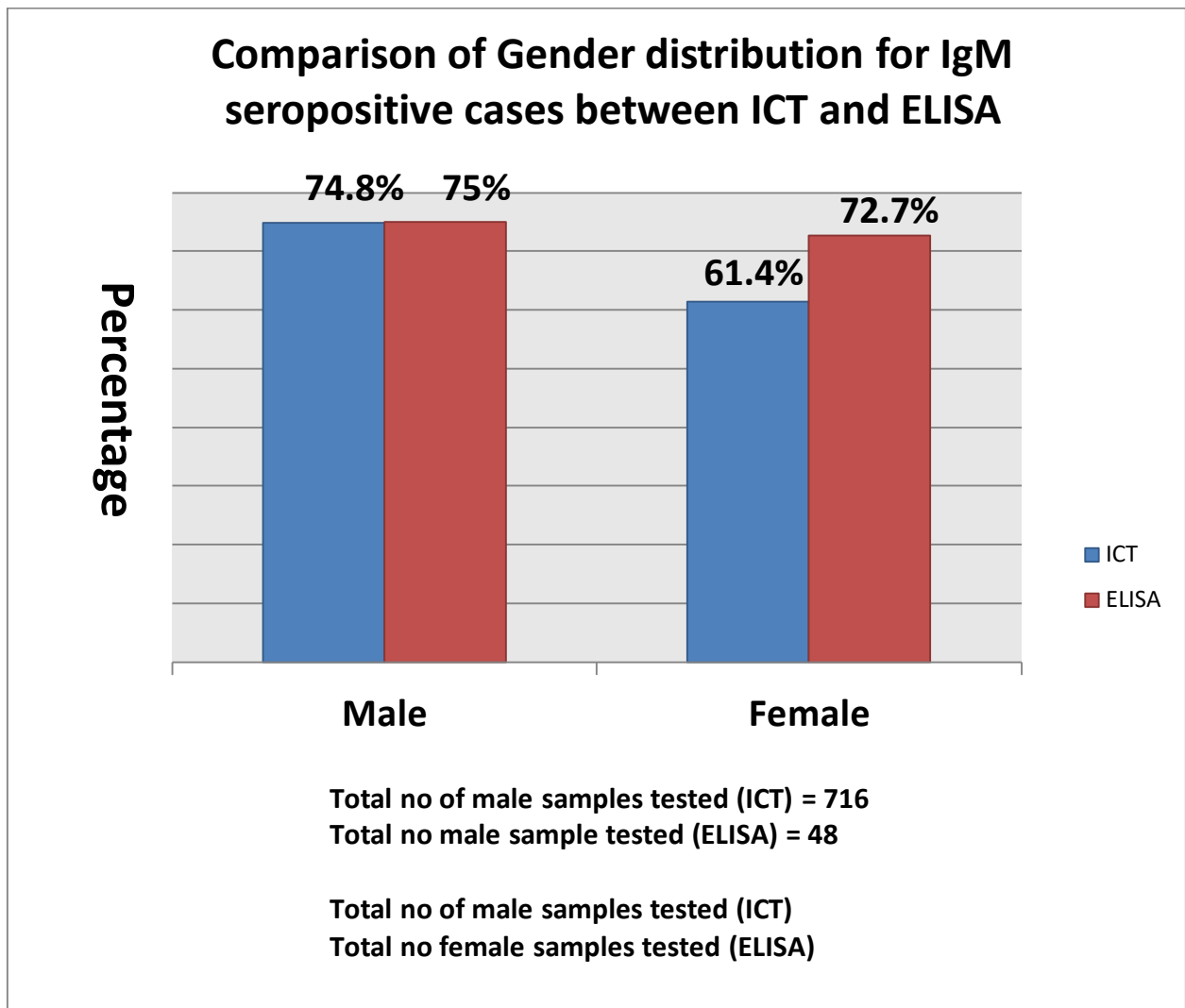
After the completion of performing ICT test in total 1215 convalescent patient blood samples, ELISA test was performed for 81 individuals who were selected through age stratified random sampling technique. The following column charts shows the comparison between ELISA and ICT test for different variables in the study.



**Figure 3.12: Comparison of IgM seropositive cases between ELISA and ICT test**

This column chart shows the comparison of seropositive cases of IgM antibody between ELISA and ICT test. The percentage of IgM seropositive cases by ELISA is slightly greater (74.1%) than the percentage of ICT IgM seropositive cases (69.5%).

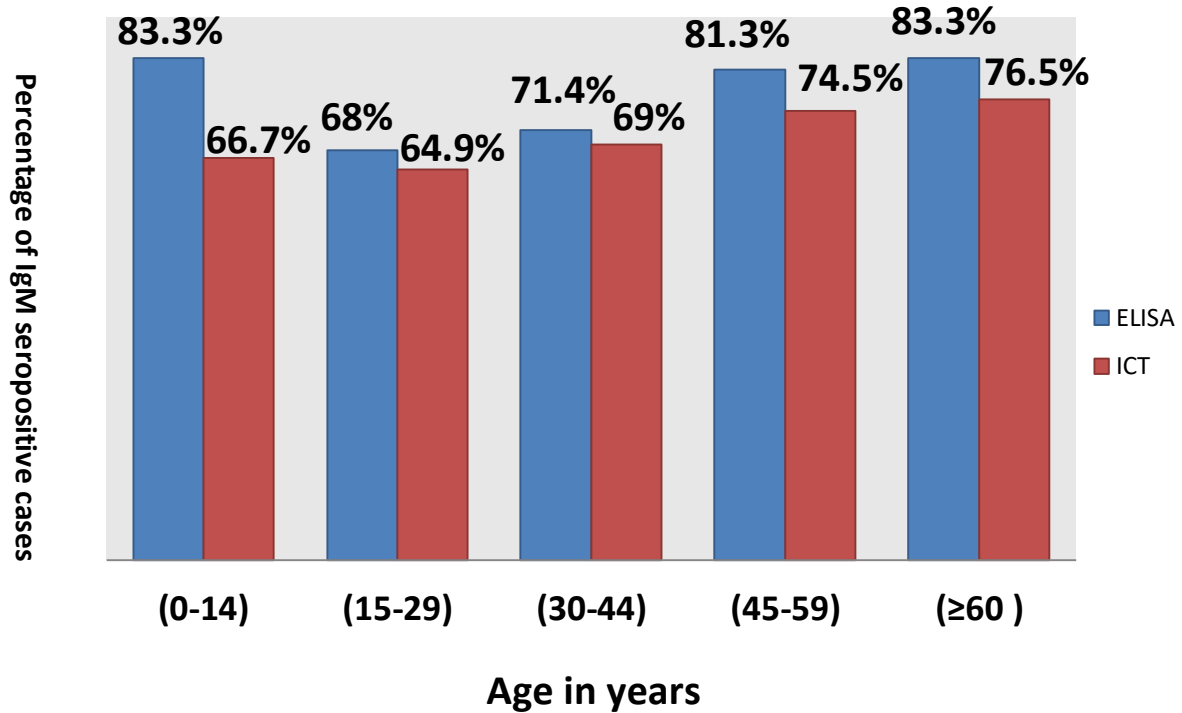




**Figure 3.13: Comparison of gender distribution for IgM seropositive cases between ICT and ELISA test**

This column chart shows the comparison of gender distribution for IgM seropositive cases between ICT and ELISA test. Male persons almost show no differences in IgM seropositivity between the two serological tests whereas the IgM seropositivity is slightly greater for female persons in the ELISA test (72.7%) than the ICT test (61.4%). The difference between male and female seropositivity rate is much smaller in ELISA test (2.1%) than the ICT test (13.4%).

### Comparison of Age distribution for IgM seropositive cases between ICT and ELISA



**Figure 3.14: Comparison of age distribution for IgM seropositive cases between ICT and ELISA test**

This side-by-side column chart represents the comparison of age distribution for IgM seropositive cases between ELISA test and ICT. Here it is apparent that the age group (0-14) years showed greater difference (16.6%) in IgM seropositivity between the two tests.

### 3.5 Determination of risk factors for CHIKV through Inferential Statistics

In order to find out whether there is any association between gender and seroprevalence, chi-square for independence test was performed in SPSS. A **chi-square test for independence** compares two variables in a contingency table to see if they are related. The chi-squared statistic is a single number that tells how much difference exists between observed counts and the counts one would expect if there were no relationship at all between the variables.

The formula for the chi-square statistic used in the chi square test is:

$$\chi_c^2 = \sum \frac{(O_i - E_i)^2}{E_i}$$

Here, O = Observed value of the variable

E = Expected value of considering no association between the variables.

Another factor to consider is the degrees of freedom which is the number of values in the final calculation of a statistic that are free to vary. It can be calculated by the following formula -

$$\mathbf{DF = Number\ of\ categories - 1}$$

The test was carried out for both IgM and IgG seroprevalence found by ICT test.

## Association between Gender and IgM seroprevalence

The following hypothesis was developed to conduct the test -

Null hypothesis:  $H_0$  = Gender and IgM seroprevalence are independent.

Alternative hypothesis:  $H_1$  = Gender and IgM seroprevalence are dependent.

**Table 8.1: Contingency table for Gender and IgM Seroprevalence**

|                           |                | IgM seroprevalence ICT |          | Total |
|---------------------------|----------------|------------------------|----------|-------|
|                           |                | Positive               | Negative |       |
| Male                      | Observed count | 531                    | 185      | 716   |
|                           | Expected count | 498                    | 218      |       |
| Female                    | Observed count | 304                    | 181      | 485   |
|                           | Expected count | 148                    | 337      |       |
| Total<br>(Observed count) |                | 835                    | 366      | 1201  |

**Table 8.2: Chi square result for Gender and IgM Seroprevalence**

|                    | Value  | Degrees of freedom | P value |
|--------------------|--------|--------------------|---------|
| Pearson Chi square | 17.990 | 1                  | 0.000   |

The p value determined from the test is 0.000 which is smaller than  $\alpha = 0.05$  at 95% confidence interval. The significance level  $\alpha$  is the probability of making the wrong decision when the null hypothesis is true. A p value of  $\leq 0.05$  is considered statistically significant here.

Therefore, it can be concluded that there is a significant association between gender and IgM seroprevalence.

## Association between Gender and IgG seroprevalence

The following hypothesis was developed to conduct the test -

Null hypothesis:  $H_0$  = Gender and IgG seroprevalence are independent.

Alternative hypothesis:  $H_1$  = Gender and IgG seroprevalence are dependent.

**Table 9.1: Contingency table for Gender and IgM Seroprevalence**

|                        |                | IgG seroprevalence ICT |          | Total |
|------------------------|----------------|------------------------|----------|-------|
|                        |                | Positive               | Negative |       |
| Male                   | Observed count | 458                    | 258      | 716   |
|                        | Expected count | 440                    | 276      |       |
| Female                 | Observed count | 280                    | 205      | 485   |
|                        | Expected count | 298                    | 187      |       |
| Total (Observed count) |                | 738                    | 463      | 1201  |

**Table 9.2: Chi square result for Gender and IgM Seroprevalence**

|                    | Value | Degrees of freedom | P value |
|--------------------|-------|--------------------|---------|
| Pearson Chi square | 4.744 | 1                  | 0.029   |

The p value determined from the test is 0.029 which is smaller than  $\alpha = 0.05$  at 95% confidence interval. Therefore, it can be concluded that there is a significant association between gender and IgG seroprevalence.

Overall, considering the chi-square test results for both IgM and IgG, it can be inferred that gender can be a risk factor for Chikungunya infection in this study.

## Association between Age and seroprevalence

In order to find out whether there is any association between gender and seroprevalence, Binary logistic regression was performed in SPSS. A binomial logistic regression (often referred to simply as logistic regression), predicts the probability that an observation falls into one of two categories of a dichotomous dependent variable based on one or more independent variables that can be either continuous or categorical.

Here in this particular case,

Age = continuous independent variable

Seroprevalence = dependent variable with two outcomes - either positive or negative.

**Table 10: Binary logistic regression result to determine the association between age and seroprevalence**

| Variable | Degrees of freedom | P-value | Exp(B) | 95.0% C.I. for EXP(B) |       |
|----------|--------------------|---------|--------|-----------------------|-------|
|          |                    |         |        | Lower                 | Upper |
| Age      | 1                  | 0.001   | 1.015  | 1.006                 | 1.024 |

The p value determined from the test is 0.001 which is smaller than  $\alpha = 0.05$  at 95% confidence interval. Therefore, it can be concluded that there is a significant association between age and Chikungunya seroprevalence.

# *Chapter 4*

## *Discussion*

## Discussion

Bangladesh recently faced its first time massive outbreak on Chikungunya particularly in the capital city Dhaka during the rainy season in 2017 where a large portion of the population was affected and a significant damage was inflicted on the lives of people. Chikungunya is a mosquito-borne viral disease that can cause debilitating arthralgia where Chikungunya virus (CHIKV) act as the aetiological agent. In this year's outbreak the total number of confirmed Chikungunya cases rose to over 750 between April and late July according to Bangladesh Government's Institute of Epidemiology, Disease control and Research (IEDCR). In addition, they also reported that 23 wards in Dhaka were considered high risk areas for contracting the virus. The infection was peaked at the month of Jun and July and started to subside after September. The reasons for this massive outbreak could be torrential rains and subsequent flooding and landslides that affect Bangladesh on a yearly basis during the rainy season which spans from April to October, leaving widespread pools of stagnant water where mosquitoes breed, thereby increasing the risk of mosquito-borne diseases. Since the disease is quite novel in this area except some minor cases found in the year of 2008 and 2011, there is a lack of sufficient data to properly manage the infection and apply protective measures.

The clinical similarity of CHIKV to other arbovirus infections makes diagnosis difficult on the basis of clinical symptoms alone, particularly as arboviruses such as dengue virus and more recent Zika virus now co-circulate in the same geographical regions and are transmitted by the same mosquito species (Kuno et al., 2015; Powers et al., 2010; Sadaho et al., 2015). Expansion and endemicity of CHIKV is likely, and large outbreaks of CHIKV infection may continue for the foreseeable future. Therefore, laboratory confirmation along with clinical manifestation is essential to accurately diagnose Chikungunya infection. Public health and hospital diagnostic laboratories throughout Bangladesh will need to build and maintain high-volume diagnostic testing capacity to manage epidemics that can appear further in the future.



Moreover, they will need validated and reliable commercial CHIKV diagnostic assays to respond to these increased diagnostic testing responsibilities.

To determine the magnitude of infection and the extent of transmission of the outbreak that occurred in Dhaka city, a total of 1487 suspected blood samples were collected from households of hundred different cluster locations in the Dhaka city by the Epidemiological team of National Institute of Preventive and Social Medicine (NIPSOM) according to case definition and clinical history. This study was conducted on the convalescent cases of these collected blood samples to highlight the importance of serological diagnostic procedures to confirm the suspected cases that can be implemented at the time of epidemic. Convalescent cases are defined as cases whose date of onset is >7 days at the time of blood collection (WHO, 2009). It aims to evaluate the immunochromatographic test (ICT) which is a rapid test to detect the presence of anti Chikungunya IgM or IgG antibody by taking the ELISA as the reference method and to see whether it can be established as a reliable technique for mass screening in the context of developing countries like Bangladesh where resource facilities are poor. In addition, a comparative statistical analysis of the seropositive cases for both ICT and ELISA test was also performed.

Immuno-chromatographic (ICT) test was performed on almost all the cases (n = 1201) where some cases are discarded as they fall into the rejection criteria such as hemolyzed and improperly labeled samples. STANDARD Q Chikungunya IgM/IgG test kits of SD biosensor Inc., Korea was used for performing the immunochromatographic test (ICT). After the completion of Immunochromatographic test for all convalescent blood samples, Enzyme linked Immunosorbent Assay (ELISA) test was performed for a total of 81 specific individuals who were selected through stratified random sampling technique and the test results were recorded. STANDARD E Chikungunya IgM ELISA (SD Biosensor, Korea) was applied for performing the ELISA test.

Out of the 1201 (N = 1201) suspected convalescent cases who provided blood samples the number of total male is 725 (60%) and the number of total female is 490 (40%). It can be concluded that more male persons participated in the study than female. Total number of

convalescent samples were divided into five age groups which are (0-14) years, (15-29) years, (30-44) years, (45-59) years and  $\geq 60$  years. The age distribution was as follows - 6% (73/1201) individuals at (0-14) years, 29.6 % (360/1201) individuals at (15-29) years, 35.2% (428/1201) people at (30-44) years, 22% (268/1201) individuals at (45-59) years and 7.1% (86/1201) individuals at  $\geq 60$  years age group. It is apparent that most of the suspected blood samples were collected from the (30-44) years age group and a lesser portion was collected from children and elderly people.

According to the ICT test, 31.8 % (166/1201) suspected cases had only anti Chikungunya IgM antibodies in their blood serum where the percentage of people having isolated IgG antibody is 5.7% (69/1201). 75.1% (903/1201) people have either IgM or IgG antibodies where the percentage of people presenting both the IgM and IgG antibodies are 55.6% (668/1201). Therefore, the seroprevalence of Convalescent Chikungunya cases is 75% which is determined by the presence of at least one antibody (IgM or IgG) in the blood sample. The percentage of people having active Chikungunya infection is 13.8% who have only IgM antibody in their blood. In addition, the percentage of people having past infection is 61.3% which can be detected by the presence of only IgG in the blood. The average days since onset of illness at the time of blood collection was detected as 36 days where most of the suspected individuals acquired infection in the Month of June (n= 629). Considering this value, the results correspond with the evidence that IgG appears only after two or three days of appearance of IgM and IgM lasts in the blood for a long period. It can be concluded that most of the individuals acquired CHIKV infection recently during the time of epidemic and developed IgM and IgG antibodies in response to the virus in their blood. The low percentage of isolated IgG shows that the outbreak occurred in a naive population where no herd immunity against this disease is yet achieved.

These results are consistent with a cross-sectional study that was conducted on Lamu Island of Kenya in a similar situation by Sergon et al., (2008) where 75% (215/288) have either IgM or IgG antibodies and 72% have IgG antibodies in the patient sera. However, the percentage of the presence of either IgM or IgG antibodies against Chikungunya was slightly lower in other studies that were conducted at Grande Comore Island and Western Cameroon by Sergon et al., (2007) and Demanou et al., (2010) respectively. 63% either IgM or IgG antibodies and 60% IgM

antibodies were reported at Grande Comore Island where the percentage of detectable IgM antibody was 51.4% at Western Cameroon.

The seroprevalence of the convalescent samples in this study by the ICT test was detected as 75% which is determined by the presence of at least one antibody (IgM or IgG) in the blood sample. This high prevalence is similar to the seroprevalence of CHIKV at Kerala (68%) on the neighbor country India after a large outbreak of CHIKV in 2007 detected by Kumar and his colleagues, (2011). Additionally, a previous serosurvey that was conducted in Bangladesh in Char Kushai village by Khatun et al. (2015) reported a high seropositivity of 80%. In contrast, inconsistent results have been found in other regions such as Emilia-Romagna Region of North-eastern Italy, Indian Ocean island of Mayotte, Managua of Nicaragua, the Caribbean island of St. Martin. and Brazil where the seropositivity rates were reported as 10.2%, 37.2%, 13.1%, 16.9% and 20% respectively (Moro et al., 2010; Sissoko et al., 2008; Kuan et al., 2016; Gay et al., 2016; Cunha et al., 2017).

The differences in seroprevalence rates between studies could be due to multiple factors. The higher seroprevalence rate reported in this study could be attributed to the exposure of the population to the infective bites of the day biting of *Aedes* mosquitoes, the vector which is responsible for transmitting the disease. Climatic conditions and environmental settings played an important factor causing the outbreak of CHIKV since the unusual heavy rainfall that occurred this year starting from mid April to September facilitates the favorable conditions required for the multiplication and activity of the vector. Vector competence can also contribute to the high rate of prevalence due to the difference in biology, ecology and capability to transmit the virus successfully to the susceptible host is different between different species. Additionally, unpreparedness of the public health authorities and other management systems and inadequate mosquito control activities failed to handle the outbreak properly. On a different note, the interval between the time the serosurvey was conducted and the peak of outbreak could have affected the seropositivity rates reported in different cross-sectional studies.

In order to find out the gender distribution of seropositive cases for convalescent Chikungunya cases, gender specific seroprevalence rates were also calculated. The seroprevalence rate which

is determined by the presence of either IgM or IgG in the blood for male and female are 79.7% and 68.5% respectively. Therefore, it is apparent that the seroprevalence rate of male is greater than female. The rate of people having active infection is also high in male (15% compared to 11% in female). However more female persons (7%) are having past infection than male persons (5%). To understand, whether this difference in male and female persons is statistically significant, chi-square test is performed. The p value determined from the test is 0.000 and 0.029 for IgM and IgG which is smaller than  $\alpha = 0.05$  at 95% confidence interval. Therefore it can be concluded that there is a significant association between gender and seropositivity where the prevalence of antibody is higher in males than females.

From age-specific distribution it was found that the age group that has maximum presentation of either antibody (IgM or IgG) is  $\geq 60$  years and the age group with least seropositive cases is (15-29) years. I gave the clear evidence that seroprevalence rate is much higher in older people than younger people. Binary logistic Regression test was performed to determine whether there is any significant association between age and seropositivity. The p-value determined from the test is 0.001 which is smaller than  $\alpha = 0.05$  at 95% confidence interval. Therefore it can be decided that there is a significant association between age and seropositivity where the prevalence of antibody is increased with age.

These findings closely matches with several other studies conducted in Emilia-Romagna Region of North-eastern Italy, Indian Ocean island of Mayotte and Kerala state of India who reported a higher Chikungunya infection rate in men compared to women and significant increase of seropositivity in older people (Moro et al., 2010; Sissoko et al., 2008; Kumar et al., 2011). On the other hand, studies conducted by Sergon et al., (2007) and Kawle et al., (2017) reported greater seropositivity in female than male in regions of Grande Comore island and Chandrapur district of India. Studies carried out in Malaysia and Thailand found no significant association between age with seropositivity and gender with seropositivity respectively. (Azami et al., 2013; Vongpunsawad et al., 2107).

The inconsistency of these findings among different studies might be related to specific

behaviors, work places and community habits which differs from places to places. According to this study female and older people are more prone to CHIKN infection than others. The likely reason is that different age groups and gender may have different attitudes and behavioral patterns towards the disease such as staying outdoors for a longer time or taking less precautions that causes higher exposure to *Aedes mosquito* bites and possible infection. In addition, immunity and genetic susceptibility can also play as important factors for acquiring the infection.

The most significant clinical features associated with Chikungunya infection are fever, debilitating arthralgia and rash. The distribution of these clinical symptoms among seropositive cases are as follows - All the laboratory confirmed cases had fever (100%), 96.6% laboratory confirmed cases reported having joint pain and 52.7% reported a rash. These results are in agreement with another study conducted by Gerardin et al., (2008) in La Reunion island where fever was present in 88.5% and joint pain was present in 87% serologically confirmed cases.

Several commercial test kits are available for performing the immunochromatographic test (ICT) for the detection of anti Chikungunya IgM or IgG antibodies in the patient serum. Among them STANDARD Q Chikungunya IgM/IgG test kits of SD biosensor Inc., Korea was evaluated in this study to see whether this technique could be established for the mass screening in the clinical settings of Bangladesh where diagnostic facilities are insufficient in different remote areas during the time of epidemic when a large number of population is involved. For the determination of the performance characteristics of ICT test, ELISA test is employed as the reference method since it is considered as one of gold standards for the serological diagnosis. The evaluation is carried out based on test performance indicator such as Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative predictive value (NPV).

The sensitivity and specificity of the ICT test is calculated as 83% and 100% respectively. Additionally the Positive Predictive Value (PPV) and Negative predictive value (NPV) of the test were found to be 100% and 67.7%. Therefore the ability of ICT test to correctly identify a person not having Chikungunya infection is absolute as it will not declare a healthy person as diseased. However considering the sensitivity of the test, it will declare some true positive cases and declare them as false negatives which is 17 out of 100 (17%). By taking account the Positive

Predictive Value (PPV), it can be concluded that positive verdicts given by this test is accurate which means the subjects with a positive screening test truly have the disease. From Negative predictive value (NPV), it can be interpreted that some of the subjects (32.3%) with the negative verdict by this test will actually carry the disease.

These findings does not corroborate with the study by Kosasih et al., (2012) where evaluation of two rapid immunochromatographic test kits namely the OnSite Chikungunya IgM Rapid Test-Cassette and the SD Bioline CHIK IgM rapid test was performed in comparison to a capture ELISA. The sensitivity of the immunochromatographic test obtained was 50.8% while its specificity was 89.2% which are much lower than the values detected by this study. Another study conducted by Prat et al., (2014) to evaluate SD Bioline Chikungunya IgM (Standard Diagnostics Inc., South Korea) also showed poor sensitivity and specificity of 30% and 73% respectively for this test where the study was done on 23 samples.

This discrepancy of the results between this study with other studies could be due to several factors. The ICT test kits utilized in the other studies could have different test principles and designs and the evaluations were performed at a much earlier period. Since then the dynamics of the test potentials could go through several alterations and manufacturer of the kits tried and provided necessary readjustments and advancements based on them. Concentration of Immunoglobulin M and environmental settings can also play as important factors. In this study the tests were done on blood samples that were collected around the peak time of outbreak which enables the confirmation of the suspected cases by the test despite the poor performance characteristics where information on the timing of sample collection was not provided in the other studies when received at the lab. Moreover the sample size in the other studies was much smaller which rises confusion for a clear indication of the test.

The immunochromatographic test was also compared with the Enzyme-linked immunosorbent assay in terms of time, cost and methodology to draw final conclusions. ELISA test requires several reagent preparations and sample dilutions for each sample which are time consuming where ICT test can provide result in a minimum amount of time of 15 minutes. Again considering the price, ICT test is much cheaper and cost effective compared to the expensive

ELISA test. Additionally, ELISA test is in need of expert technicians to perform as the methodology is complex and calls for precision where on the other hand the ICT test can be executed by any individual with little training and knowledge. Therefore, considering every findings in this study, it can be conferred that the Immunochromatographic (ICT) can be implemented as a point of care test to manage epidemic like situations in resource limited countries like Bangladesh where diagnostic capacity is insufficient. However it should utilized only for initial screening purpose to rapidly distinguish the individuals with Chikungunya infection from other arboviral disease patients and provide necessary treatments based on that. The overall conclusion must be drawn by considering other confirmatory factors for the disease.

## Conclusion and Future perspectives

There is no documentation of large outbreak of CHIKV in Dhaka city before 2017. Thus, the population in the city being immunologically naive, the recent CHIKV outbreak caused tremendous agony in the social and economic lives of people which also gave rise to much alert in the research community. This investigation performed an evaluation on the immunochromatographic (ICT) test which is a rapid test for the serological diagnosis of Chikungunya patients to find out whether it can be applied on large scale for initial screening in epidemic like situations in resource limited countries like Bangladesh. It also explored the statistical information available from the study to identify the possible risk factors associated with Chikungunya infection based on laboratory results. The result from the study indicates that ICT test can be in fact applied as a point of care test in remote locations where equipments are limited and diagnostic facilities are poor to primarily differentiate the disease from other arboviral infections. It was also found that gender and age are significantly associated with Chikungunya infection. The findings of this research may help to clarify about the techniques available for diagnosis, provide valuable information for further research and what counter measures should be taken to prevent widespread outbreaks of Chikungunya in developing countries like Bangladesh. This study will also help to provide support and services to public health science which will eventually contribute the country for the diagnosis, prevention and control of Chikungunya and similar viral diseases. Overall, it necessitates the importance of utilizing appropriate and reliable diagnostic methods, proper surveillance system and effective control measures that must be implemented to manage the disease outbreak situations.



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# Appendices

## APPENDIX I

### ACTIVE INGREDIENTS AND REAGENT CONTENTS OF ELISA KIT, SD

The following components are provided with the STANDARD E Chikungunya IgM ELISA, SD Biosensor, Korea

| Component                     | Composition   |
|-------------------------------|---|
| Chikungunya IgM plate         | 96 wells coated with monoclonal anti-human IgM              |
| 101 X Enzyme conjugate        | monoclonal anti-chikungunya to horseradish peroxidase (HRP) |
| Conjugate Diluent             | Phosphate buffered saline                                   |
|                               | Bovine Serum Albumin (BSA)                                  |
|                               | Preservative: Proclin 300                                   |
| Chikungunya IgM NC            | BSA   |
|                               | Preservative: Proclin 300                                   |
| Chikungunya IgM PC            | Inactivated Chikungunya IgM positive specimen               |
|                               | BSA   |
|                               | Preservative: Proclin 300                                   |
| Sample Diluent                | Casein  |
|                               | Tris  |
|                               | Preservative: Sodium azide                                  |
| Chikungunya antigen           | Inactivated Chikungunya virus                               |
|                               | Gelatin   |
|                               | Preservative: Proclin 300                                   |
| Wash buffer (20X concentrate) | Phosphate buffer solution                                   |
|                               | Tween 20  |
|                               | Preservative: Proclin 300                                   |
| TMB Substrate                 | Tetramethylbenzidine (TMB)                                  |
| Stop solution                 | 1N sulfuric acid  |

## APPENDIX II

### Ethical Approval

The blood samples utilized in this study was collected by the Epidemiology Department of National Institute of Preventive and Social Medicine for the project proposal entitled **"Chikungunya outbreak 2017 and its biometeriological relationship"** which had been reviewed and approved by the Institutional Review Board (IRB) of National Institute of Preventive and Social Medicine (NIPSOM). The following page contains the document of Ethical clearance. Written consent was also obtained from all the participants after providing them with a detailed explanation of the study and procedures. The ethical guidelines were maintained strictly throughout the whole study to ensure privacy and confidentiality of every participant.

## APPENDIX III

### Facilities

The significant equipment's that used during the study are listed below

| Name of the Instrument        | Company                       |
|-------------------------------|-------------------------------|
| Biosafety cabinet Class-2     | ESCO, Singapore               |
| - 20 <sup>0</sup> Freeze      | Equitek, USA                  |
| 4-8 <sup>0</sup> Refrigerator | Toshiba, Japan                |
| Incubator,                    | Memmert, Germany              |
| ELISA Reader                  | Biotek, USA                   |
| ELISA Automatic washer        | Biotek, USA                   |
| Vortex mixer                  | Eppendorf , Germany           |
| Centrifuge machine            | Thermo Fisher Scientific, USA |
| Pipette 1000 $\mu$ l          | Eppendorf , Germany           |
| Pipette 20-200 $\mu$ l        | Eppendorf , Germany           |