DEVELOPMENT OF A SEROLOGICAL ASSAY FOR CHIKUNGUNYA VIRUS

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ABBREVIATIONS

BSA	bovine serum albumin
CHAPS	3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate
CHIKV	Chikungunya virus
CPE	Cytopathic effect
C6/36	*Aedes albopictus* mosquito gut cells
C6/36 CHIKV	CHIKV-infected C6/36 cells lysate antigen
C6/36 ELISA	ELISA using CHIKV-infected C6/36 cells lysate antigen
C6/36 Mock	Mock-infected C6/36 cells lysate antigen
DAB	3,3'-Diaminobenzidine
EDTA	Ethylene diamine tetracetic acid
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
HRP	Horseradish peroxidase
IF	Immunofluorescence assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LD	Lethal dose
NEAA	Non-essential amino acid
NPV	Negative predictive value
nsP	Non-structural protein
OD	Optical density

OPD	o-phenyl amine
ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PPV	Positive predictive value
RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase polymerase chain reaction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
TCID	Tissue culture infecting dose
UMMC	University Malaya Medical Center
Vero	African green monkey kidney cells
Vero CHIKV	CHIKV-infected Vero cells lysate antigen
Vero ELISA	ELISA using CHIKV-infected Vero cells lysate antigen
Vero Mock	Mock-infected Vero cells lysate antigen

1.0 ABSTRACT

Chikungunya virus (CHIKV) is a RNA virus that belongs to the Alphavirus genus of the family *Togaviridae*. It is transmitted by *Aedes albopictus* and *Aedes aegypti*, and causes clinical symptoms like fever with acute fever, skin rash and athralgia which mimics dengue fever. With current outbreaks of CHIKV in Malaysia, it is essential to develop a serological assay as a tool for laboratory diagnosis and seroprevalence study. An IgG indirect ELISA was developed and used to validate nine samples collected from Bagan Panchor residents one year after an outbreak occurred in 2006. A variety of factors like varying cell culture types (C6/36 and Vero) to prepare the virus lysate antigen, different serum dilutions and the cut-off value determination methods were studied to optimize an IgG indirect ELISA assay. Neutralization assay was used as the gold standard. The IgG indirect ELISA using CHIKV-infected Vero cell lysate antigen performed better compared to CHIKV-infected C6/36 cell lysate antigen. The developed assay had a sensitivity of 100%, poor specificity of 25%, positive predictive value of 62.5%, negative predictive value of 100%, and concordance of 66.7%, compared to neutralization. The poor specificity and non-specific background readings are likely due to the crude total cell lysate used in the assay. Western blot identified the capsid protein as the immunogenic protein, which maybe used as a CHIKV recombinant antigen for further development of a more specific ELISA assay.

2.0 INTRODUCTION

Chikungunya (CHIKV) is an infectious viral disease transmitted by *Aedes* mosquitoes. First isolated from the serum of febrile humans in Tanganyika (Tanzania) in 1953, the word “Chikungunya”, which is used for both the virus and the disease means “to walk bent over” in the African dialect Makonde, reflecting the severe joint pains that are an element of the disease (Pialoux *et al*., 2007). Since then, CHIKV has caused many well-reported outbreaks and epidemics in both African and Asian countries, namely India, Indonesia, Malaysia and Thailand involving millions of people (Powers and Logue, 2007).

CHIKV disease epidemics are distinguished by sporadic outbreaks with disappearance which may last from a few years to a few decades (Mohan, 2006). Since the first recorded CHIKV epidemic, which occurred in Tanzania in 1952-1953, human CHIKV infection has been documented in Asia with an outbreak in Bangkok in 1958, which was followed by outbreaks in countries such as Burma, Thailand, Cambodia, Vietnam, India, Sri Lanka and the Philippines. CHIKV also occurred in many parts of Africa. In India, the virus was first isolated in Calcutta in 1963 with many other reports soon after. Between the years 2001-2003 a re-emergence of CHIKV outbreak in Indonesia was documented almost 20 years after the first documented case between 1950s to the 1960s (Sourisseau *et al*., 2007). It was observed that in both Africa and Asia, the re-emergence was erratic with intervals of 7-8 years to 20 years between consecutive epidemics (Schuffenecker *et al.*, 2006).

CHIKV caused a large outbreak in the Indian Ocean starting at the end of 2004. It is presumed that the CHIKV epidemic in the Indian Ocean region started first in Kenya, before it reached Comoros between January and March 2005 with more than 5000 reported

cases. Then CHIKV spread to other islands namely Seychelles in March 2005, followed by Mauritius. Finally in March till April 2005 CHIKV reached Réunion Island with a population of 760,000 and about 266,000 cases had been diagnosed (Schuffenecker *et al.,* 2006). CHIKV then spread to India where, since December 2005 it is estimated that more than 1,400,000 cases have occurred (Pialoux *et al*., 2007). By the beginning of 2007, the contagion was on the decline in La Réunion and Seychelles but it seems to have been reported to be continuing in areas of India. Strict mosquito-control actions in 2006 ameliorated the epidemics on the Indian Ocean islands, but the spread of the same strain of CHIKV to India confirms that the virus is not easy to contain (Panning *et al.,* 2009).

Four known outbreaks have occurred in Malaysia, the first was between December 1998 and February 1999 when more than 51 people in Port Klang were infected with CHIKV (Lam *et al*., 2001). CHIKV recently re-emerged in Malaysia after seven years of non detection. From March through April 2006, a second outbreak of CHIKV infection was reported in Bagan Panchor, an isolated coastal town in Perak, in which at least 200 villagers were infected with no deaths reported. Genomic sequences of the recovered isolates of the second outbreak were highly similar to those of the previous 1998 outbreak in Port Klang (AbuBakar *et al*., 2007).

The clinical manifestations of the two previous outbreaks are considered classical. These two outbreaks were due to CHIKV virus of Asian genotype. There were also reports of a third outbreak in Ipoh between December 2006 and January 2007. The third outbreak however involved two areas of the suburb of Ipoh City within the Kinta district, whereby around thirty seven people were affected in the main outbreak area, and another two

patients were affected in the secondary outbreak area. Based on the molecular study conducted, it was confirmed that the CHIKV that caused the Kinta outbreak was of the Central or East African genotype, which occurred for the first time in Malaysia (Noridah *et al.*, 2007). A fourth outbreak was reported in Johor in April 2008 with almost 2000 cases. CHIKV outbreak then eventually spread to different parts of Peninsula Malaysia with effected areas such as Melaka, Selangor, Perak and Pahang involving over 8920 cases to date (Malaysia Ministry of Health, 2009; Sam *et al*., 2009). The number of reported cases declined in 2009 but persisting outbreaks continuing in several states.

2.1 CHIKUNGUNYA OUTBREAK CASE STATISTICS

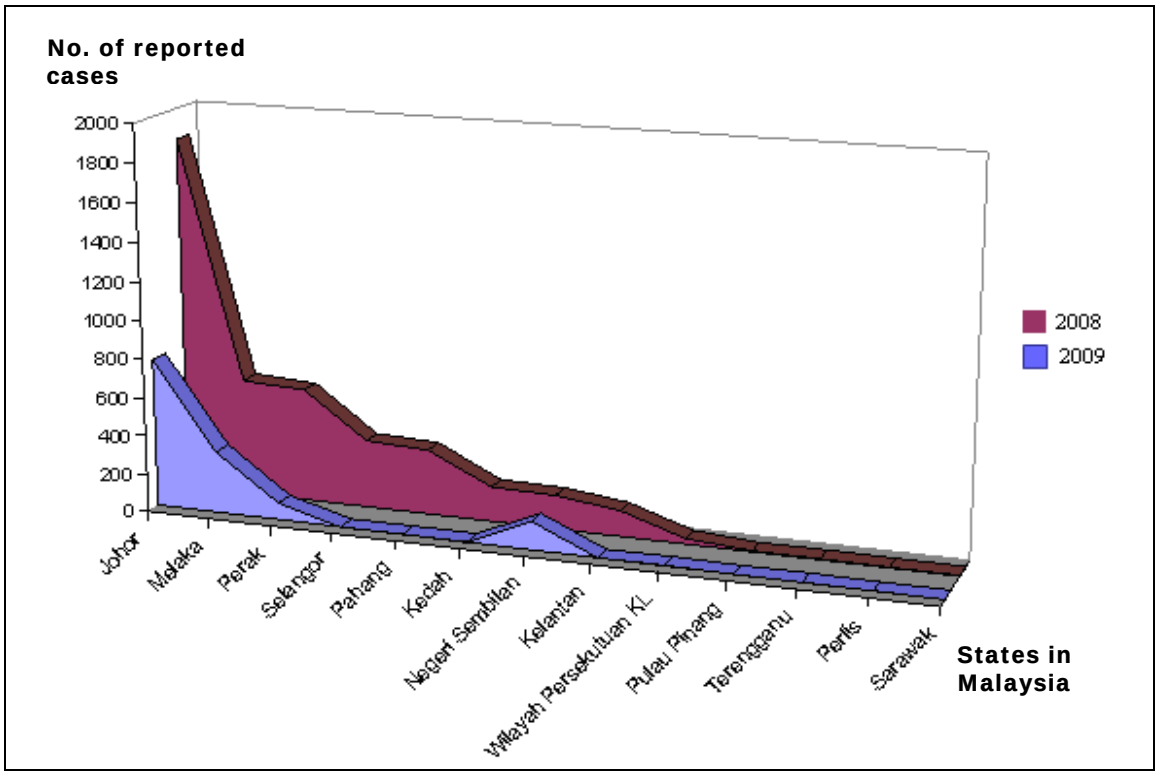


Figure 1: Reported Chikungunya cases of 2008-2009.

Figure 1 shows an 80% decrease in the total number of cases throughout Malaysia in 2009, compared to the 4271 reported cases in 2008. (Data collected from Malaysian Ministry of Health, www.moh.gov.my).

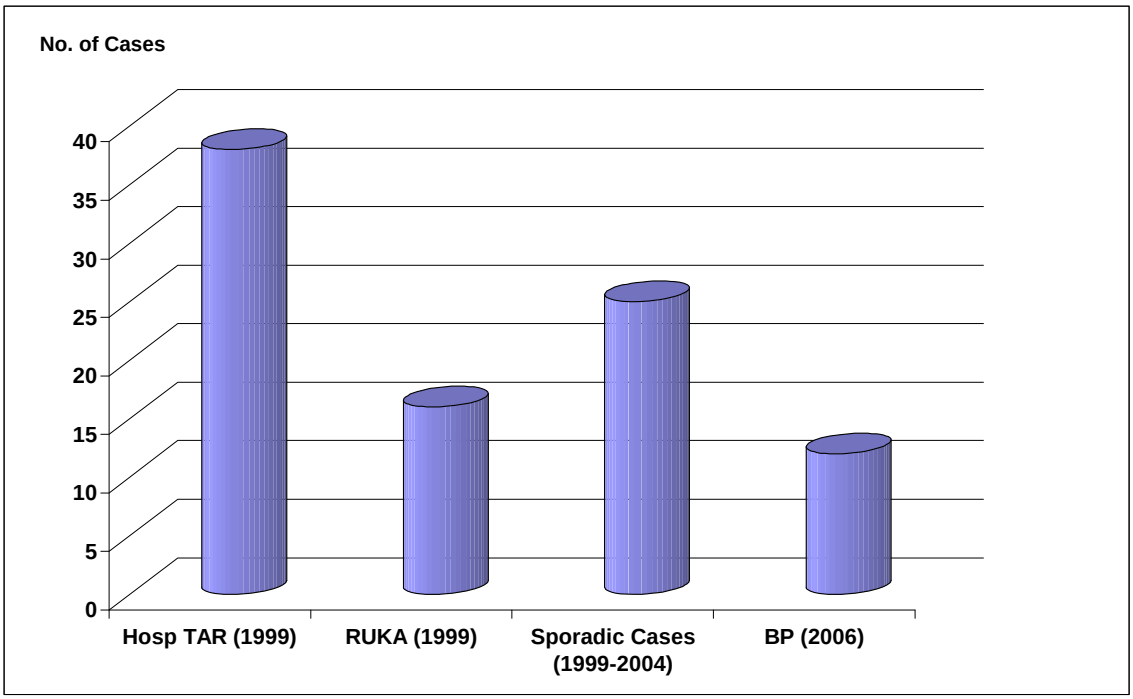


Figure 2: Cases tested for CHIKV in UMMC

Hosp TAR: Hospital Tengku Ampuan Rahimah, Klang.

RUKA: Klinik Rawatan Utama Klinik Am

BP: Bagan Panchor

Figure 2 shows all the UMMC cases tested for CHIKV. During the CHIKV outbreak in Klang in 1999, most of the patients test serum sent to University Malaya Medical Centre (UMMC) were from the Tengku Ampuan Rahimah (TAR) Hospital. Most of these samples were only tested using indirect immunofluorescence staining (IF) to confirm the presence or absence of CHIKV IgM and IgG, due to a lack of proper diagnostic method available at such short notice. Most of the samples were kept for further study.

Apart from the samples sent from TAR Hospital in Klang, samples sent in 1999 also included samples obtained from patients from *Rawatan Utama Klinik Am* or also known as Primary Care Clinic in UMMC. There were also some sporadic cases seen between 2000 and 2004 involving patients seen at UMMC and local private hospitals. However it is still uncertain as to how and why CHIKV has the ability to disappear and then re-emerge some time later. During the 2006 outbreak in Bagan Panchor about 10 serum samples were sent to UMMC by the Perak state health authorities for further analysis.

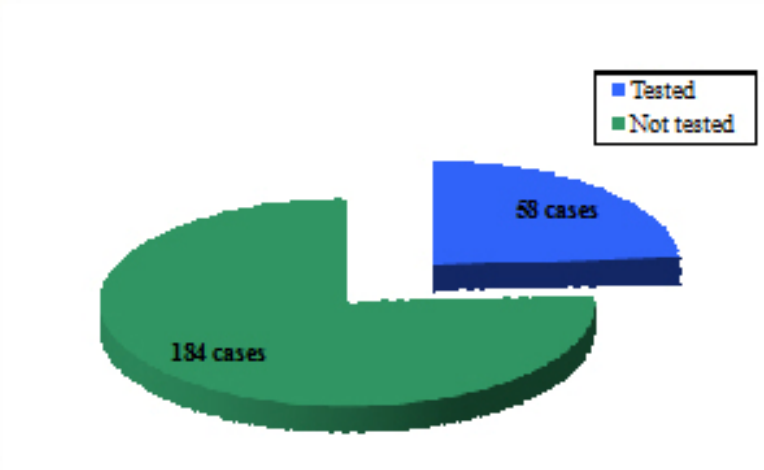


Figure 3: Reported CHIKV cases in Bagan Panchor, 2006

Figure 3 shows the 242 suspected cases reported in Bagan Panchor in 2006. These patients reported the symptoms consistent with CHIKV such as fever, joint pains and rash. However only 58 of these suspected cases had blood samples taken, and had laboratory confirmation of CHIKV infection by presence of IgM, CHIKV virus isolation, or detection by PCR.

2.2 VIROLOGY OF CHIKUNGUNYA VIRUS

CHIKV is a mosquito-borne Togavirus belonging to the genus Alphavirus. The closest antigenic related species to CHIKV is the O'nyong nyong virus (ONN) (Blackburn *et al.*, 1995). In Asia CHIKV is transmitted by *Aedes albopictus* (Figure 4) and *Aedes aegypti* (Figure 5). However in Africa it can also be transmitted by other mosquitoes such as *Aedes furcifer, Aedes fulgens, Aedes luteocephalus, Aedes dalzieli, Aedes vigilax, Aedes camptorhynchites, Culex sp.*, and *Mansonia sp.*, especially in certain regions in Africa (Pialoux *et al*., 2007). Humans serve as the CHIKV reservoir during epidemic periods. And outside this period in Africa, non-human primates such as baboons and *Cercopithecus* monkeys (Soon *et al*., 2007), rodents, birds and other unidentified vertebrates become the main animal reservoirs (Pialoux *et al.*, 2007).

Figure 4: *Aedes albopictus* - vector of Chikungunya virus

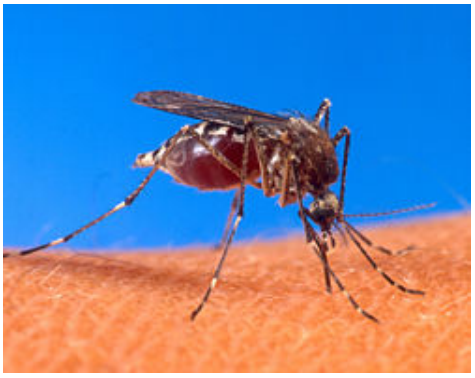


Figure 5: *Aedes aegypti* – vector of Chikungunya virus

(Figures 4 & 5 obtained from Pialoux *et al.*, 2007).

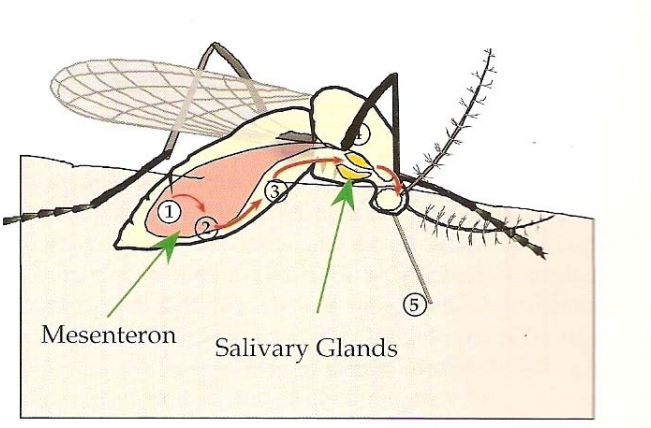


Figure 6: Cutaway view of the mosquito showing steps in the replication and transmission of an arbovirus (Schlesinger and Schlesinger, 1996)

Figure 6 shows the sequential step necessary for a mosquito to transmit an arbovirus. (1) A female mosquito ingests an infectious blood meal and virus enters the mesenteron. (2) Virus infects and multiplies in the mesenteronal epithelial cells. (3) Virus is released across the basal membrane of the epithelial cells and replicates in other tissues. (4) Virus infects salivary glands. (5) Virus is released from the epithelial cells of the salivary glands and is transmitted in the saliva during feeding.

Based on Schlesinger and Schlesinger (1996), most *Alphaviruses* are sensitive to desiccation and to temperatures above 58ºC (Pialoux *et al*., 2007). CHIKV is an arbovirus (arthropod-borne) which infects arthropods that ingest vertebrates' blood. They multiply in the arthropods' tissues and can be transmitted by bite to susceptible hosts.

A CHIKV virion is about 50-70 nm, spherical and composed of evenly distributed glycoprotein spikes on the phospholipid bilayer (Sourisseau *et al*., 2007). The structure consists of an icosahedral nucleocapsid containing linear positive sense single strand RNA of 11,800 nucleotides enclosed within a lipoprotein envelope. The arrangement of the nucleocapsid and the protein constituent of the envelope are consistent with T=4 icosahedral symmetry.

2.2.1 GENOMIC STRUCTURE OF CHIKUNGUNYA VIRUS

The 11.8 kb long Chikungunya genome is capped at the 5' end and has a poly(A) tail in its 3' end. It is made of two open reading frames (ORFs) fixed between nontranslated regions (3' NTR and 5' NTR). The ORF located at the 5' end of the genome encodes a polyprotein precursor of nonstructural proteins (nsP1, nsP2, nsP3 and nsP4) with replicative or proteolytic activities (or both). As for the second ORF, it encodes the polyprotein precursor of the structural proteins (C, E1, E2, E3 and 6K) forming the viral nucleocapsid and envelope (Chevillon *et al*., 2008). Figure 7 shows the *Alphavirus* genome and its products.

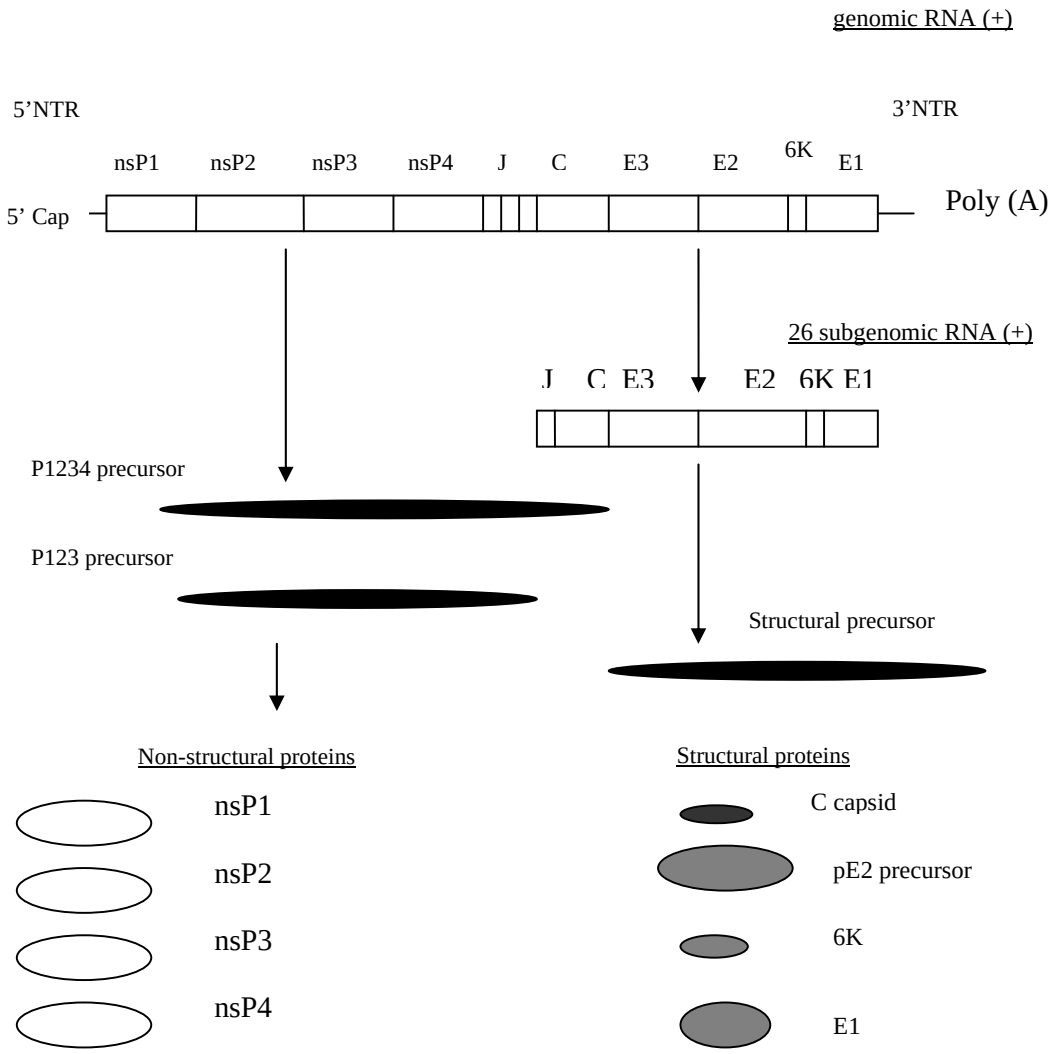


Figure 7: *Alphavirus* genome and its products. (Adapted from Chevillon *et al., 2008*).

The CHIKV envelope protein E1 and E2 are components of spikes which are composed of triplets of heterodimers of E1 and E2 glycoproteins and cover the viral surface in the form of membrane-anchored types. The viral spikes aid attachment to cell surfaces and viral entry into cells. E1 protein is known to be a class II fusion peptide which mediates low pH-triggered membrane fusion during virus infection, while the E2 envelope protein is a type I transmembrane glycoprotein responsible for receptor binding (Cho *et al.*, 2008).

2.3 CLINICAL MANIFESTATIONS OF CHIKV

CHIKV is serologically and genetically closely related to O'nyong nyong, Igbo Ora and to some extent Mayaro and Ross River viruses, all of which are associated with acute epidemic polyarthalgia (Lanciotti *et al.,* 2007).

Following a bite from an infectious mosquito, there is an incubation period lasting 2-4 days on average (Pialoux *et al*., 2007). CHIKV causes an abrupt onset of fever, headache, nausea, vomiting, myalgia, skin rash, incapacitating arthralgia and occasional frank arthritis. The articular symptoms resolve within days to a few weeks, but in some severe cases joint pain and stiffness can last for months (Porter *et al.,* 2004).

The clinical symptoms of CHIKV infection often imitate those of dengue fever, and since CHIKV virus is present in the regions where dengue virus is endemic, it is possible that many cases of CHIKV infection have been misdiagnosed as dengue and that CHIKV infection could be much more common than reported (Powers *et al.*, 2000).

CHIKV also results in complications including respiratory failure, cardiovascular decompensation, meningoencephalitis, severe acute hepatitis and kidney failure. More than half (53%) of serious cases involved patients above 65 years of age and more than one third (35%) died due to CHIKV (Renault *et al*., 2007).

2.4 LABORATORY DIAGNOSIS OF CHIKUNGUNYA INFECTION

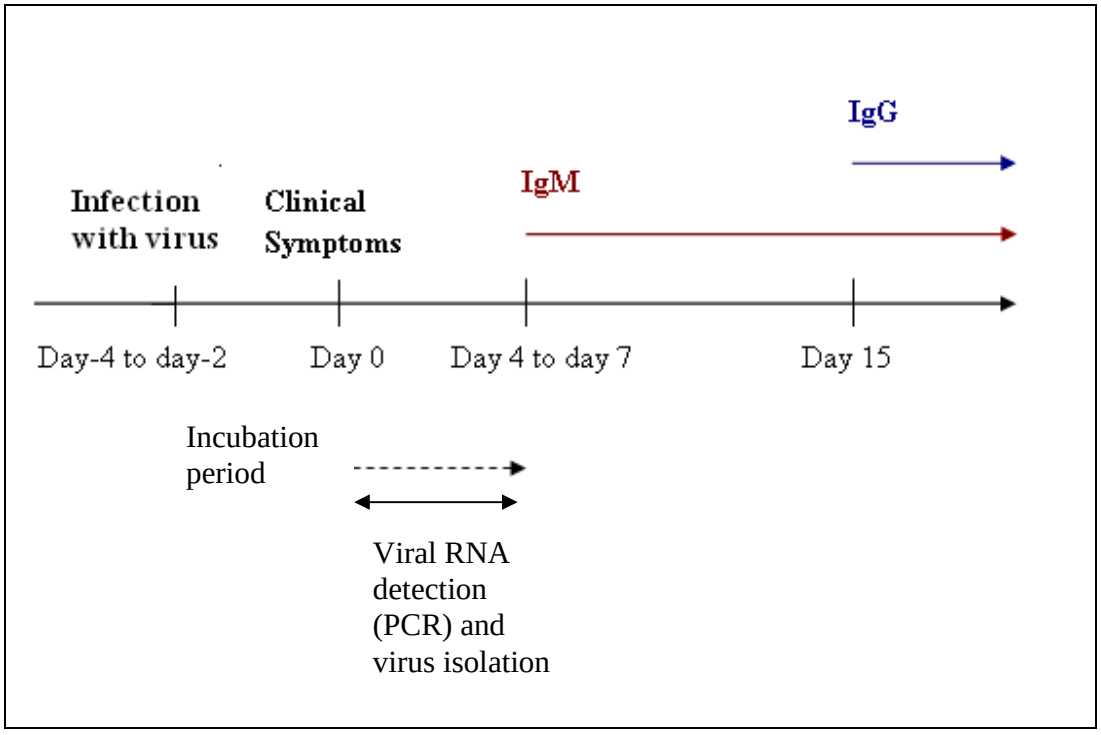


Figure 8: Laboratory diagnosis of Chikungunya (Pialoux *et al.*, 2007)

The diagnosis of CHIKV can be made by virus isolation, reverse transcriptase polymerase chain reaction (RT-PCR) and classical serology methods, which include neutralization assay, haemagglutination inhibition, complement fixation, indirect immunofluorescence (IF) and enzyme linked immunosorbent assay (ELISA) (Figure 8).

2.4.1 VIRUS ISOLATION

Considered the gold standard for diagnosis, CHIKV virus isolation is based on inoculation of mosquito cell cultures, mosquitoes, mammalian cell cultures or mice. Commonly used cell lines for isolating CHIKV include Vero (African green monkey kidney cells) and C6/36 (*Aedes albopictus* mosquito gut cells). Successful cell culture results in cytopathic effect (CPE). CPE is the morphological or the degenerative changes host cells go through due to the growth of infecting virus. This may not be visible until three to four days of incubation, and further passaging may be required. Nevertheless cell culture is still an important method to harvest CHIKV virus for further study. Identification of CHIKV virus can then be confirmed by indirect IF with specific antisera, or by PCR, but IF is more subjective (Panning *et al*., 2009).

2.4.2 POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is used widely in molecular biology. After extraction and purification, a DNA polymerase is used to amplify a piece of DNA using *in vitro* enzymatic replication. The original DNA molecule or molecules are replicated by the DNA polymerase enzyme thus doubling the number of DNA molecules copied. Then each of the copied molecules will replicate in a second cycle of replication resulting in four times the number of the original molecule. This continual cycle of replication is known as a "chain reaction" in which the original DNA molecule is exponentially amplified. However to amplify, isolate and identify a known sequence of RNA, reverse transcriptase is required to generate the complementary cDNA strand from the RNA sample. Reverse transcriptase PCR or RT-PCR is widely used in many different applications such as expression profiling, expression of genes or to identify the sequences of an RNA transcript. Being a

single stranded RNA virus, CHIKV RNA is amplified to construct cDNA for further analysis (Powers *et al*., 2000). The PCR products were analyzed by performing gel electrophoresis with ethidium bromide-stained agarose gel.

Although CHIKV virus analysis based on virus isolation is considered as the gold standard, it requires time and sensitivity and depends on many factors such as quality of maintenance of cell culture. RT-PCR is the most sensitive technique for mRNA detection and quantification (Rohani *et al*., 2005).

2.4.3 SEROLOGICAL TECHNIQUES

Serology is the scientific method of study involving the use of blood serum mainly. However there are studies involving the use of other bodily fluids such as semen or saliva. It is utilized as means of diagnostic identification or detection of the presence of antibodies in response to other foreign proteins or one's own proteins. There are a few known serological techniques most frequently used such as enzyme linked immunosorbent assay (ELISA), haemagglutination, precipitation, complement-fixation and immunofluorescence assay. A classic serological algorithm involves testing acute and convalescent serum specimens for immunoglobulin M (IgM) and IgG antibody, followed by a neutralization test. Virus isolation and RT-PCR are commonly used with early acute-phase specimens (before day 7) before appearance of IgM (Lanciotti *et al., 2*007).

(a) ENZYME LINKED IMMUNOSORBENT ASSAY

Enzyme linked immunosorbent assay (ELISA) is an immunological technique for the detection of the presence of an antibody or antigen in a sample. There are a few different types of ELISA such as indirect ELISA, sandwich ELISA and competitive ELISA, the choice of which depends on the aims of the assay. In indirect ELISA for CHIKV antibodies, CHIKV antigen is coated onto a surface of a microtitre plate, followed by addition of the serum to be tested. A secondary anti-human antibody bound to an enzyme is then added, which will allow binding with the presence of the right antibody. When a substrate is added, the linked enzyme catalyses detectable colour change, allowing the measurement of the primary antibodies using an ELISA microplate reader.

Many serological tests like indirect IF assay, plaque reduction neutralization test, hemagglutination-inhibition test and complement fixation test are technically demanding, making them complicated to apply reproducibly and are often poor measures of early antibody IgM. Furthermore, performing these tests might take several days and requires paired serum samples or live cell culture. Virus isolation is hardly ever a viable option even in epidemic situations, due to inadequately timed specimens and large numbers of specimens (Martin *et al*., 2000). IgM capture enzymed linked immunosorbent assay (MAC-ELISA) was designed to detect IgM antibody, which is a valuable tool for quick diagnosis of acute viral infection. It appears early in infection and rises rapidly in the disease course and is usually less virus cross-reactive than IgG. Indirect ELISA however is a very sensitive, reliable, and widely-used method for identifying the presence of IgG antibodies of many infectious diseases that are present long after an infection, making it easier to work with.

(b) INDIRECT IMMUNOFLUORESCENCE ASSAY

IF is a technique, commonly used to visualize the subcellular distribution of biomolecules of interest. It is the labeling of antibodies with fluorescent dyes. The immunofluorescent-labelled tissue or cultures are then analyzed using a fluorescence microscope or a confocal microscope. Although IF is useful for serological diagnosis of many viruses and bacteria, this method is cumbersome, laborious, subjective and requires a trained microscopist equipped with an expensive microscope (Lao *et al*., 2004).

(c) NEUTRALIZATION ASSAY

Neutralization of a virus is defined as the loss of infectivity through reaction of the virus with specific antibody. Virus and serum are mixed under suitable conditions and then inoculated into cell culture, eggs or animals depending on the virus. The presence of non-neutralized virus may be detected by reactions such as CPE, haemadsorption/haemagglutination, plaque formation or as disease in animals. The loss of infectivity is bought about by interference by the bound antibody with any one of the steps leading to the release of the viral genome into the host cells (Wu *et al*., 2004). There are two types of neutralization which are reversible neutralization and stable neutralization. Reversible neutralization is when the process can be reversed by diluting the antibody-antigen mixture within a short time of the formation of the antibody-antigen complexes. It is thought that reversible neutralization is due to the interference with attachment of virions to the cellular receptors which requires the saturation of the surface of the virus with antibodies. Stable neutralization is when the antibody-antigen complexes become more stable with time and it is irreversible by dilution.

Stable neutralization has a different mechanism to that of reversible neutralization. It has been shown that neutralized virus can attach and that already attached virions can be neutralized. The number of antibody molecules required for stable neutralization is considerably smaller than that of reversible neutralization. Kinetic evidence shows that even a single antibody molecule can neutralize a virion. Such neutralization is generally produced by antibody molecules that establish contact with 2 antigenic sites on different monomers of a virion, greatly increasing the stability of the complexes. An example of stable neutralization is the neutralization of polioviruses, whereby, the attachment of the antibody to the viral capsid stabilizes the capsid and inhibits the uncoating and release of viral nucleic acid (Hanon and Mayhew, 1970).

Viral evolution must tend to select for mutations that change the antigenic determinants involved in neutralization. In contrast, other antigenic sites would tend to remain unchanged because mutations affecting them would not be selected for and could even be detrimental. Because of its high immunological specificity, the neutralization test is often the standard against which the specificity of the other serological techniques is evaluated.

To proceed with the neutralization protocol, known components that are to be used must be standardized. To identify a virus isolate, a known pre-titred antiserum is used. Conversely, to measure the antibody response of an individual to a virus, a known pre-titred virus is used. To titrate a known virus, serial tenfold dilutions of the isolate are prepared and inoculated into a susceptible host system such as cell culture or animal. The virus endpoint titre is the reciprocal of the highest dilution of virus that infects 50% of the host system eg. 50% of cell cultures develop CPE, or 50% of animals develop disease. This endpoint

dilution contains one 50% tissue culture infecting dose ($TCID_{50}$) or one 50% lethal dose (LD_{50}) of virus per unit volume. The concentration of virus generally used in the neutralization test is 100 $TCID_{50}$ or 100 LD_{50} per unit volume (Hanon and Mayhew, 1970).

The antiserum is titrated in the neutralization test against its homologous virus. Serial twofold dilutions of serum are prepared and mixed with an equal volume containing 100$TCID_{50}$ of virus. The virus and serum mixtures are incubated for 1 hour at 37°C. The time and temperature for incubation varies with different viruses. The mixtures are then inoculated into a susceptible host system. The endpoint titration contains one antibody unit and is the reciprocal of the highest dilution of the antiserum protecting against the virus. Generally 20 antibody units of antiserum are used in the neutralization tests (Wu *et al*., 2004).

2.4.4 COMPARISON BETWEEN PCR, ELISA AND NEUTRALIZATION ASSAY

RT-PCR is the most sensitive for detection of CHIKV during the initial viraemic phase, which is between day zero to day seven (Rohani *et al*., 2005; Pialoux *et al*., 2007). PCR is less useful in the later stages, due to the patient's antibody production and clearance of viraemia. PCR is also an expensive method to work with especially as a rapid and regular technique, since the equipment used such as the thermal cycler and gel electrophoresis system are expensive. Viral culture is also less useful outside the viraemic phase, as the presence of antibody prevents the virus isolation. Culture is also labour-intensive and technically demanding.

IgM antibodies are detectable after about 4-5 days and persist for up to three months. IgG antibodies are detectable in convalescent sera and persist for years. Thus it is easier to retrospectively identify infected patients from known outbreaks even from the earlier 1999 Klang outbreak to the current 2006 Bagan Panchor outbreak, which is not possible with PCR or isolation methods. ELISA is rapid and sensitive (after the viraemic phase), and can be introduced as an in–house diagnostic method to detect CHIKV IgM or IgG. Apart from this ELISA is a method that is relatively easily conducted even with large numbers of samples. ELISA can be automated and is not subjective unlike certain methods like indirect IF (Wu *et al.,* 2004).

Neutralization test is also used to measure the antibody response of an individual to a CHIKV virus. Because of its high immunologic specificity, the neutralization test is often the standard against which other serologic procedures are evaluated. As neutralization is technically difficult, it is not used as a routine diagnostic method.

The main reason neutralization assay was chosen as a gold standard was due to its ability to detect biologically active antibodies. It is the most specific method for detection of virus-specific neutralizing antibodies. Before beginning the neutralization assay, it is important to determine the effect of a single variable of interest within the particular system, a scientific control is used to minimize the unintended influence of other variables on the same system. Therefore, positive controls confirm that the procedure is effective, thus minimizing false negatives, and negative controls confirm that the procedure is not observing an unrelated effect, thus minimizing false positives. Therefore Bagan Panchor patients' sera which was IF negative were pooled and used as the negative control, while

the CHIKV confirmed patients' sera was used as positive control. Pearson's correlation is used to find a correlation between at least two continuous variables. The value for a Pearson's can fall between 0 (no correlation) and 1 (perfect correlation). Other factors such as group size will determine if the correlation is significant. Generally, correlations above 0.8 are considered good. The correlation between the neutralization assay titres and ELISA optical density readings was measured and recorded.

2.5 OBJECTIVES

The main objective of this research is to develop a serological assay to detect IgG antibodies against CHIKV.

Specific aims:

1. To examine the kinetics of CHIKV propagation at different time points in different cell lines.
2. To perform neutralization assay for use as gold standard for CHIKV IgG indirect ELISA.
3. To develop a serological ELISA assay to detect IgG antibodies against CHIKV.

3.0 MATERIALS AND METHODS

3.1 BAGAN PANCHOR SERUM SAMPLES

Nine serum samples were picked from the collection of serum samples obtained with the approval of Bagan Panchor residents in 2007, following the outbreak a year earlier. These included people with and without the known clinical symptoms of CHIKV infection. Table 1 shows the list of patients indicating presence and absence of symptoms. Rubella confirmed patients' sera were used as a negative control and CHIKV known positive as positive control.

Patients	Symptoms
1	YES
2	YES
3	YES
4	YES
5	NO
6	YES
7	NO
8	NO
9	NO

Table 1: List of serum samples obtained from Bangan Panchor

3.2 VIRUS ISOLATE

The virus isolate chosen for the present study (MY/0306/BP37348) was from the Bagan Panchor outbreak in 2006, and was provided by University of Malaya Medical Center (UMMC) Diagnostic Virology Laboratory. It has been subcultured more than 20 times.

3.3 CELL CULTURE

Vero (African green monkey kidney) cells and C6/36 (*Aedes albopictus* mosquito) cells were used for the propagation of the CHIKV. These cells were grown in monolayers in 75 cm^2 tissue culture flasks (Corning, USA) with 1X Eagle's Minimum Essential Medium (EMEM, from Flowlab, Australia) supplemented with 10% heat inactivated foetal bovine serum (FBS, from Flowlab, Australia). The Vero cells were incubated at 37˚C in a 5% CO_2 incubator, and C6/36 cells were incubated at 28˚C in a 5% CO_2 incubator. These cells were monitored on a daily basis for healthy growth.

3.4 VIRUS PROPAGATION

Healthy Vero and C6/36 cells growth were maintained up to 70% confluence in 75 cm^2 tissue culture flasks before inoculation of the CHIKV. As the initial step, the medium in the tissue culture flask was discarded before inoculation of the virus. 50µl of CHIKV viral stock were used as the inoculum for each 75 cm^2 tissue culture flask. This was followed by gently rocking of the tissue culture flask for one hour. After an hour, the virus inoculum was removed and 10 ml of maintenance medium (2% FBS, 1X EMEM) was added into each of the infected flasks. These flasks of infected Vero cells and C6/36 cells were later incubated at their respective incubating temperatures and carefully monitored daily for CPE before considered ready for harvest. The harvesting time points are at 0 hour, 24 hours, 48 hours, and 72 hours.

3.5 VIRUS HARVESTING

Once there was 70-80% CPE in the Vero and C6/36 cells, CHIKV was harvested by centrifuging the virus suspension at 40,000 x g for 30 minutes to remove the cell debris. Once centrifuged, the virus supernatant was sterile filtered using 0.20 μm syringe filter, and then used as virus inoculum for other procedures.

As a control, mock-infected cells for both Vero and C6/36 cells were prepared by replacing the viral inoculum with equal volume of maintenance medium. These mock-infected cells were incubated at their respective temperatures and processed together with the infected cells.

3.6 CONFIRMATION OF CHIKV INFECTION

3.6.1 MICROSCOPIC OBSERVATION

CHIKV infected Vero and C6/36 cells were observed regularly using an inverted microscope (Nikon, Japan) for their CPE. Pictures were taken on a daily basis to compare between infected and uninfected cells and their distribution of infectivity in relation to incubation day.

3.6.2 IMMUNOFLUORESCENCE STAINING

As a confirmatory assay to detect the presence of CHIKV infection IF staining and reverse transcriptase polymerase chain reaction (PCR) were performed. The Vero and C6/36 cells, CHIKV-infected and mock-infected, were harvested once there was 70-80% CPE. These cell suspensions were washed with phosphate buffer saline (PBS) three times before being coated onto a glass slide precoated with poly-L-lysine. These slides were air-dried for several minutes then fixed with acetone for 15 minutes and kept in 4˚C for later use.

Using a known CHIKV IgG-positive patient serum as primary antibody, presence of CHIKV antigen was detected. The serum was diluted 1:400 in PBS and 10 µl of the diluted serum were added onto each coated well. These slides were then incubated in a moist chamber at 37˚C for 30 minutes. The slides were then washed with PBS for 10 minutes several times. Subsequently the slides were incubated with 10µl of rabbit polyclonal anti-human IgG conjugated with fluorescein isothiocyanate (FITC) at 37˚C in a moist chamber for 30 minutes. These slides were then washed with PBS for 10 minutes several times, followed by a wash in milli-Q water for 10 minutes. The slides were then air-dried and observed under an UV microscope (Zeiss, Germany).

3.6.3 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

3.6.3 (a) RNA EXTRACTION OF CHIKV-INFECTED VERO CELLS

The CHIKV-infected cells were harvested once 70-80% CPE was reached. Cells were scraped and the cell suspension was transferred into a 15ml Falcon tube which was then centrifuged at 800 x g for 10 minutes. The cell pellet was then lysed with 1 ml of TRI REAGENT (Molecular Research Center Inc., USA)

It was then homogenized in the TRI REAGENT by 5 minutes incubation at room temperature. 200 µl chloroform was then added into each tube and mixed vigorously. The mixture was further incubated at room temperature for 15 minutes followed by 15 minutes centrifugation at 4˚C, at 12000 x *g*.

The aqueous phase was transferred into a fresh 1.5ml microcentrifuge tube. RQ1 RNase free DNase (5U/ml TRI REAGENT) was added, mixed and incubated at 37˚C for 30 minutes. The total RNA was then precipitated at room temperature for 10 minutes by adding 500µl isopropanol. Then the mixture was centrifuged 12000 x *g* at 4˚C for 10 minutes. The supernatant was discarded and the pellet was washed by mixing the pellet with ice cold 75% ethanol. The tube was later centrifuged 7500 x *g* at 4˚C for 5 minutes. The supernatant was discarded and the pellet was dissolved in nuclease free water. The extracted total RNA was then incubated at 60˚C for 10 minutes to remove the secondary structures of RNA. The extracted RNA was kept in -80˚C until use.

3.6.3 (b) RT-PCR

Specific primers targeting the nsP1 region (Hasebe *et al,*. 2002; Schuffenecker *et al.,* 2006) were utilized for the PCR reaction to confirm the presence of CHIKV RNA in the extracted total RNA. The RT-PCR was carried out using the Access RT-PCR system (Promega Corporation, USA) following the manufacturer's protocol. The reverse transcription was carried out at 45˚C for 45 minutes followed by 94˚C for 2 minutes for inactivation of reverse transcriptase and RNA. It then went through 30 cycles of denaturation at 94˚C for 30 seconds, annealing at 55˚C for 1 minute and extension at 72˚C for 2 minutes. Then finally the elongation step was carried out at 72˚C for 7 minutes. This protocol was based on work by Hasebe *et al*. (2002). The PCR product was then loaded into 1.5% agarose gel for electrophoresis at 80V for an hour. The gel was then stained with ethidium bromide (ETBr) and visualized using a digital gel documentation system (Bio-Rad, Hercules, USA).

3.6.4 WESTERN BLOT

3.6.4.1 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL (SDS-PAGE)

To proceed with the SDS-PAGE protocol, the Mini-Protean Tetra Cell (Bio-Rad, China) was cleaned with methanol and tightly assembled to ensure no leakage. A 10% separating polyacrylamide gel suspension was prepared according to the protocol, which was carefully mixed and pipetted gently into the casting set. Once at the proper level a layer of water was introduced to enhance the polymerization of the gel. After polymerization, the water was discarded, then a layer of 4% stacking polyacrylamide gel was pipetted and followed by gently securing the comb in place. While waiting for the stacking gel to polymerize, the samples to be tested were mixed in a 2:1 ratio with the loading dye (SDS buffer) and boiled for 10 minutes to denature the protein. After complete polymerization, the comb was gently removed, then the 30µg of the boiled samples and 10 µl of protein marker were introduced into respective wells. Broad range marker (New England Biolabs, USA) was utilized for the Coomassie blue staining while prestained marker (New England Biolabs, USA) was utilized for the Western Blotting. This protein electrophoresis was run in tank buffer in a gel tray at 160V for one hour.

3.6.4.2 SEMI-DRY TRANSFER

The electrophoresed polyacrylamide gel was soaked in the chilled cathode buffer for 20 minutes. The Hybond-P polyvinylidene fluoride membrane was cut to the desired size then soaked in 100% methanol for 10 seconds. The membrane was washed in water for 5 minutes, then equilibrated in anode buffer (II) for 20 minutes. The filter papers (Bio-Rad, USA) were also prewetted in chilled cathode buffer, anode buffer (I) and anode buffer (II). Once ready, the Trans-Blot Semi Dry Transfer Cell (Bio-Rad, USA) was assembled. The SDS-PAGE gel was stacked in between the filter papers together with the membrane, and preceded with transfer was carried out using 10V for one hour. After the transfer the membrane was trimmed to a proper size and marked for better identification. The transferred membrane was stored at 2-8˚C.

3.6.4.3 IMMUNODETECTION

The blotted membrane was pre-wet in 100% methanol then washed with water for 5 minutes. 5% skimmed milk in 0.1% Tween 20 (Merck, Germany) was added to the membrane and incubated for one hour at room temperature. It was then washed with PBS Tween 20 for 5 minutes. Subsequently, the membrane was probed with 100X diluted serum from seropositive patients which was further diluted in 5% skimmed milk by incubation for one hour on a shaker. Later, excess serum was washed and bound antibody was detected by incubation with 1000X diluted rabbit anti-human IgG horseradish peroxidase (Dako, Sytomation, Denmark) in 5% skimmed milk for an hour.

After that, the secondary antibody conjugated with HRP was added to the membrane and further incubated for an hour. After further washing, metal enhanced DAB substrate working solution (Thermo Scientific, USA) was applied according to manufacturer's

instructions and incubated for 5-15 minutes for the colorimetric reaction that produces dark brown precipitate when horseradish peroxidase enzyme reacts with cobalt chloride and nickel chloride.

This was followed by a rinse and wash with water for 10 minutes at least three times. Once completed, metal enhanced DAB substrate was added to the membrane, which was incubated for 5-15 minutes. The membrane was then twice washed in PBS Tween 20 for 10 minutes. The washed membrane was then blotted dry and viewed for presence of bands, and could be kept in 2-8 ˚C for several months.

3.7 CHIKV CULTURE AND TITRATION

Vero and C6/36 cells with 70% confluence in 75 cm^2 tissue culture flasks were observed under the microscope daily for optimum CPE of 70%. The optimum CHIKV harvest was obtained at day 3 for C6/36 cells and day 2 for Vero cells. The C6/36 and Vero flasks for their respective time points were then incubated and frozen. When all samples were obtained, the flasks were thawed and centrifuged at 40,000 x g for 30 minutes to remove cell debris. The supernatant was then used as the inoculum for virus titration.

CHIKV assay titration was carried out to quantify the concentration of virus stock harvested. A 96 well microplate (Falcon, Australia) was seeded with 10^4 C6/36 and Vero cells in 100 µl of inoculum in each cell. The cells were grown overnight in a humidified incubator at 37°C with 5% CO_2. Ten-fold serial dilution of the harvested CHIKV was added to the plate. Mock-infected C6/36 and Vero cells were inoculated with serum free media used as control. The microplate was shaken for one hour, then the residue discarded and EMEM with 2% FBS were added for maintenance. The cells were observed for seven days to determine determining the highest dilution of the virus suspension which produced 100% CPE.

3.8 QUANTITATING EXTRACTED PROTEIN

3.8.1. PREPARATION OF CELL LYSATE

Vero and C6/36 cells with 70% confluence in 75 cm^2 tissue culture flasks were inoculated with 50 µl CHIKV. Based on the daily observance for optimum CPE of 70%, these infected flasks were then harvested on day two for Vero cells and day three for C6/36 cells by gently scraping the cells off the tissue culture flasks. Anti-protease was added to the cell suspension, before centrifuging at 800 x *g* for 10 minutes. The pellet was then resuspended in PBS, then centrifuged again at 800 x *g* for another 10 minutes. This step was repeated several times before finally resuspending the pellet with R2 lysis buffer containing detergent 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS) (Bio-Rad, USA). To prevent degradation the mixture was continually kept on ice and rotationally vortexed while maintaining the temperature. After vortexing, the mixture was spun down to wash off the suspension, then kept in 4˚C and gently rocked overnight. The overnight rocked suspension was then centrifuged at 40,000 x *g* for 30 minutes. Once centrifuged, the liquid suspension was removed gently then mixed with glycerol (final 10%) and kept in -80˚C for later use.

3.8.2. PROTEIN ASSAY

Protein BCA assay kit (Pierce, USA) was used to prepare a protein standard curve. Different concentrations of albumin were prepared using the albumin from the kit. These different concentrations were then pipetted into the 96 well U-bottom microtiter plate, followed by adding working reagent, which is a mixture of Reagents A, B and C. The prepared plates were then covered with foil and incubated in a moist chamber at 37˚C for two hours. After two hours the optical density (OD) was measured at or near 562 nm on a plate reader. Then a standard curve was plotted based on the OD, and the protein concentration obtained from the CHIKV culture was measured.

3.8.3 COOMASSIE BLUE STAINING

Once the electrophoresis is complete, the SDS-PAGE gel was then removed from its chamber and placed into Coomassie Blue G-250 staining solution. The gel was then immersed in the solution for one hour on a shaker or overnight. Once stained, the gel was further destained for 10-15 minutes with destaining I (40% methanol, 7% acetic acid). The gel was finally washed and destained with milliQ water overnight until it was cleared.

3.9 NEUTRALIZATION TEST

About 1 x 10^4 Vero cells were seeded onto 96 well flat bottom tissue culture plate in 10% FBS 1X EMEM. CHIKV stock titer (10^{-4}) was diluted 10,000 X. Two-fold serial dilutions of patients' serum were prepared. These patients' serum have been heated at 56°C for 30 minutes to inactivate complement that may inhibit the neutralization test. The mixture was then incubated for one hour at 37˚C. After an hour the medium from the overnight seeded plate with 70-80% confluent cells was discarded, then 100 µl of the virus-serum mixture was added. As controls, CHIKV mixed with a patient's serum known to be negative for

anti-CHIKV antibodies, CHIKV without serum, and non-inoculated cells were also prepared. The infected plate was incubated at 37˚C in 5% CO_2 and observed for a minimum of five days for CPE.

3.10 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Once the protein concentration was measured, it was then used as an antigen to coat the ELISA 96 well U-bottom plate. For optimal optimization, the antigen was prepared based on a few different concentrations, 0.312 µg, 0.625 µg, 1.25 µg, 2.5 µg , 5 µg and 10 µg. These coated plates were kept at 4˚C overnight before use. As an initial step, the excess antigen was removed by washing with PBS mixed with 0.05% Tween 20 three times. Once complete, the plate was blocked by adding a blocking buffer (3% bovine serum albumin mixed with 0.05% PBS Tween 20 and incubated for one hour at room temperature. The buffer was then removed and the plate was washed three times. The known positive and negative patients' serum samples were pooled for a more precise measurement, and were then diluted to 100 and 1000 times respectively and incubated for two hours at 37˚C. After two hours, the plates were again washed three times with PBS Tween 20.

Next, 100 µl of 6000X diluted rabbit anti-human antibody (IgG) conjugated with Horse Radish Peroxidase (HRP) was added to each of the wells and incubated for two hours at 37˚C. This was followed by washing the plates with PBS Tween 20 three times before adding 100 µl of O-phenyl amine (OPD) substrate as a colour substance, and incubating for 30 minutes. 1.5N Sulphuric acid (H_2SO_4) was then added to each of the wells to stop the process, and then the absorbance values for each of the wells were read using the ELISA microplate reader at 410 nm, with a reference of 630 nm. The antigen concentration giving the optimal absorbance value was used for the subsequent ELISA.

3.11 CALCULATION OF CUT-OFF VALUES FOR ELISA

Two methods were used to calculate the cut-off values for ELISA. The first method used negative control absorbance ± 3 standard deviations (SD) (Laras *et al*., 2005; Porter *et al*., 2004). A sample is deemed negative when the optical density (OD) ratio is less than three SD below the negative control. A sample is deemed positive when the OD ratio is greater than three SD above the negative control. A sample is borderline when the OD ratio lies within ± 3 SD of negative control. These results were expressed as an OD ratio of serum OD over negative control OD value. Using this method the cut-off value for both CHIKV-infected C6/36 cell lysate (C6/36 ELISA) and CHIKV-infected Vero cell lysate (Vero ELISA) were calculated. In the second method, the cut-off value was determined based on the ratio of OD of serum sample over negative control OD value. With this, any serum samples with ratios > 2 were considered positive, and < 2 were considered negative (Johnson *et al*., 2000).

3.11.1 METHOD ONE

Negative control absorbance ± 3 SD :

Ratio of $\left(\frac{\text{Test Serum Absorbance}}{\text{Negative Control Absorbance}}\right)$	Result of test serum
$< \left(\frac{\text{Negative Control Absorbance - 3 SD}}{\text{Negative Control Absorbance}}\right)$	Negative
Between $\left(\frac{\text{Negative Control Absorbance ± 3 SD}}{\text{Negative Control Absorbance}}\right)$	Borderline
$> \left(\frac{\text{Negative Control Absorbance ± 3 SD}}{\text{Negative Control Absorbance}}\right)$	Positive

3.11.2 METHOD TWO

Ratio of: $\frac{\text{Sample Absorbance}}{\text{Negative Control Absorbance}}$

Ratio	Result
$\left(\frac{\text{Sample Absorbance}}{\text{Negative Control Absorbance}}\right) > 2$	Negative
$\left(\frac{\text{Sample Absorbance}}{\text{Negative Control Absorbance}}\right) < 2$	Positive

3.12 CALCULATION OF SENSITIVITY, SPECIFICITY, POSITIVE PREDICTIVE VALUE, NEGATIVE PREDICTIVE VALUE AND CONCORDANCE

The performance of C6/36 and Vero ELISA were calculated via both cut-off value methods with neutralization assay as reference. The sensitivity of ELISA was defined as proportion of neutralization assay-positive samples which tested positive with the ELISA. The specificity of ELISA was defined as the proportion of neutralization assay-negative sample which tested negative with ELISA. The probability that a sample is truly positive by neutralization when a positive result is observed by ELISA is known as the positive predictive value (PPV). The negative predictive value (NPV) is the probability that a sample is truly negative by neutralization when a negative result is obtained by ELISA. Concordance is the overall agreement between ELISA and the neutralization assay. A borderline result is recorded as a negative result.

ELISA	Gold Standard (Neutralization Assay)	
	Positive	Negative
Positive	A	B
Negative	C	D

Sensitivity = A / (A+C)

Specificity = D/ (B+D)

PPV = A / (A+B)

NPV = D / (C+D)

Concordance = (A+D) / (A+B+C+D)

4.0 RESULTS

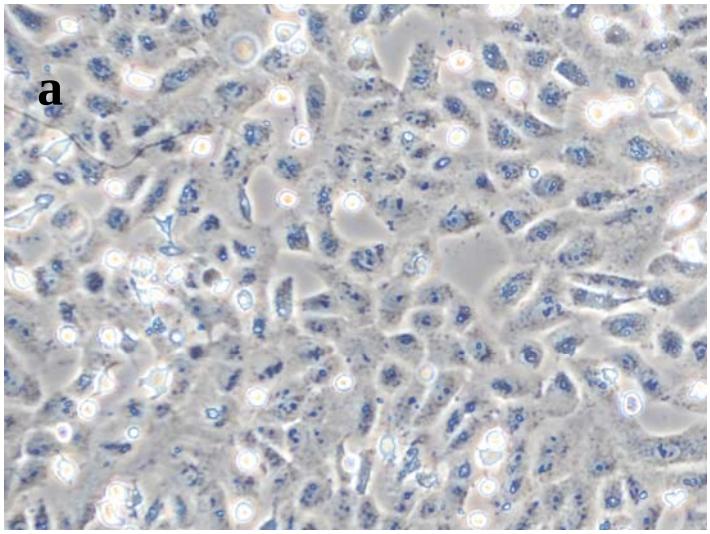
4.1 CONFIRMATION OF INFECTIVITY

4.1.1 OBSERVATION OF INFECTED CELL MORPHOLOGY

Two cell lines C6/36 and Vero were used to study the infectivity of CHIKV. Based on the coverage of the flask by the C6/36 cells infected with CHIKV resulted in more than 70% CPE three days after inoculation while the flask covered by Vero cells resulted in about 70% only two days after inoculation. The infected cells in tissue culture flasks were observed under microscope to appear as round floating cells with a distinct red colour compared to the healthy Vero (Figure 9) or C6/36 (Figure 10) cells. However all the mock-infected cells appeared as a monolayer of intact epithelial cells.

Figure 9: Morphology of Vero cell lines two days after virus inoculation.

The mock-treated cell divided rapidly while forming a monolayer on the tissue culture flask surface (a) while there were floating cells observed in the BP37348 infected cell line (b).


a

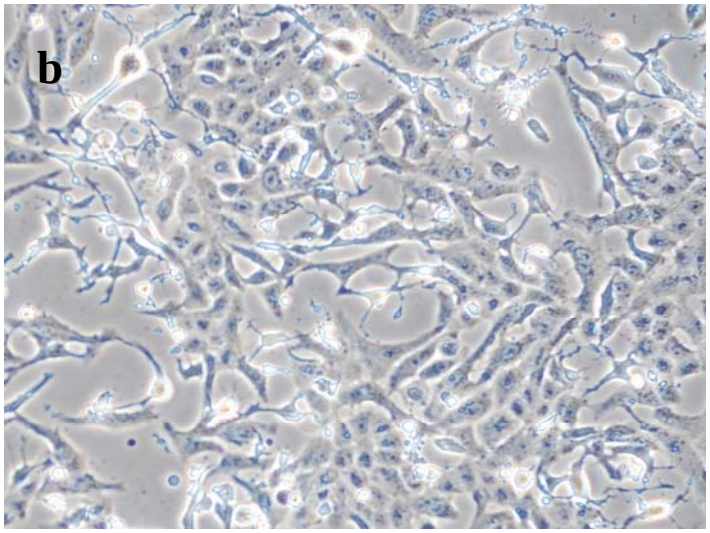
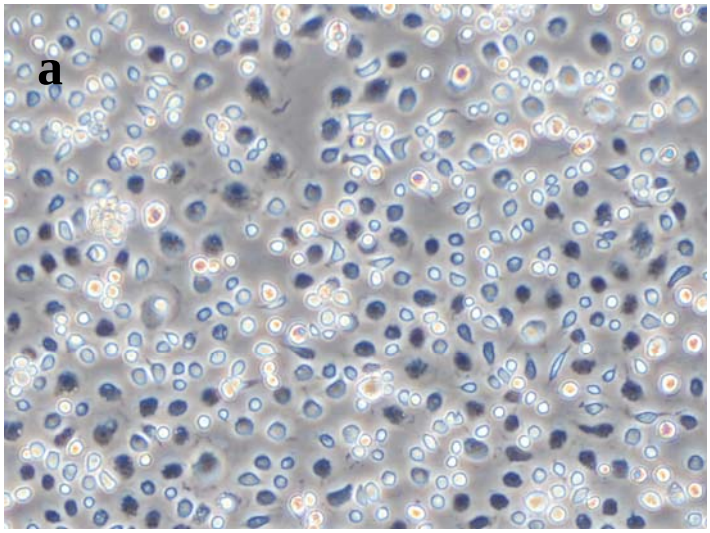
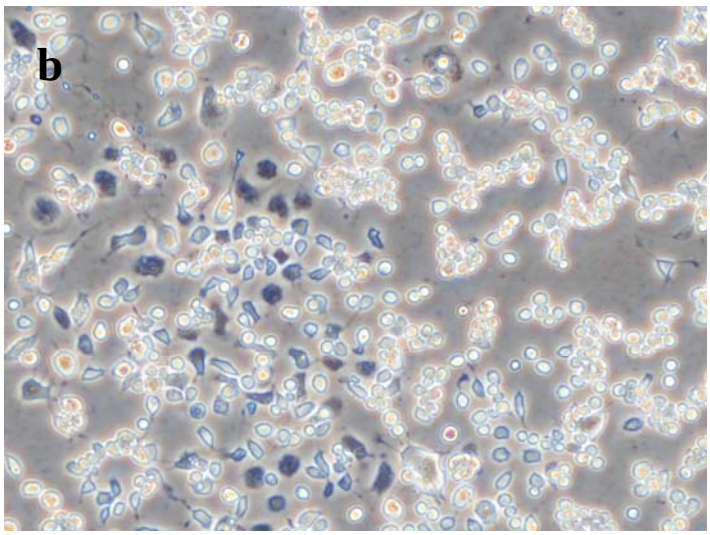

b

Figure 10: Morphology of C6/36 cell lines two days after virus inoculation. The mock-treated cell divided rapidly while forming a monolayer on the tissue culture flask surface (a) while there were floating cells observed in the BP37348 infected cell line (b).


a

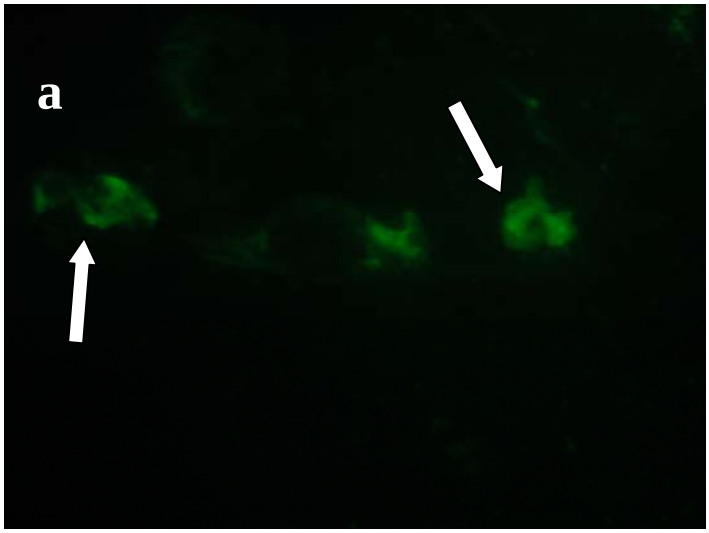

b

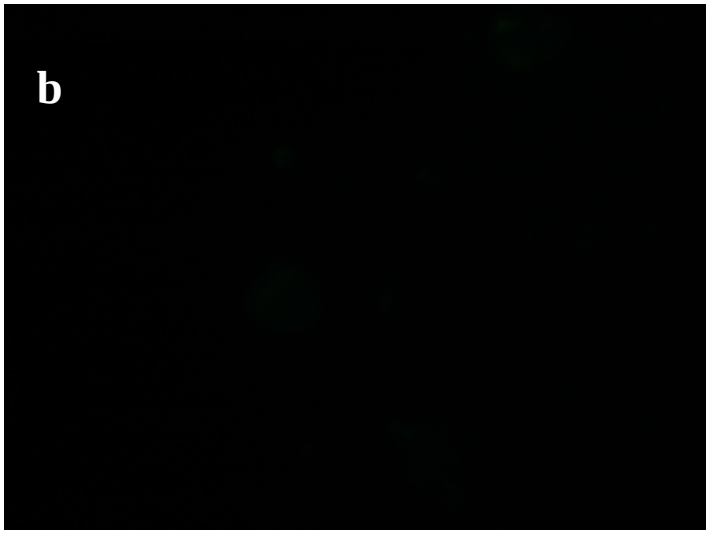
4.1.2 IMMUNOFLUORESCENCE STAINING

Vero cells infected with the CHIKV isolates used in the study (MY/0306/BP37348) were stained with IgG from known seropositive human serum followed by FITC-conjugated rabbit polyclonal anti-human IgG which was the positive serum. The CHIKV-infected Vero cells showed green fluorescence under a UV microscope (Figure 11). The stained mock-treated cells were later used as the negative control, with no fluorescence observed.

Figure 11: Confirmation of CHIKV infectivity in Vero cells using immunofluorescence staining.

Vero cells were infected with MY/0306/BP37348 stained with FITC-conjugated anti-human IgG and viewed at magnification of 400 x using a UV microscope (Zeiss, Germany). CHIKV-infected cells are shown with the help of the arrow (a) and mock-treated cells were used as negative control (b). The image shown is the negative of the photograph.


a

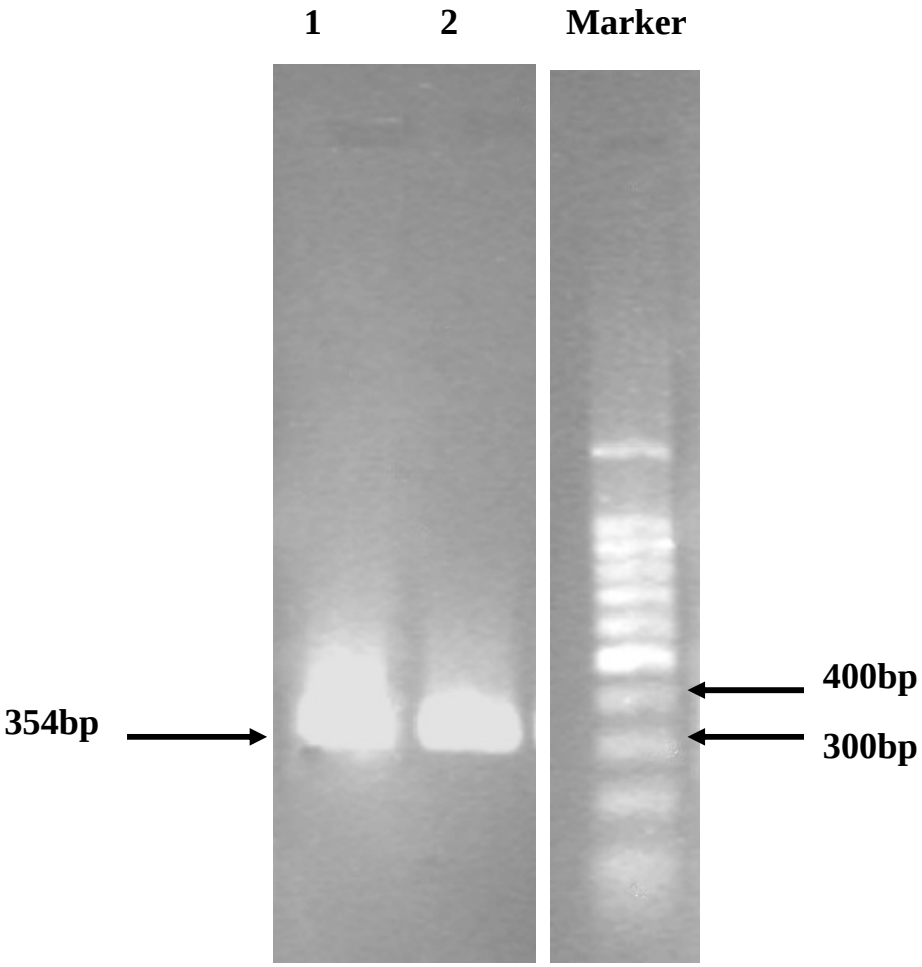

b

4.1.3 RT-PCR confirmation of CHIKV RNA

The RNA extracted from CHIKV-infected Vero cells were used as a template for the RT-PCR confirmatory test. The nsP1-S primers specifically targeted and amplified the nsP1 region within the viral genome was used. The CHIKV RNA presence was confirmed in the RNA sample extracted from the isolate used in this study (BP37438). The RT-PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide (Figure 12).

Figure 12: Confirmation of CHIKV virus from the RNA extracted from CHIKV-infected cells.

Primers utilized targeted the nsP1 region. The first lane (1) illustrates the DNA amplified from a CHIKV positive control with a fragment size of 354bp, while the second lane (2) was the amplification product from the CHIKV isolate (BP37348).


1
2
Marker
354bp
400bp
300bp

4.1.4 WESTERN BLOT

Early CHIKV Western Blot analysis had recognized a few regions of viral protein that were deemed to be immunogenic. The 52kDa E1 envelope glycoprotein, the 62kDa precursor for the envelope protein E1, and the capsid C protein of 36kDa were determined as the viral proteins which caused antigenic response (Sourisseau *et al*., 2007). Using the Bagan Panchor known positive pooled serum samples, Western blot analysis only managed to distinguish the capsid protein of approximately 30kDa region, which is closest to the expected 36kDa (Figure 13). For both the mock and infected cell lysate antigen, the presence of non-specific bands were seen at the 52kDa and 62kDa immunogenic band regions.

Figure 13: Western Blot detection using pooled Bagan Panchor human serum.

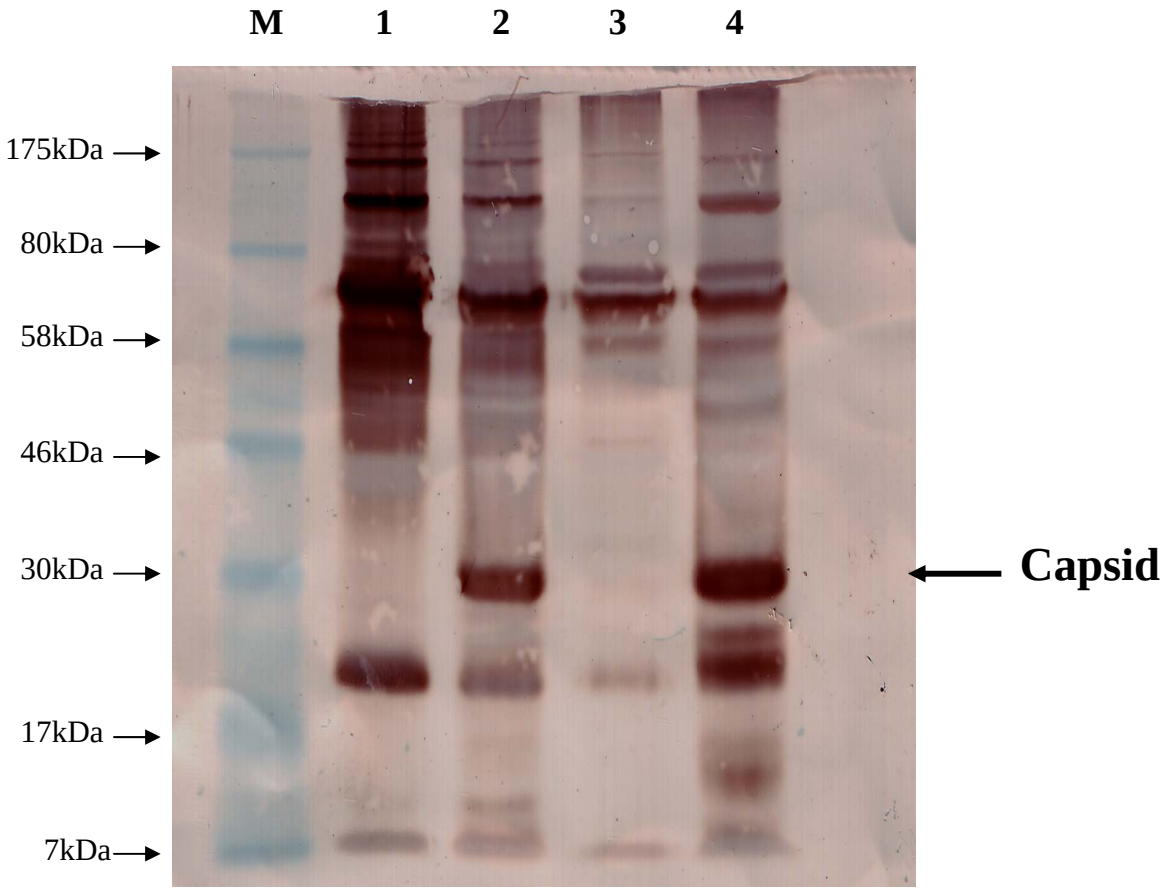
Lane 1, cell lysate of mock-infected C6/36 cells

Lane 2, cell lysate of CHIKV-infected C6/36 cell

Lane 3, cell lysate of mock-infected Vero cells

Lane 4, cell lysate of CHIKV-infected Vero cells

The western blot marker (M) used was Prestain Marker. The putative 36kDa capsid C protein was identified in both lane 2 and 4 respectively.


M
1
2
3
4
175kDa
80kDa
58kDa
46kDa
30kDa
17kDa
7kDa
Capsid

4.2 CHIKV CULTURE AND TITRATION

Two infected cell lines, C6/36 and Vero were observed daily to determine the optimum incubation time for harvesting virus. The C6/36-infected cells gave 70% CPE after the third day of inoculation (Figure 14), while the Vero-infected cells resulted in 70% CPE within two days of inoculation (Figure 15). For each cell line the CPE increased as each day progressed until it reached maximum CPE with most cells dead or floating. Therefore the optimum CHIKV harvest was obtained at at day 3 for C6/36 cells and day 2 for Vero cells. The end point titration value which caused 100% CPE was 10^{-4} for C6/36 cells and 10^{-5} for Vero cells.

Figure 14: Microscopic observation of Chikungunya virus-infected C6/36 cells at different time point.

The C6/36 cells inoculated with Chikungunya virus at different time point: 0 hour (1a), 24 hour (2a), 48 hour (3a) and 72 hour (4a) with its respective mock treated C6/36 cells as negative controls (1b, 2b,3b and 4b) for the time points studied.

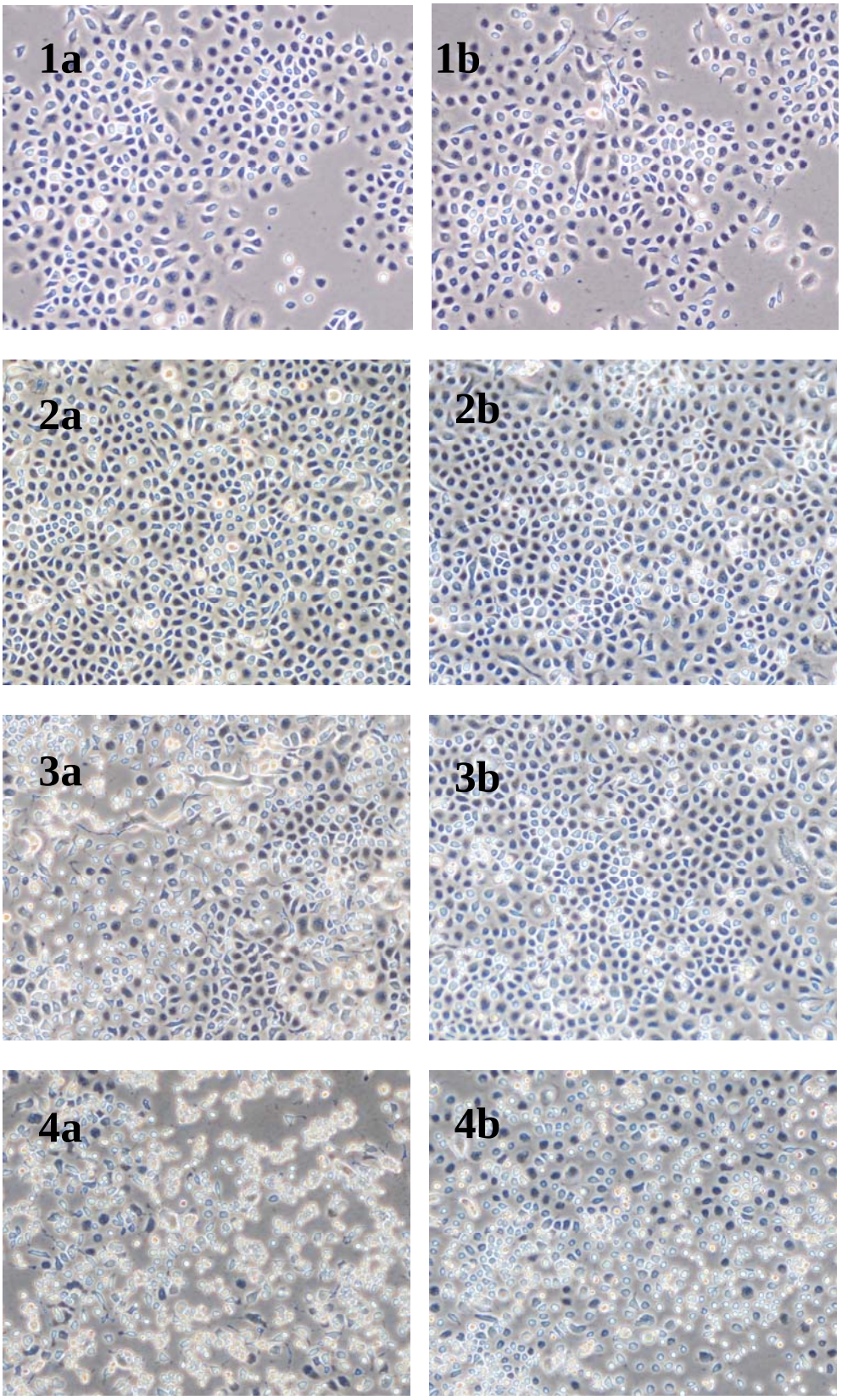
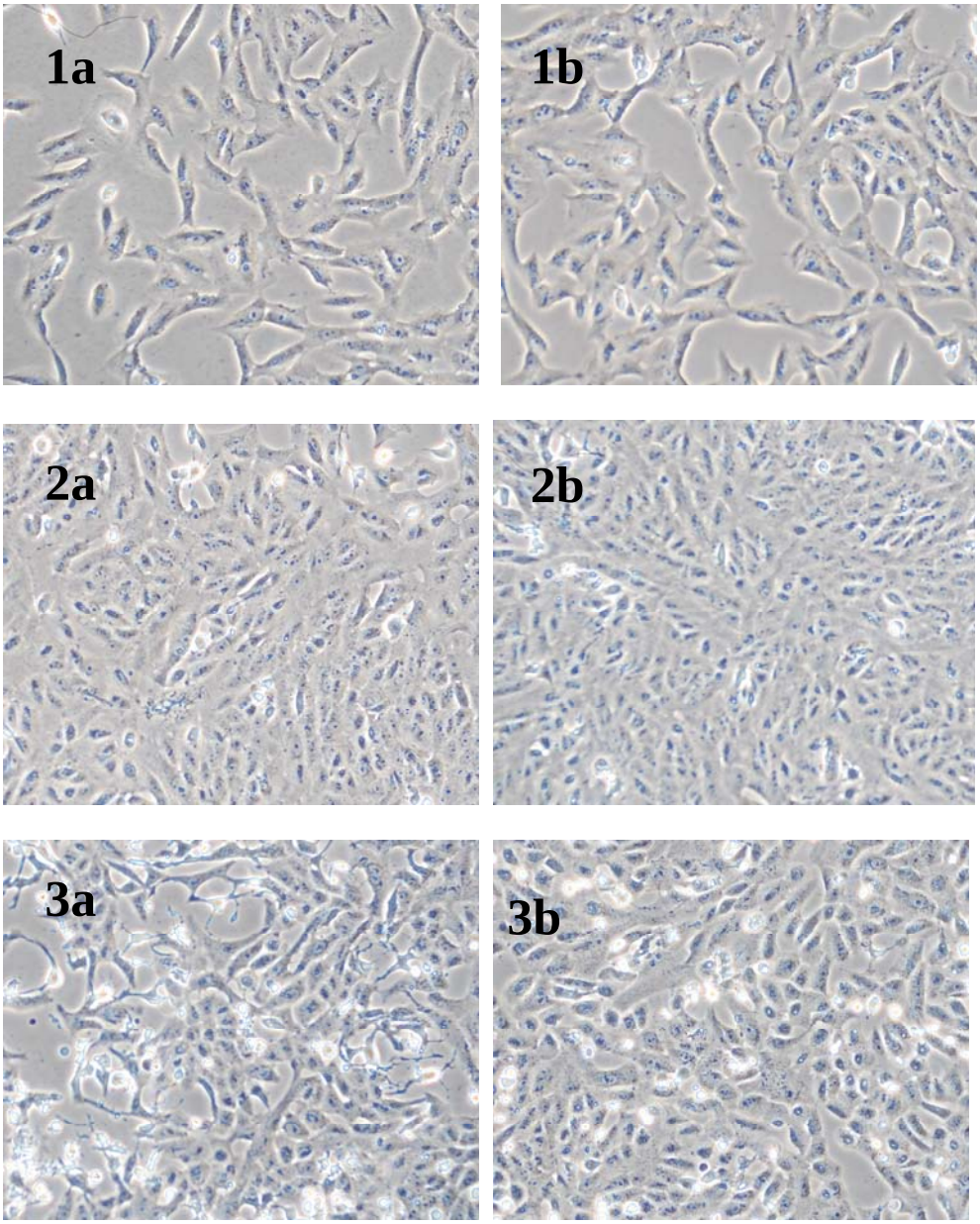

1a
1b
2a
2b
3a
3b
4a
4b

Figure 15: Microscopic observation of Chikungunya virus-infected Vero cells at different time point.

The Vero cells inoculated with Chikungunya virus at different time point: 0 hour (1a), 24 hour (2a) and 48 hour (3a), with its respective mock-treated Vero cells as negative controls (1b, 2b, 3c) for the time points studied.


1a
1b
2a
2b
3a
3b

4.3 QUANTITATING EXTRACTED PROTEIN

4.3.1 PROTEIN MICRO-BCA ASSAY

The cell lysate containing viral antigen extracted from CHIKV infected C6/36 and CHIKV-infected Vero cells were quantitated using micro BCA protein assay kit (Pierce, USA). Concurrently while harvesting the viral antigen protein, mock protein consisting of uninfected tissue culture for both C6/36 and Vero cells were also harvested. The concentrations for the extracted protein were calculated based on the curve of BCA protein assay (Figure 16) using the absorbance reading obtained (Table 1).

4.3.2 SDS-PAGE GEL

The quantitation was further validated via the Coomassie Blue method (Figure 17) as compared to the C6/36 mock and infected protein (lanes 3 and 4 of Figure 17). Many bands were observed in all the samples. However, no distinct band differences were observed between the samples (Figure 17).

Figure 16: BCA Protein Assay Curve

Protein is diluted 500X and protein concentration calculated based on the standard BCA protein Assay curve:

e.g. Absorbance of CHIKV-infected C6/36 cell lysate antigen = 0.101

Using Y= 0.0075X – 0.0016

From the graph:

x = protein concentration (µg/ml)

y = absorbance value

Curve slope = 0.0075 with y intercept value of 0.0016

Correlation coefficient (R^2) = 0.9799

This gives the equation y = 0.0075x – 0.0016 on y = mx + c for a linear curve.

Concentration µg/ml $= \frac{0.101 + 0.0016}{0.0075}$

$= 13.68 \times 500$

$= 6840$ µg/ml

$= \frac{6840}{1000}$ µg/ml

$= 6.840$ µg/µl

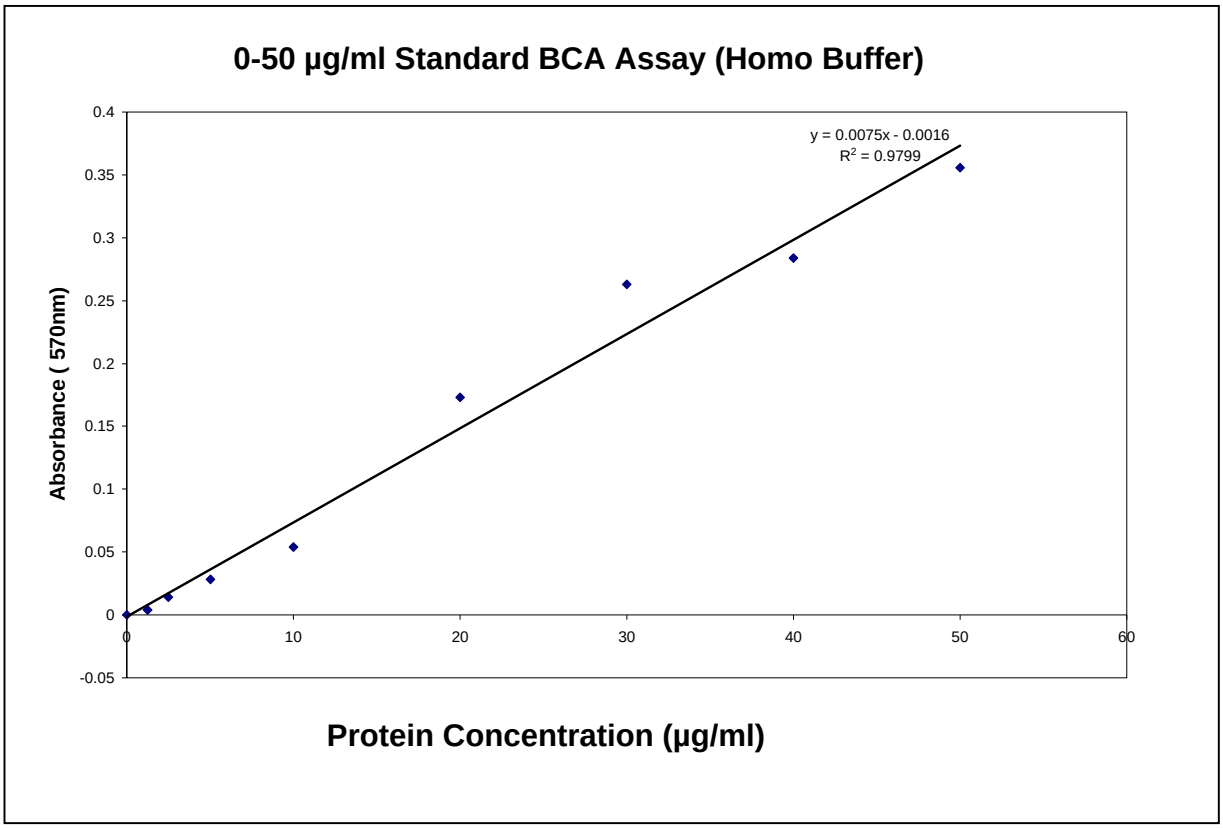

0-50 µg/ml Standard BCA Assay (Homo Buffer)
y = 0.0075x - 0.0016
R2 = 0.9799
Absorbance (570nm)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
0
10
20
30
40
50
60
Protein Concentration (µg/ml)

Table 2: Protein concentration calculated using standard Micro BCA assay curve.

	500 X diluted absorbance reading	Concentration (µg/µl)
C6/36 CHIKV	0.101	6.840
C6/36 Mock	0.074	5.040
Vero CHIKV	0.052	3.573
Vero Mock	0.030	2.106

C6/36 CHIKV: CHIKV-infected C6/36 cell lysate antigen

C6/36 Mock : Mock-infected C6/36 cell lysate antigen

Vero CHIKV : CHIKV-infected Vero cell lysate antigen

Vero Mock : Mock-infected Vero cell lysate antigen

Figure 17: Coomassie Blue-stained SDS-PAGE gel

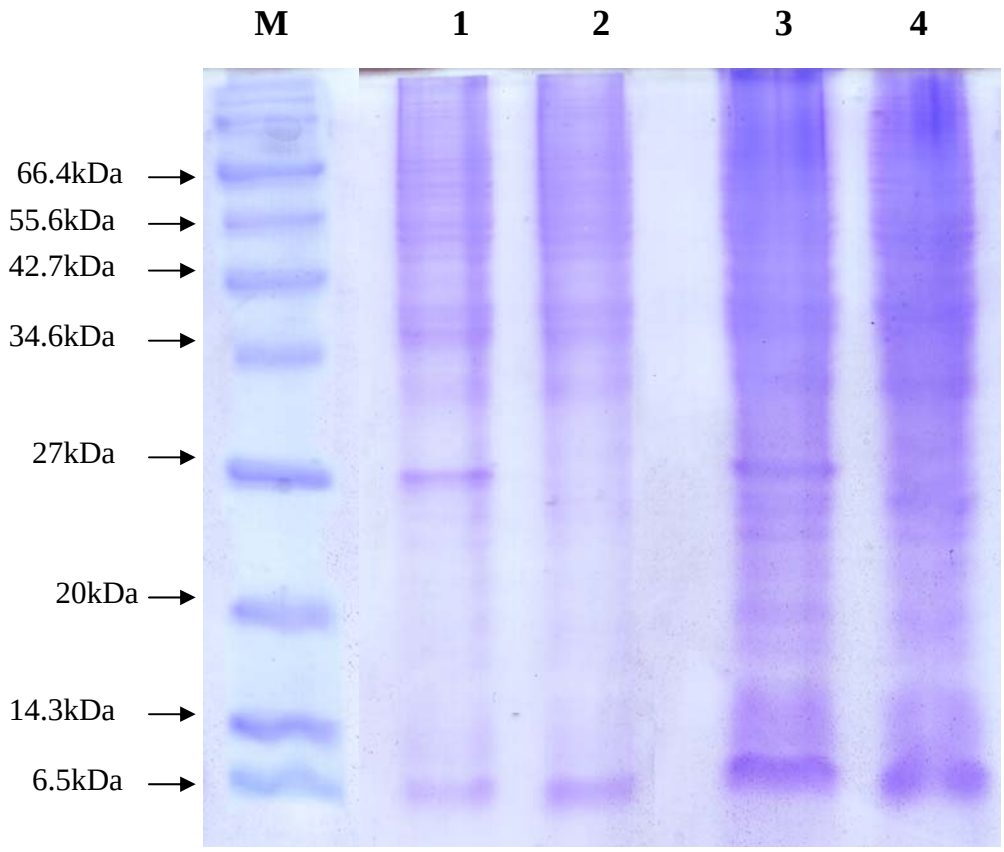
Lane 1, cell lysate of CHIKV-infected Vero cells

Lane 2, cell lysate of mock-infected Vero cells

Lane 3, cell lysate of CHIKV-infected C3/36 cells

Lane 4, cell lysate of mock-infected C6/36 cells

Broad range marker was used as the marker (M).


M
1
2
3
4
66.4kDa
55.6kDa
42.7kDa
34.6kDa
27kDa
20kDa
14.3kDa
6.5kDa

4.4 OPTIMIZATION OF IgG ELISA

4.4.1 SERUM DILUTION

Two pooled of positive and negative (Rubella confirmed patient) serums were used for optimization. For the ELISA optimization, two different serum dilutions 100X and 500X were tested. The former resulted in much higher absorbance value of 1 to 1.4 for C6/36 (Figure 18) and 0.8 to 1.2 for Vero (Figure 19) compared to the later which resulted with only 0.2 to 0.3 for C6/36 cells and 0.1 to 0.25 for Vero cells. Based on Figure 18 and Figure 19, 500X diluted serum resulted in a very low absorbance reading, 100X serum dilutions was chosen for the subsequent experiments for the current study.

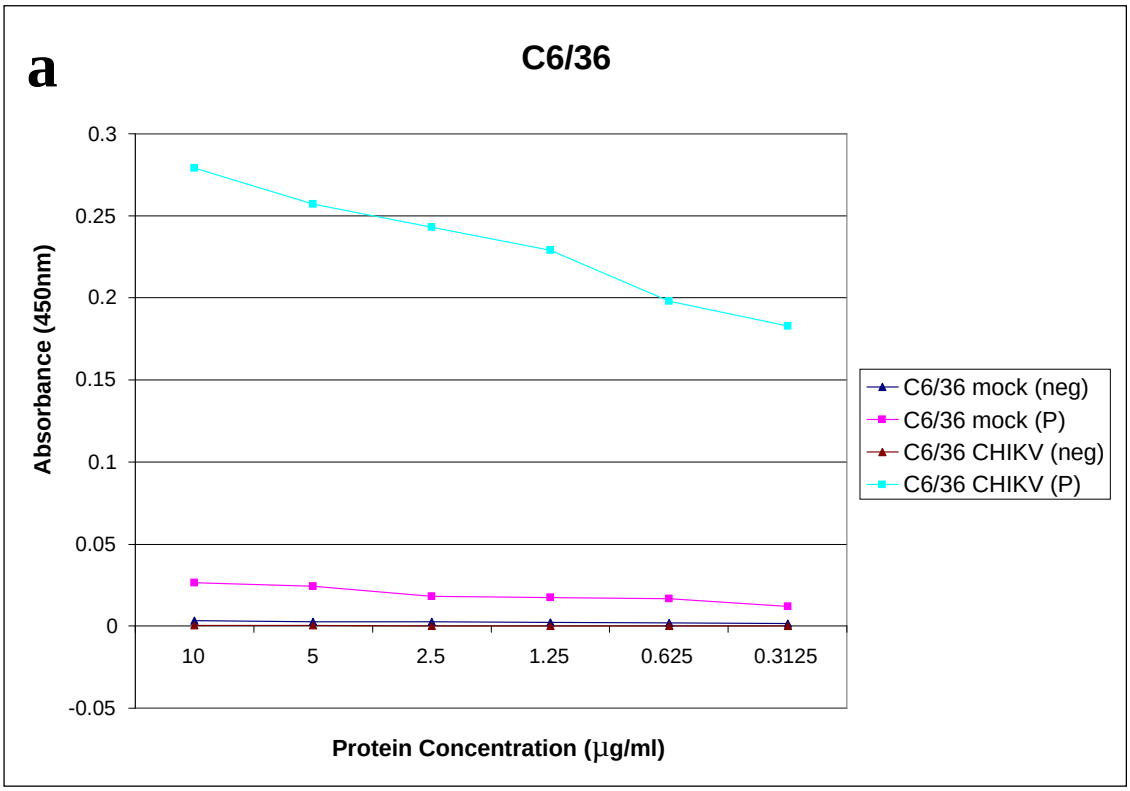
4.4.2 DOSE TRITRATION CURVE

The dose titration for plate coating was conducted with a range of protein concentration which was measured with 100X serum dilution. From the dose titration curve, it is clear that 10 µg/µl , 5 µg/µl and 2.5 µg/µl gave relatively good absorbance results, and with that 2.5 µg/µl were chosen as the optimal concentration for ELISA plate coating (Figure 20).

Figure 18: Graph of C6/36 ELISA absorbance with different serum dilutions against different protein concentrations in μg/ml.

Assay measured serum dilution of 500X (a) serum dilution of 100X (b).

The negative control serum was measured against mock cell lysate (mock (neg)) and CHIKV-infected cell lysate (CHIKV (neg)). Absorbance of CHIKV-seropostive serum as determined by neutralization assay were also measured against mock cell lysate (mock (P)) and CHIKV-infected cell lysate (CHIKV (P))


a
C6/36
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
Absorbance (450nm)
10
5
2.5
1.25
0.625
0.3125
Protein Concentration (µg/ml)
C6/36 mock (neg)
C6/36 mock (P)
C6/36 CHIKV (neg)
C6/36 CHIKV (P)

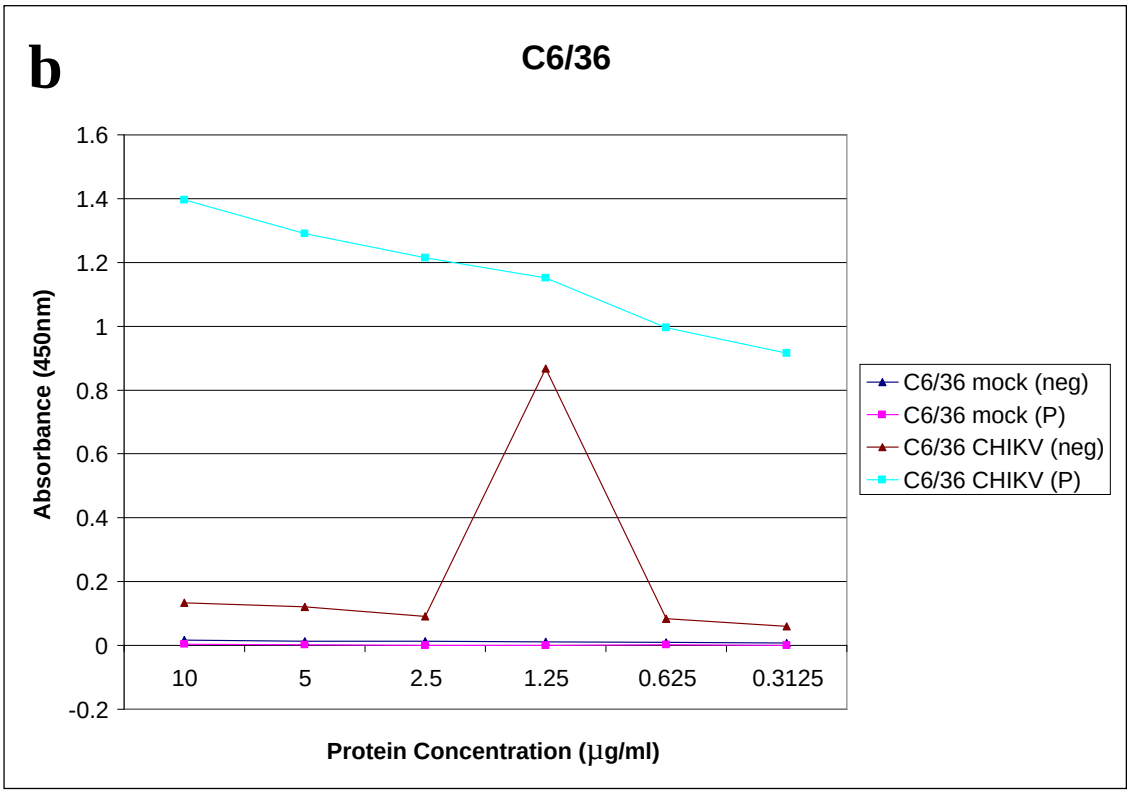
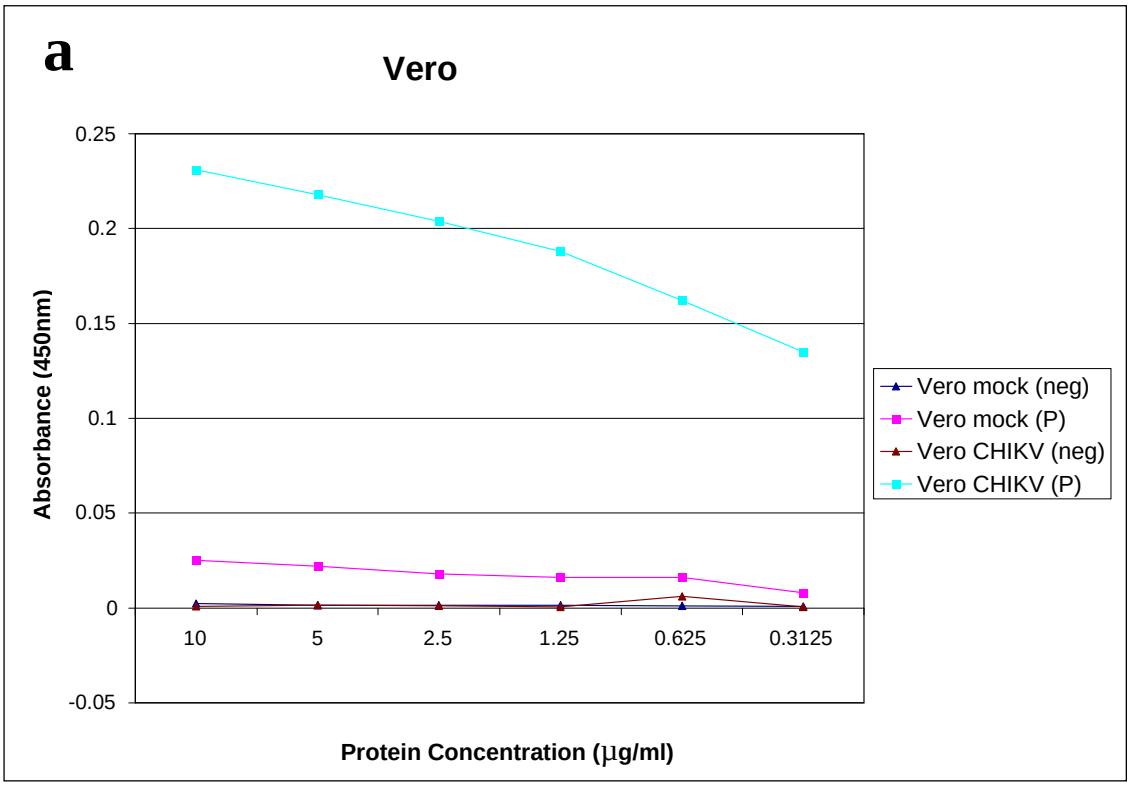

b
C6/36
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Absorbance (450nm)
10
5
2.5
1.25
0.625
0.3125
Protein Concentration (µg/ml)
C6/36 mock (neg)
C6/36 mock (P)
C6/36 CHIKV (neg)
C6/36 CHIKV (P)

Figure 19: Graph of Vero ELISA absorbance with different serum dilutions against different protein concentration in µg/ml.

Assay using serum dilution of 500X (a) and serum dilution 100X (b). The negative control serum absorbance was measured against mock cell lysate (mock (neg)) and CHIKV-infected cell lysate (CHIKV (neg)). Absorbance of CHIKV-seropostive serum as determined by neutralization assay were also measured against mock cell lysate (mock (P)) and CHIKV-infected cell lysate (CHIKV (P)).


a
Vero
0.25
0.2
0.15
0.1
0.05
0
-0.05
Absorbance (450nm)
10
5
2.5
1.25
0.625
0.3125
Protein Concentration (µg/ml)
Vero mock (neg)
Vero mock (P)
Vero CHIKV (neg)
Vero CHIKV (P)

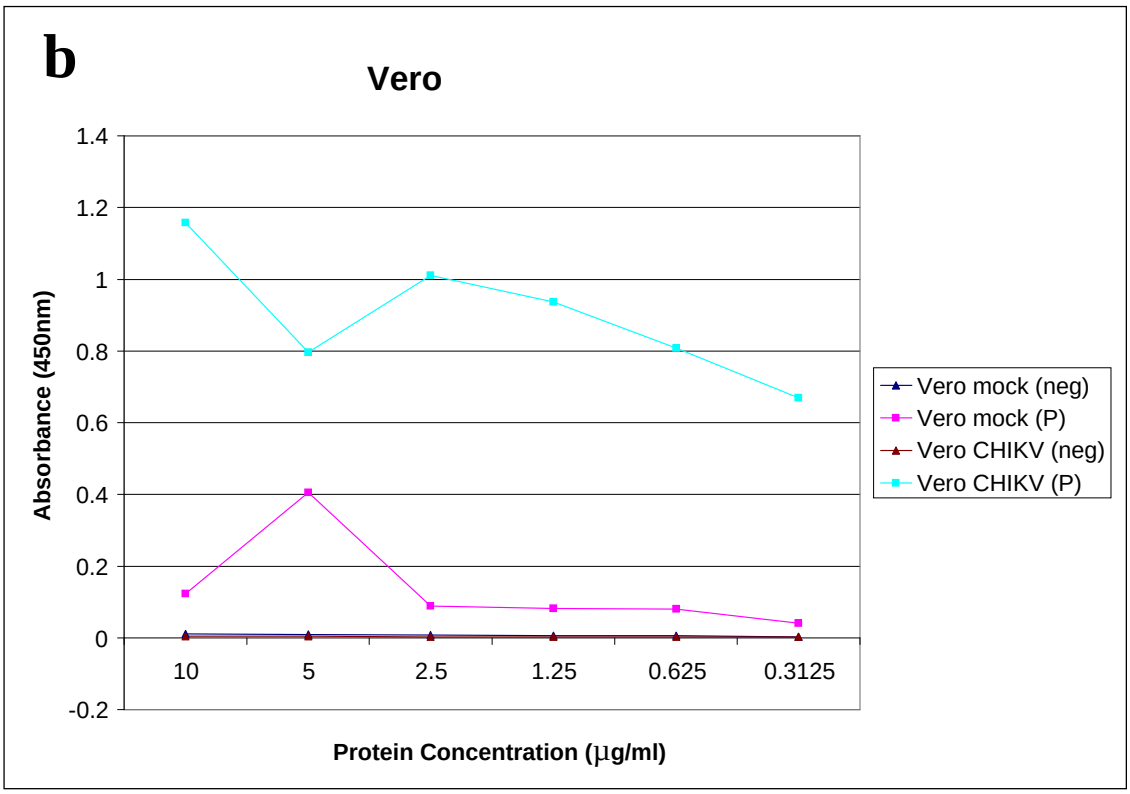
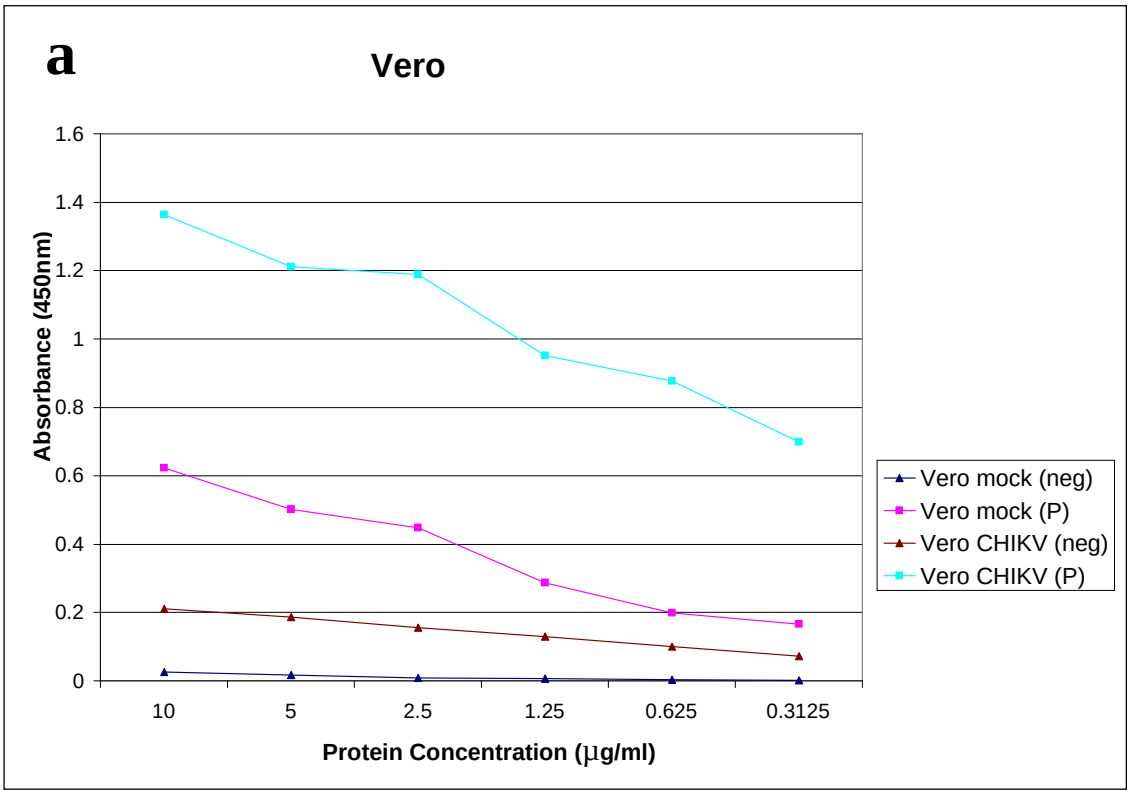
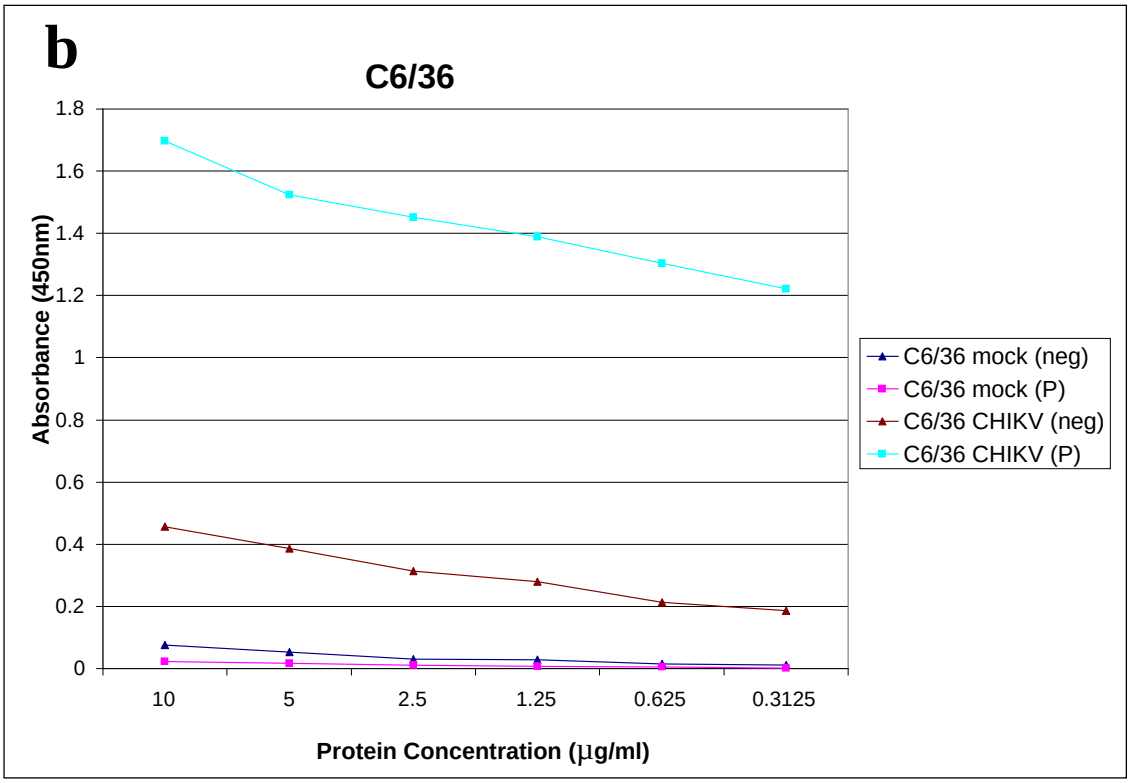

b
Vero
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Absorbance (450nm)
10
5
2.5
1.25
0.625
0.3125
Protein Concentration (µg/ml)
Vero mock (neg)
Vero mock (P)
Vero CHIKV (neg)
Vero CHIKV (P)

Figure 20: Graph of Dose titration for ELISA absorbance with 100X serum dilution against different protein concentration in µg/ml.

Dose titration was conducted for C6/36 ELISA (a) and Vero ELISA (b). The negative control serum absorbance was measured against mock cell lysate (mock (neg)) and CHIKV-infected cell lysate (CHIKV (neg)). Absorbance of CHIKV-seropostive serum as determined by neutralization assay were also measured against mock cell lysate (mock (P)) and CHIKV-infected cell lysate (CHIKV (P)).


a
Vero
Absorbance (450nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10
5
2.5
1.25
0.625
0.3125
Protein Concentration (µg/ml)
Vero mock (neg)
Vero mock (P)
Vero CHIKV (neg)
Vero CHIKV (P)


b
C6/36
Absorbance (450nm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10
5
2.5
1.25
0.625
0.3125
Protein Concentration (µg/ml)
C6/36 mock (neg)
C6/36 mock (P)
C6/36 CHIKV (neg)
C6/36 CHIKV (P)

4.5 THE INDIRECT IgG ELISA CUT-OFF VALUE

The cut-off value was calculated and determined for ELISA using two methods. Method one was using the negative control absorbance ± 3 standard deviation (SD) (Laras *et al*., 2005; Porter *et al*., 2004). The absorbance result was expressed and interpreted in ratio form of OD of serum sample over negative control optical density (OD) value. For the ELISA using the CHIKV-infected C6/36 viral antigen ('C6/36 ELISA'), a sample was considered positive if the OD ratio value was greater than 1.012. A sample was considered negative if the OD ratio was less than 0.978. It was considered as borderline, if the OD ratio lay between 0.978 and 1.012 (Table 2). In ELISA using CHIKV-infected Vero viral antigen ('Vero ELISA'), a sample was considered positive if the OD ratio value is greater than 1.010. However it is considered negative if the OD ratio is less than 0.990. A sample was considered borderline between 0.990 and 1.010 (Table 3).

In the second method, the cut-off value was determined based on the ratio of OD of serum sample over negative control OD value. Any serum sample with the ratio greater than 2 was considered positive and any ratio less than 2 was considered negative (Johnson *et al*., 2000).

Table 3: Cut off values for C6/36 ELISA using negative control absorbance ± 3 SD

Negative control absorbance ± 3 SD = 0.243 ± 3 (0.003)

This gives an absorbance range of 0.240 to 0.246 for a borderline result.

Test serum absorbance reading (in ratio)	Result of test serum
< 0.978	Negative
0.978 – 1.012	Borderline
> 1.012	Positive

Table 4: Cut off values for Vero ELISA using negative control absorbance ± 3 SD.

Negative control absorbance ± 3SD = 0.205 ± 3 (0.002)

This gives an absorbance range of 0.203 to 0.207 for a borderline result.

Test serum absorbance reading (in ratio)	Result of test serum
< 0.990	Negative
0.990 – 1.010	Borderline
> 1.010	Positive

4.6 NEUTRALIZATION TEST AFTER HEAT INACTIVATION

Nine samples of the 2006 outbreak in Bagan Panchor were chosen for the neutralization study. These serums were tested for neutralization activity after heat inactivation with 56°C for 30 minutes. Then highest neutralization titer obtained was 80. Another 4 samples had neutralization titers ranging from 10 to 40. Four samples showed no neutralization titer.

Table 5: Neutralization titres of 9 serum samples after heat-inactivation of serum.

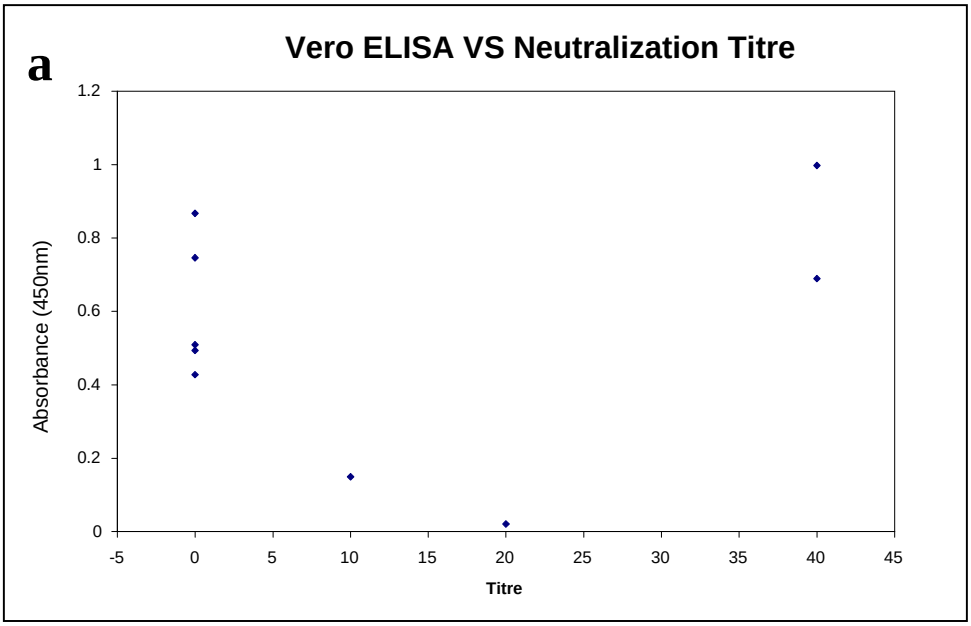
Patients	Symptoms	Neutralization Titer
1	YES	40
2	YES	20
3	YES	40
4	YES	10
5	NO	0
6	YES	80
7	NO	0
8	NO	0
9	NO	0

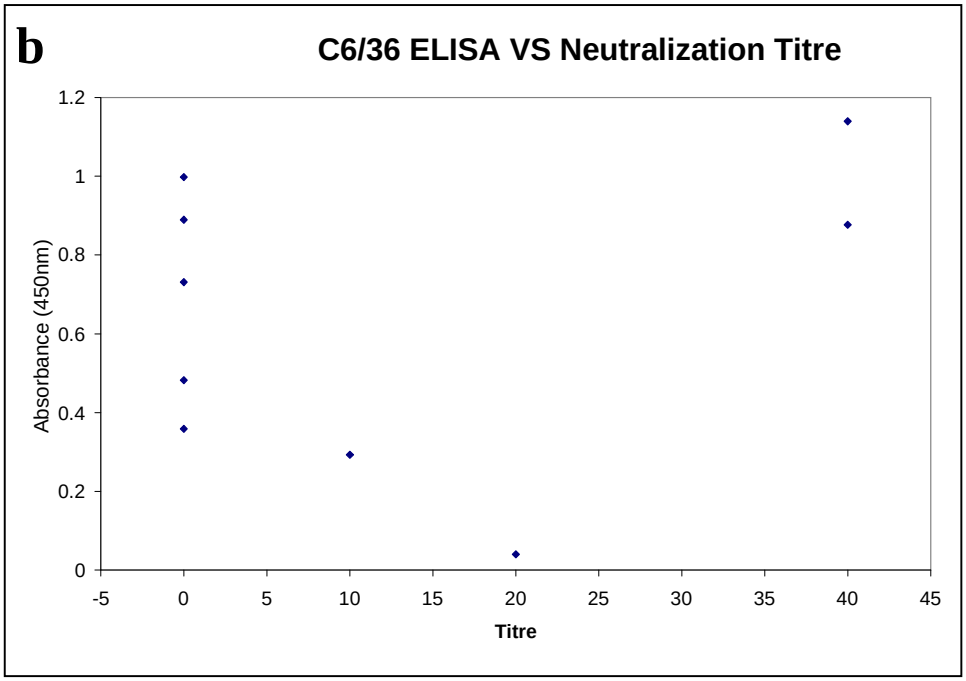
4.7 ASSOCIATION OF IgG INDIRECT ELISA WITH NEUTRALIZATION TITER

The C6/36 and Vero ELISA absorbance reading for the nine Bagan Panchor serum samples were compared with their respective neutralization titre (Figure 21). The associations of the values were calculated using Pearson correlation. Both Vero and C6/36 ELISA absorbance readings exhibited low correlation value with neutralization titre. Vero ELISA showed a correlation value of r=0.216 with a *p*-value of value of 0.22 while C6/36 ELISA showed a correlation of r=0.264 with a *p*-value of 0.27.

Figure 21: Graph of ELISA absorbance value against neutralization titre.

Association of ELISA absorbance reading measured against the neutralization titre for both Vero ELISA (a) and C6/36 ELISA (b) for nine samples.


a
Vero ELISA VS Neutralization Titre
Absorbance (450nm)
1.2
1
0.8
0.6
0.4
0.2
0
-5
0
5
10
15
20
25
30
35
40
45
Titre


b
C6/36 ELISA VS Neutralization Titre
Absorbance (450nm)
1.2
1
0.8
0.6
0.4
0.2
0
-5
0
5
10
15
20
25
30
35
40
45
Titre

4.8 SUMMARIZED IgG ELISA RATIO, NEUTRALIZATION TITER AND CLINICAL STATUS OF SERUM SAMPLES FROM 9 BAGAN PANCHOR RESIDENTS

The neutralization titres, ELISA reading (expressed as ratio of sample OD value over negative control OD value), ELISA results interpreted using the two cut-off methods, and clinical status for 5 laboratory-confirmed cases and 4 non-infected contacts were summarized (Tables 5 and 6). ELISA results which are highlighted in red are results discordant with neutralization titre, the gold standard.

Using cut-off method 1 all 5 laboratory-confirmed cases were detected by C6/36 ELISA and Vero ELISA (Table 5). Four cases were listed as ‘uninfected contacts’ who had no observation of clinical symptoms or neutralization titer. However, both Vero ELISA and C6/36 ELISA gave positive results for at least 75% of the samples. These false-positives could only mean that ELISA absorbance reading were a result of background reading and non-specific binding of antigens.

Table 6: Neutralization titre, ELISA readings interpreted using two cut-off methods, and clinical status for 5 laboratory-confirmed cases.

			VERO			C6/36		
Patient	Synptoms	Neutralization Titer	Absorbance ratio	Method 1	Method 2	Absorbance ratio	Method 1	Method 2
1	YES	40	10.579	Positive	Positive	11.111	Positive	Positive
2	YES	20	1.240	Positive	Negative	1.388	Positive	Negative
3	YES	40	8.474	Positive	Positive	9.656	Positive	Positive
4	YES	10	3.068	Positive	Positive	4.368	Positive	Positive
6	YES	80	11.081	Positive	Positive	9.318	Positive	Positive

Table 7: Neutralization titres, ELISA readings interpreted using two cut-off methods, and clinical status of four non-infected contact

			VERO			C6/36		
Patient	Synptoms	Neutralization Titer	Absorbance ratio	Method 1	Method 2	Absorbance ratio	Method 1	Method 2
6	NO	0	7.332	Positive	Positive	9.213	Positive	Positive
7	NO	0	0.833	Negative	Negative	1.023	Positive	Negative
8	NO	0	7.852	Positive	Positive	4.339	Positive	Positive
9	NO	0	9.887	Positive	Positive	10.980	Positive	Positive

4.9 SENSITIVITY, SPECIFICITY, POSITIVE PREDICTIVE VALUE, NEGATIVE PREDICTIVE VALUE AND CONCORDANCE OF IgG ELISA COMPARED TO NEUTRALIZATION ASSAY

With the neutralization assay as the reference standard, the performance for both Vero and C6/36 ELISA assay was evaluated for both the cut-off methods. Out of the nine samples tested, four patients with no neutralization titer had no clinical symptoms. The sensitivity, specificity, positive predictive value and negative predictive value were measured for the reported clinical symptoms with neutralization as the reference. Sensitivity, specificity, positive and negative predictive value were all 100% (Table 7).

Table 8: Neutralization titer versus clinical symptoms

	Neutralization Assay	
Symptoms	Positive	Negative
Yes	5	0
No	0	4

Sensitivity	100.0%
Specificity	100.0%
Positive Predictive Value	100.0%
Negative Predictive Value	100.0%
Concordance	100.0%

4.9.1 C6/36 ELISA VS NEUTRALIZATION TITER

The sensitivity, specificity, positive predictive value and negative predictive value were measured for the ELISA results with neutralization as the reference for seropositivity. Both ELISA using Vero and C6/36 cell lysate showed very similar sensitivity values ranging from 80-100% while the specificity was 0-25%. The positive and negative predictive values were 57.1-100%, respectively. The concordance for the ELISA assays ranged from 55.6-66.7% (Tables 8-12)

4.9.1.1 METHOD ONE

Table 9: C6/36 ELISA (Method one) vs neutralization titer

	Neutralization	
ELISA	Positive	Negative
Positive	5	4
Negative	0	0

Sensitivity	100.0%
Specificity	0%
Positive Predictive Value	55.6%
Negative Predictive Value	0%
Concordance	55.6%

4.9.1.2 METHOD TWO

Table 10: C6/36 ELISA (Method two) vs neutralization titer

	Neutralization	
ELISA	Positive	Negative
Positive	4	3
Negative	1	1

Sensitivity	80.0%
Specificity	25.0%
Positive Predictive Value	57.1%
Negative Predictive Value	50.0%
Concordance	55.6%

4.9.2 VERO ELISA VS NEUTRALIZATION TITER

The sensitivity, specificity, positive predictive value and negative predictive value were measured for the ELISA result with neutralization as the reference for seropositivity.

4.9.2.1 METHOD ONE

Table 11: Vero ELISA (Method one) vs neutralization titer

	Neutralization	
ELISA	Positive	Negative
Positive	5	3
Negative	0	1

Sensitivity	100%
Specificity	25.0%
Positive Predictive Value	62.5%
Negative Predictive Value	100.0%
Concordance	66.7%

4.9.2.2 METHOD TWO

Table 12: Vero ELISA (Method two) vs neutralization titer

	Neutralization	
ELISA	Positive	Negative
Positive	4	3
Negative	1	1

Sensitivity	80.0%
Specificity	25.0%
Positive Predictive Value	57.1%
Negative Predictive Value	50.0%
Concordance	55.6%

Table 13: Summary of sensitivity, specificity, positive predictive value, negative predictive value and concordance rates of Vero and C6/36 ELISA using both cut-off methods against neutralization assay as the gold standard.

	Vero		C6/36		Clinical Symptoms
	Method 1	Method 2	Method 1	Method 2	
Sensitivity	100.0%	80.0%	100.0%	80.0%	100.0%
Specificity	25.0%	25.0%	0.0%	25.0%	100.0%
Positive Predictive Value	62.5%	57.1%	55.6%	57.1%	100.0%
Negative Predictive Value	100.0%	50.0%	-	50.0%	100.0%
Concordance	66.7%	55.6%	55.6%	55.6%	100.0%

5.0 DISCUSSION

5.1 CONFIRMATION OF CHIKV

5.1.1 IMMUNOFLUORESCENCE ASSAY

Immunofluorescence assay (IF) in the current study was used to detect presence of CHIKV in cell cultures. The Vero and C6/36 cells which were grown and infected with the Bagan Panchor isolate BP37348, were tested for the presence of CHIKV using seropositive patients' sera collected from Bagan Panchor residents. Based on the indirect IF method, the immunoglobulin G (IgG) which are present in the positive patients' sera would react with the secondary anti-human antibodies conjugated with FITC to give a fluorescent effect. However the negative patients' sera would not exhibit such an effect due to absence of the antibody (AbuBakar *et al*., 2007). The mock-infected Vero and C6/36 cells were also tested as negative controls using both positive and negative patients' sera in which they did not give any fluorescent effect.

5.1.2. POLYMERASE CHAIN REACTION (PCR)

With the CHIKV RNA extracted either directly from the patients sera or from infected cell cultures, nucleic acid amplification was performed with 3 pairs of primers designed specifically for the non-structural protein 1 (nsP1) (AbuBakar *et al*., 2007). Following agarose electrophoresis, the correct sized 354bp DNA was obtained from the positive pools after amplification, confirming the presence of CHIKV infection.

5.1.3 IMMUNODETECTION OF CHIKV PROTEINS

The expression of the proteins were observed and analyzed using SDS-PAGE which was stained with Coomassie Blue. Banding patterns (Figure 17) observed showed the lanes with the presence of CHIKV viral lysate harvested from both Vero and C6/36 managed to exhibit a viral capsid protein of approximately 30-46kDa which is the closest to the expected CHIKV capsid protein of 36kDa (Sourisseau *et al*., 2007). A similar band was not detected in the mock infected cells in lanes 1 and 3 for both cells, which confirms that the corresponding protein band belongs to the CHIKV. The present analysis revealed the presence of CHIK-specific protein band of approximately 30-46 kDa. Based on the size, this protein is likely to be the 36 kD capsid protein reported previously (Shimizu et al., 1984).

Based on an earlier study, it has been reported that the Western blotting of CHIKV seropositive serum samples managed to detect 43.3% of capsid protein while 50% detected the E2 protein region and only 13% managed to detect the E1 region from the viral lysate mixture (Kowalzik *et al*., 2008). Some studies only managed to detect the structural protein E2 and capsid for CHIKV positive serum sample (Deshmane and Banerjee, 1983). This may be explained by the fact that CHIKV envelope protein E1 is hidden within the viral structure which reduces the likelihood of E1 exposure to host immune system (Cho *et al*., 2008). More likely, the differing detection of proteins between studies may reflect different methods of extracting protein lysate.

5.2 CHIKV INFECTIVITY IN C6/36 AND VERO CELLS

There are a few commonly used cells to propagate CHIKV such as BEAS (bronchial epithelial cell), MRC5 (primary lung fibroblasts), C6/36 (*Aedes albopictus*, mosquito cells) and Vero (African green monkey kidney cell) (Sourisseau *et al.,* 2007;Powers *et a*l., 2000; Hasebe *et al*., 2002; Shuffenecker *et al*., 2006; AbuBakar *et al*., 2007). All these cells were susceptible to infection and allowed viral production. Being the only known vector species transmitting CHIKV, C6/36 cells was chosen for this study together with Vero cells being the animal reservoir known for the CHIKV sylvatic transmission cycle in Africa. The virus infectivity observed on the cells exhibited the replication pattern of CHIKV. Vero cells revealed much higher sensitivity to CHIKV infection with fast and vital CPE display in several studies (Cunningham *et al*., 1975) and increased binding efficiency towards the host receptor compared to C6/36 cells (invertebrates) (Sourisseau *et al*., 2007). The difference between the invertebrate and vertebrate cells infection has been observed repeatedly among alphaviruses and expected to occur for CHIKV nonetheless. As individual host, vertebrate's immune system usually clears infections within days whereas an invertebrate supports lifelong infection. Alphavirus replication usually results in host cell apoptosis in vertebrates as in CHIKV in some human cells but not in invertebrates (Chevillon *et al*., 2008). The present study further proved the theory with much less significant CPE in C6/36 infected cells even after four days of inoculation compared to CHIKV-infected Vero cells which projected substantial CPE with just two days of inoculation (Figure 9 and 10).

5.3 INDIRECT ELISA IgG

Immunoglobulin G (IgG) is detectable in convalescent samples about 7-10 days after onset of symptoms and persists for years (Pialoux *et al*., 2007). Anti-arboviral IgG may be detected in patients using the ELISA format in which the inactivated antigen is applied as a direct coating onto plate. Non-specific background reaction maybe minimized with the use of ELISA capture method. The disadvantage of anti-IgG capture method is a lack of sensitivity since the anti-human IgG captures all IgG present in a serum sample which would cause competition from non-specific IgG (Johnson *et al*., 2000).

With the application of monoclonal antibodies, the background due to extraneous antibodies is eliminated, resulting in less frequent nonspecific reaction and further remove false-positive reaction caused by rheumatoid factor and less competition between antibody-antigen binding (Martin *et al*., 2000). However, due to time constraint, an in-house ELISA was designed to detect anti-CHIKV IgG antibodies from serum samples using CHIKV cell lysates.

ELISA is highly sensitive to conditions, and with slight changes in pH, incubation time, temperature and reagent concentrations, ELISA absorbance reading can be affected. In the current study, the serum dilution and antigen concentration for plate coating were optimized. The patients sera were diluted 100X and 500X while antigen concentrations used were 0.3125 µg, 0.625 µg, 1.25 µg, 2.5 µg, 5µg and 10µg.

The ELISA readings for CHIKV antigens prepared using C6/36 and Vero cells were compared using sera diluted 100 and 500 times, in which the 100X dilution was chosen as the optimal serum dilution (Figure 18 and 19). The 500X dilution however was too dilute

to measure as the absorbance reading was low, which was difficult to eliminate background readings. Identifying optimum serum dilution is crucial to retain standard absorbance measurement while eliminate the phenomenon known as the prozone effect, which is associated with the binding efficacy of antibodies and viral antigens. The lack of binding to antigen at high concentration of antibodies due to excess antibodies present resulting in the formation of complexes that hinder binding has been reported (Johnson *et al*., 2000)

For ELISA plate coating, achieving an optimal concentration of antigen is crucial as too little antigen will cause the ELISA to be less sensitive to identify low level of antibodies, while too much of antigen will give background reading. Thus the dose titration graph illustrates that the use of antigen concentrations of 10µg, 5 µg and 2.5 µg produces good performance (Figure 20). With factors like cost and reagent wastage, the antigen concentration 2.5 µg was chosen as the optimum concentration to coat the ELISA microtitre plate.

C6/36 and Vero mock protein in ELISA resulted in no specific binding towards the positive serum, in which the absorbance reading acquired for the serum binding towards mock protein was low with no significant variation between the mock proteins concentrations measured. However in the CHIKV-infected cells 6/36 and Vero cells lysate resulted in a dose dependent effect, in which the absorbance was improved with increasing protein concentration that further demonstrated serum binding to CHIKV antigen specifically in the indirect IgG ELISA assay.

5.4 THE CORRELATION BETWEEN NEUTRALIZATION TITER AND INDIRECT ELISA

Close linear correlation between neutralization assay and ELISA is crucial to develop a quantitative ELISA to measure antibody titre responses. Linear correlation is less critical for most commonly used diagnostic IgG ELISAs, which are quantitative assays which give positive, equivocal or negative results. In the current study, the IgG indirect ELISA was developed as a qualitative assay to examine seroprevalence and restrospective diagnosis. Correlation between neutralization and IgG indirect ELISA assays for the nine serum samples were measured using Pearson's correlation. Both Vero and C6/36 IgG indirect ELISA exhibited a weak correlation with neutralization assay titres.

The correlation study showed some serum samples with higher neutralization titre but with lower ELISA absorbance value. The crude viral lysate used as antigen in the ELISA assay could be a factor for the inconsistency of the result. There is also a lack of sensitivity of ELISA assay to measure serum samples with higher antibody concentration, which exhibits prozone effect due to improper use of serum dilution for the assay (Johnson *et al*., 2000). Factors like auto-immune antibodies and multiple non-neutralizing antibodies present within the serum samples displayed low neutralization titres but higher ELISA absorbance readings, due to non-specific binding with crude viral lysate antigen (Johnson *et al*., 2000).

5.5 THE CUT-OFF VALUE FOR INDIRECT ELISA

Cut-off values are important in ELISA assays, as they allow distinction between positive and negative results, as determined by the gold standard (in this case neutralization). It is essential to identify the appropriate cut-off value to eliminate non-specific cross-reaction (Akerstedt, 2002).

Cut-off values in the current study were calculated using two methods. In the first method, negative absorbance ± 3 SD was taken as the intermediate range, with absorbance values above this range considered positive, and values below this range taken as negative. The positive and negative cut-offs were converted into ratio of absorbance of test serum versus absorbance of negative control values (Laras *et al*., 2005).

The cut-off value determined using the second method was based on the ratio of absorbance of test serum against absorbance of negative control, in which the serum samples with a ratio greater than two were considered positive, and those with a ratio less than two were considered negative (Johnson *et al*., 2000). Based on the present study, method one was chosen over method two because it yielded greater sensitivity.

5.6 CHIKV SENSITIVITY, POSITIVE PREDICTIVE VALUE, NEGATIVE PREDICTIVE VALUE AND CONCORDANCE

Nine patients' serum samples with clinical data were tested to calculate the sensitivity, positive predictive value, negative predictive value and concordance for both the Vero and C6/36 ELISA measured with two cut-off methods against the gold standard, the neutralization assay. The serum samples were collected from residents of Bagan Panchor in 2007, some of who were related to CHIKV confirmed patients and some without any confirmation based on the 2006 outbreak. Presence of clinical symptoms had only 50% sensitivity as a diagnostic method for CHIKV infection (Pialoux *et al*., 2007). The presence of other possible causes of fever in some patients, for example dengue, explained the low positive predictive value of 80%. A negative value of 25% suggested the possibility of primary asymptomatic case or patients without proper recollection of previous symptoms. A previous study conducted in two separate areas in Indonesia also confirmed that the ratio of symptomatic to asymptomatic with CHIKV infection was 1.2:1 and 1.3:1 (Porter *et al*., 2004).

For both Vero and C6/36 ELISA, and with both cut-off methods used, resulted in very low (0-25%) specificity, indicating high false positivity rates. This data could be explained by the high background reading in ELISA, which could be the result of poor quality of crude viral lysate antigen used. Hence for future studies, pure viral antigens will be more beneficial.

In the current study, sensitivity was the primary concern because the IgG ELISA assay was originally proposed to employ as a screening assay. The higher the affinity of antibodies, the more sensitive the assay. Presence of background signals from the assay could also

result in poor sensitivity (Andreotti *et al*., 2003). Specificity and sensitivity have an inverse relationship and adjustment of cut-off method can be performed to boost the specificity and sensitivity rates to fit in with the aim of the study (Cuzzubbo *et al*., 1999). Concordance values of 55.7% for C6/36 ELISA and 66.7% for Vero ELISA based on method one and concordance value of 55.6% for both C6/36 and Vero ELISA measured based on method two. Thus concordance rates were marginally better using method one.

Based on the data collected, Vero ELISA gave more accurate result compared to C6/36 ELISA. Vero ELISA using the first cut-off method of negative control ± 3 SD gave the best performance, although specificity was still poor at 25% and as a result PPV was also low (57.1%). Low PPV means, that the ELISA assay might not be able to distinguish clearly samples which are truly positive that from negative ones, which could be a result of small number of samples that were tested against.

Time was the main constraint preventing further improvement of the low sensitivity and specificity of IgG ELISA. However, for future work, this indirect ELISA could be enhanced with the usage of a much better purified viral protein extraction to reduce contaminants that may give background readings. The assay should be further developed with more positive and negative patient sera. Developing an ELISA capture assay which is capable of detecting low levels of antibodies present in patients' serum can also enhance specificity and sensitivity. ELISA capture assay can be further improved by using recombinant protein of the CHIKV viral protein and anti-CHIKV monoclonal antibodies that could result in higher specificity with reduced non-specific binding compared to indirect ELISA.

6.0 CONCLUSION

The in-house indirect IgG ELISA designed was an appropriate technique as a qualitative detection tool. Although the use of Vero ELISA was more sensitive, overall specificity rates were low, meaning that further optimization is required before this assay may be considered acceptable for use.

To improve specificity, future work could focus on the development of an ELISA capture assay, using monoclonal anti-CHIKV antibodies. The serum samples obtained from Bagan Panchor outbreak exhibited a detectable immunological response against the 32kDa viral capsid protein. Therefore, the capsid protein would be possible candidate as a recombinant protein for use in the assay, which would offer much higher specificity with less cross-reactivity. A large amount of serum samples should have been tested to further validate the assay instead of using a small number. The ELISA assay should be tested for cross-reactivity against other vector-borne viruses such as dengue. Development of monoclonal or polyclonal antibodies for the ELISA detection would improve the specificity as well as the sensitivity of the method. And this can further enhance the development of ELISA capture method which is much preferred over indirect ELISA. The application of recombinant CHIKV antigen would also offer more specificity with less cross-reactivity.

APPENDIX

SOLUTIONS AND MEDIA

1. CELL CULTURE

a. Growth media for Vero and C6/36 cells (10% FBS EMEM)

1 X EMEM

EMEM powder (Flowlab, Australia)	10.5g
Milli-Q Water	to 1000.0ml

EMEM (2X)	50.0ml
FBS	10.0ml
NEAA (100X) (Flowlab, Australia)	1.0ml
Penicillin Streptomycin (5000IU/ml: 5000µg/ml)	1.0ml
L-glutamine (100X) (Flowlab, Australia)	1.0ml
Milli-Q water	to 100.0ml

b. Maintenance media for Vero and C6/36 cells (2% FBS EMEM)

EMEM (2X)	50.0ml
FBS	2.0ml
NEAA (100X) (Flowlab, Australia)	1.0ml
Penicillin Streptomycin (5000IU/ml: 5000µg/ml)	1.0ml
L-glutamine (100X) (Flowlab, Australia)	1.0ml
Milli-Q water	to 100.0ml

c. Serum free media (for dilution and virus stock)

EMEM (2X)	60.0ml
NEAA (100X) (Flowlab, Australia)	1.0ml
Penicillin Streptomycin(5000IU/ml: 5000µg/ml)	1.0ml
L-glutamine (100X) (Flowlab, Australia)	1.0ml
Milli-Q water	to 100.0ml

(All media solutions kept at 4ºC)

2. Polymerase Chain Reaction (PCR)

a. Solutions for RNA extraction

TRI-REAGENT (Molecular Research Center, Inc., USA)

Chloroform (Merck, Germany)

GR for analysis, 99.0-99.4 %

Isopropyl alcohol (Amresco, USA)

Biotech grade

75% ethanol (Merck, Germany)

Absolute ethanol, GR for analysis	75.0ml
Milli-Q water	to 100.0ml

RQ1 RNase-free DNase (Promega, USA)

1u/1µl

Nuclease free water (Promega, USA)

b. Solutions for agarose gel electrophoresis

0.5 X TAE buffer

10 X TAE buffer	50ml
RO water	to 1000.0ml

Ethidium bromide (10mg/ml)

Ehtidium bromide	100.0g
RO water	to 10.0ml

Loading dye (Promega, USA)

DNA ladder 100bp (Promega, USA)

3. ELISA

a. 10X Phosphate buffer saline (PBS) pH 7.5

Na_2HPO_4, 8mM (Merck,Germany)	114g
KH_2PO_4, 1.5mM (Merck, Germany)	2.0g
NaCl, 0.14M (Merck, Germany)	80.0g
KCl, 2.7 mM (Merck,Germany)	2.0g
Milli-Q water	to 1 L

(pH titrated to pH 7.5. Autoclaved for 20 minutes at 121ºC. Kept at room temperature. Fresh 1X PBS was prepared by diluting with autoclaved mili-Q water when required. Solution kept at 4ºC)

b. Homogenization buffer

Tris-HCl, pH 7.5, 2M (invitrogen, USA)	5.0 ml
KCl, 1.5M (Merck, Germany)	100.0ml
$MgCl_2$, 0.2M (Merck, Germany)	10.0ml
$CaCl_2$, 0.2M (Merck,Germany)	10.0ml
Milli-Q water	to 1 L

(Solution autoclaved for 20 minutes at 121ºC. Kept at 4ºC.)

c. Carbonate bicarbonate buffer (0.05M, pH 9.6)

Sodium carbonate anhydrous (Sigma,USA)	0.53g
Sodium hydrogen carbonate (Sigma,USA)	0.42g
Milli-Q water	100.0ml

(pH titrated to pH 9.6. Autoclaved for 20 minutes at 121ºC. Kept at 4ºC)

4. WESTERN BLOT

a. SDS-PAGE 10% separating gel

1.5M Tris-HCl, pH 8.8 (Invitrogen, USA)	4.0ml
10% SDS (Bio-Rad,USA)	160.0µl
Milli-Q water	6.36ml
30% Bis/acrylamide (GE Healthcare, USA)	5.34ml
10% Ammonium Persulphate	160.0µl
TEMED (Invitrogen, USA)	12.0µl

b. SDS-PAGE 4% stacking gel

0.5MTris-HCl; pH 8.8 (Invitrogen, USA)	1.25ml
10% SDS (Bio-Rad,USA)	50.0µl
Mili-Q water	2.98ml
30% Bis/acrylamide (GE Healthcare, USA)	0.67ml
10% Ammonium Persulphate	40.0µl
TEMED (Invitrogen,USA)	3.0µl

c. 3X SDS buffer

Tris-HCl, 0.5M, pH 6.8 (Invitrogen, USA)	3.75ml
SDS (Bio-Rad,USA)	0.6g
100% Glycerol (GE Healthcare, USA)	3.0ml
0.1% Bromophenol blue (Bio-Rad, Sweden)	3.0ml
Dithiothreitol (GE Healthcare, USA)	0.465g
Milli-Q water	to 100.0ml

(Kept at -20ºC)

d. 10X Tank buffer

Tris-base (Invitrogen, USA)	30.0g
Glycine (Sigma,USA)	144.0g
Mili-Q water	to 1 L

(Autoclaved for 20 minutes at 121°C then 10g SDS added. Kept at room temperature. Fresh 1X tank buffer prepared by diluting with autoclaved mili-Q water.

e. 10X Anode buffer I (0.3M Tris-base, 10% methanol, pH 10.4)

3M Tris-base (Invitrogen, USA)	181.71g
Milli-Q water	to 500.0ml

(pH titrated to pH 10.4. Autoclaved for 20 minutes at 121ºC. Kept at 4ºC. Fresh 1X Anode buffer I prepared by diluting with mili-Q water and added with methanol when needed. Solution kept at 4ºC)

f. 10X Anode buffer II (25 mM Tris, 10% methanol, pH 10.4)

Tris-base (Invitrogen, USA)	15.14g
Milli-Q water	to 500.0ml

(pH titrated to pH 10.4. Autoclaved for 20 minutes at 121ºC. Kept at 4ºC. Fresh 1X Anode buffer II prepared by diluting with mili-Q water and added with methanol when needed. Solution kept at 4ºC)

g. 10X Cathode buffer

Tris-base (Invitrogen, USA)	15.14g
Glycine (Sigma, USA)	15.01g
Milli-Q water	to 500.0ml

(pH titrated to pH 10.4. Autoclaved for 20 minutes at 121ºC. Kept at 4ºC. Fresh 1X Cathode buffer prepared by diluting with mili-Q water and added with methanol when needed. Solution kept at 4ºC)

h. Coomassie blue stain

Coomassie blue G250 (Bio-Rad, USA)	0.25g
Methanol (Merck, Germany)	400.0ml
Acetic Acid (Merck, Germany)	70.0ml
Milli-Q water	to 1 L

(Solution kept at room temperature)

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