Review of Literature
Group B: Chikungunya
2. Review of literature

2.1 Chikungunya Virus (causative agent of Chikungunya)

Chikungunya Virus (CHIKV), also known as Buggy Creek Virus belongs to the Family - Togoviridae, Group: Group IV ((+) ssRNA), Genus - Alpha virus and Species: Chikungunya virus. It was first isolated from the blood of an infected patient in Tanzania in 1953. CHIKV was originally described in the early 1950s after an outbreak in a Swahili village on the Makonde plateau that lies between Tanzania and Mozambique (Envis newsletter). The translation of Chikungunya from Makonde means "illness of the bent walker." In India, it is known as Aakyda, meaning "stiff man" and Maakyda meaning "monkey-like". These words refer to the arthritic condition that occurs in some patients which gives rise to a stooped posture. The virus was first found in Asia, isolated in Bangkok, Thailand in 1958. Chikungunya virus is an alpha virus closely related to the o'nyong'nyong virus (Vanlandingham DL et al., 2005), the Ross River virus in Australia, and the viruses that cause eastern equine encephalitis and western equine encephalitis (Martin Enserink et al., 2007). Three genotypes of this virus have been described: West African, East/Central/South African and Asian genotypes (Powers AM et al., 2000). The major clinical symptom of CHIKV infection is febrile illness, which is clinically similar to symptoms of Dengue virus infection (Karabatsos N et al., 1975). Both these viral diseases are transmitted by the same species of the mosquitoes Aedes aegypti and Aedes albopictus, and mixed outbreak of CHIKV with sporadic cases of Dengue virus (DENV) has been reported in Andhra Pradesh state, India (Mourya DT et al., 2006). However, unlike DENV infection, CHIKV infection is rarely fatal and usually does not require close clinical supervision. Therefore, the ability to distinguish CHIKV infection from Dengue virus infection would be important to launch control measures, particularly in areas where Dengue virus infection is endemic or epidemic.

2.1.1 Transmission

Chikungunya is generally spread through bites from Aedes aegypti mosquitoes, but recent research by the Pasteur Institute in Paris has suggested Chikungunya virus strains in the 2005-2006 Reunion Island outbreak incurred a mutation that facilitated transmission by Asian tiger mosquito (Aedes albopictus) (Martin E et al., 2007). Concurrent studies by arbovirologists at the University of Texas Medical Branch in Galveston, Texas, confirmed definitively that enhanced Chikungunya virus infection
of *A. albopictus* was caused by a point mutation in one of the viral envelope genes (E1) (Tsetsarkin KA et al., 2007). Enhanced transmission of Chikungunya virus by *A. albopictus* could mean an increased risk for Chikungunya outbreaks in other areas where the Asian tiger mosquito is present. A recent epidemic in Italy was likely perpetuated by *A. albopictus*. Although in some cases Culex has also been reported for the transmission of virus (Schuffenecker I et al., 2006, Diallo M et al., 1999, Vanlandingham DL et al., 2005). Transmission of Chikungunya virus by Anopheles Stephens is also reported in a recent Indian study (Yadav P et al., 2003). The common reservoirs for Chikungunya virus are monkeys and other vertebrates but the role of cattle and rodents has also been reported in the transmission of the virus in some cases (Diallo M et al., 1999). In the current outbreak macaque monkeys, lemurs and bald mouse have reported to be suspected reservoirs.

In Africa, Chikungunya is spread via a sylvatic cycle in which the virus largely resides in other primates in between human outbreaks (Martin E et al., 2007). The physicians presumed the virus might be able to be transmitted from a mother to her fetus, but without laboratory confirmation (CDC) (2006). Chikungunya virus is transmitted by mosquitoes of the genus *Aedes* such as *A.aegypti* and *A. albopictus* in Asia (Turell MJ et al., 1992; Tesh RB et al., 1992; Banerjee K et al., 1988) and *A. frucifer, A. hutocephalus, A. taylori* in Africa (Diallo M et al., 1999) (Fig 2.1). *A. albopictus* was the principal vector in the outbreaks in the Indian Ocean islands and *A. aegypti* in the 2006 Indian epidemic (Yergolkar PN et al, 2006). Regional differences in the mosquito species exist: *Anopheles* is a predominant circulating vector species in the rural areas of Orissa and Madhya Pradesh (Sharma SK et al., 1999; Singh N et al., 2006) and *A. albopictus* in Tamil Nadu (Paramasivan R et al., 2006) and Southeast Asia (Gratz NG et al., 2004). Several attributes make *A. aegypti* an efficient vector for the Chikungunya virus: it is highly susceptible to the virus, prefers to live close to people, seeks a blood meal during the day time and bites almost painlessly several people in a short period for one blood meal (Gibbons RV et al., 2002). The mosquito, well adapted to life in urban settings (Wilder Smith A et al., 2005).

### 2.2 Virus Genome

The Chikungunya virion consists of an envelope and a nucleocapsid. Virions are spherical and measure about 70nm in diameter (Fig: 2.2). Surface projections are glycoprotein spikes covering the surface evenly. Alpha viruses have conserved domains that play an important role in the regulation of viral RNA synthesis.
Life cycle of Chikungunya virus in Africa showing the interconnection between the sylvatic cycle and the urban cycle.
These domains are found at the 5’ and 3’ ends as well as at the intergenic region. Virions located on the surface of the cell membrane enter the host cells by fusion and endocytosis of the viral envelope. The uncoating of the virion occurs in the cytoplasm. The site of mRNA transcription is in the cell cytoplasm. Replication is not restricted to a particular tissue or organ of the host so the virus replication occurs in various organs. The insect host initiates the virus replication. The genome replication is done in the cytoplasm.

Chikungunya is caused by an enveloped, positive-single strand RNA molecule of approximately 12000 nucleotides long. A subgenomic positive-strand RNA referred to as 26S RNA, identical to the 3’ of the genomic RNA, is transcribed from a negative-stranded RNA intermediate. This RNA serves as the mRNA for the synthesis of the viral structural proteins.

**Figure 2.2 Structure of Chikungunya viruses**

According to the genomic organization of other alpha viruses, the genome of CHIK is considered to be: 5’ capnsP1-nsP2-nsP3-nsP4-(junction region)-C-E3-E2-6KE1-poly(A) 3’ (Fig 2.3). The 5’ end is capped with a 7-methylguanosine while the 3’ end is polyadenylated. The virus encodes 9 genes, consisting of coding sequences for non-structural polyproteins (precursor for nsP1–nsP4 proteins), structural polyproteins (precursor for C, E1–3 and 6K proteins), and polyadenylation site, flanked by 5’ and 3’ sequences (Khan AH et al., 2002).

**Fig 2.3 Genome Structure of Chikungunya virus**
An extensive genomic analysis of recent clinical CHIKV isolates from the Indian Ocean outbreak identified unique molecular features when compared to the few previously available sequences from laboratory-adapted CHIKV (Schuffenecker I et al., 2006). A specific mutation in E1 (Ala226Val) was absent in the initial viral strains but was observed in >90% of the later strains (Schuffenecker I et al., 2006). Interestingly, in the related alpha virus SFV the amino acid residue at position 226 regulates cholesterol dependency during the virus host cell fusion process (Chatterjee PK et al., 2002). The CHIKV envelope protein E1 and E2 are components of spikes, which composed of triplets of a heterodimer of E1 and E2 glycoproteins, and cover the viral surface in the form of membrane anchored types. The viral spike proteins facilitate attachment to cell surfaces and viral entry into the cells.

The E1 envelope protein is a class II fusion protein that mediates low pH-triggered membrane fusion during virus infection. The E2 envelope protein is a type I transmembrane glycoprotein and has been known to be responsible for receptor binding during the course of the alpha virus cycle (Kielian M et al., 2006; Brehin AC et al., 2008). The efficiency of alpha viral entry depends on the host cell membrane composition (including the levels of cholesterols, which mosquitoes obtain through blood meals). A mutation that affects cholesterol dependency could improve the ability of CHIKV to infect insect cells by providing a better adaptation to the lipid composition of these cells. Indeed, experimental infection of A. albopictus showed that the early viral strains were not as successful at replicating in this mosquito as later, mutated viruses (Tssetarkin KA et al., 2007; Vazeille M et al., 2007). The E1 Ala226Val mutation is directly responsible for a substantial increase in CHIKV infectivity for A. albopictus and leads to more efficient viral dissemination into mosquito secondary organs and transmission to suckling mice (Tssetarkin KA et al., 2007). Both early and late viruses invaded salivary glands in a similar pattern, but the crossing of the mid gut epithelium, one of the primary sites of infection (Tssetarkin KA, 2007; Vazeille M et al., 2007; Vanlandingham DL et al., 2005) was a crucial step that made A. albopictus particularly susceptible to later CHIKV isolates (Vazeille M et al., 2007). Interestingly, this mutation has no effect on viral replication in A. aegypti (Tssetarkin KA., 2007). Moreover, the E1 Ala226Val mutation facilitates viral replication in cholesterol-depleted C6/36 mosquito cell (Vazeille M et al., 2007). Other mutations that have been identified recently in E2 also regulate CHIKV adaptation to its mosquito hosts (Tssetarkin KA et al., 2009). Whether the enhanced ability of later CHIKV isolates to invade A. albopictus relates to cholesterol dependency has not been proved yet, but these observations strongly suggest that the rapid evolution of CHIKV conferred a selective advantage on the virus to infect and replicate in A. albopictus. Of note, both early and late CHIKV isolates replicated similarly in various human cells (Sourisseau M et al., 2007) and in the non-human BHK21 cell line (Vazeille M et al., 2007). In summary, the adaptive mutation of the
virus to replicate in *A. albopictus*, which is more common than *A. Aegypti* in some geographical regions and can act as an efficient vector for CHIKV, facilitated the spread of CHIKV. The fact is that the human population had not previously encountered CHIKV therefore immunologically naïve (Gould EA et al., 2009) contributed to the magnitude of the La Reunion CHIKV epidemic.

E1 and E2 protein correlates with the serological response in human hosts (Simizu B et al., 1984) and E1 modulates penetration of the virus in the mosquito species. On partial E1 sequences from African and Asian isolates revealed the existence of three distinct Chikungunya virus phylogroups: first containing all isolates from West Africa, the second containing isolates from Asia, and third corresponding to East, Central, and South African isolates (Higgs S, 2006). They have a worldwide distribution and all alpha viruses are antigenetically related. The viruses are inactivated by acid pH, heat, lipid solvent, detergents, bleach, phenol, 70% alcohol and formaldehyde. Most of the viruses possess haemagglutination activities.

Genetic analyses and historical accounts suggest that the Chikungunya virus originated in tropical Africa (Powers AM et al., 2008), and subsequently evolved into 3 distinct genotypes the East African, the West African and the Asian genotypes. The Asian genotypes have a high degree of nucleic acid sequence homology among themselves, but the African strains exhibit wider sequence diversity (Power AM et al., 2008), and have been shown to undergo genetic micro-evolutions even during the course of an epidemic (Schuffenecker I et al., 2006). Prior to the 2006 epidemic in India, the three genotypes were restricted to the geographical areas denoted by their names. Experts suggest that virus strains isolated from the 2005 epidemic in the Indian Ocean islands, and the strains that are currently circulating in India (Yergolkar PN et al., 2006) have evolved from the East African genotype. The past outbreaks in India were caused by the Asian genotypes.

Several hypotheses have been put forward to explain the 2006 epidemic in the Indian Ocean islands and India. First, French researchers have recently detected a mutation (at position 226 of the E1 gene) in 90% of viral sequences from the Indian Ocean strains (Outbreak news, 2006). The mutation allows the virus to acquire an ability to invade and thrive in cells which lack cholesterol (e.g. Mosquito cells) (Schuffenecker I et al., 2006). In addition, the virus gained the ability to infect a new vector, *A. albopictus*, enhancing the opportunity for transmission to humans. Second, experimental evidence suggests that mosquitoes concurrently infected with microfilaria transmit arbo viruses more efficiently (Zytoon EM et al., 1993). Because a large proportion of reported cases of Chikungunya from India belong to areas where the prevalence of filarial parasitic infection is high, researchers speculate that filarial parasitic infections could be modulating the re-emergence of Chikungunya (Mishra B
et al., 2006). Third, the affected population lacked herd immunity. Introduction of the virus into a non-immune population could have contributed to the present outbreak (Higgs S., 2006; Mourya DT et al., 2006).

2.3 Life cycle of Chikungunya virus in infected cells

The Alpha virus life cycle is depicted in the Fig 2.4. Alpha viruses enter target cells by endocytosis (Griffin et al., 2007). A few receptors (for example, dendritic cell-specific ICAM3-grabbingnon-integrin 1 (DC-SIGN; also known as CD209), liver and lymph node-SIGN (L-SIGN; also known as CLEC4M), heparan sulphate, laminin and integrins) have been implicated in this process, but their precise roles have not been firmly established (Griffin et al., 2007). Following endocytosis, the acidic environment of the endosome triggers conformational changes in the viral envelope that expose the E1 peptide which mediates virus–host cell membrane fusion. This allows cytoplasmic delivery of the core and release of the viral genome (Schuffenecker I et al, 2006; Powers AM et al., 2001; Chevillon C et al., 2008). Two precursors of non-structural proteins (nsPs) are translated from the viral mRNA, and cleavage of these precursors generates nsP1–nsP4. nsP1 is involved in the synthesis of the negative strand of viral RNA and has RNA capping properties (Griffin et al., 2007, Salonen, A et al., 2005), nsP2 displays RNA helicase, RNA triphosphatase and protease activities and is involved in the shut-off of host cell transcription (Garmashova N et al., 2007), nsP3 is part of the replicase unit and nsP4 is the viral RNA polymerase (Griffin et al., 2007). These proteins assemble to form the viral replication complex, which synthesizes a full-length negative-strand RNA intermediate. This serves as the template for the synthesis of both subgenomic (26S) and genomic (49S) RNAs. The subgenomic RNA drives the expression of the C–pE2–6K–E1 polyprotein precursor, which is processed by an autoproteolytic serine protease. The capsid (C) is released, and the pE2 and E1 glycoproteins are generated by further processing. pE2 and E1 associate in the Golgi and are exported to the plasma membrane, where pE2 is cleaved into E2 (which is involved in receptor binding) and E3 (which mediates proper folding of pE2 and its subsequent association with E1). Viral assembly is promoted by binding of the viral nucleocapsid to the viral RNA and the recruitment of the membrane-associated envelope glycoproteins. The assembled alpha virus particle, with an icosahedral core, buds at the cell membrane.
Figure: 2.4 Life cycle of Chikungunya virus in infected cells
2.3.1 Innate immune control of Chikungunya virus:

Chikungunya virus (CHIKV) is a single-stranded RNA (ssRNA) virus and may generate double-stranded RNA intermediates during replication. The double stranded RNAs have the potential to engage the pathogen recognition receptors Toll-like receptor 3 (TLR3), TLR7 and TLR8 and the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) melanoma differentiation-associated protein 5 (MDA5) and RIG-I. These receptors activate a signaling cascade that leads to the activation of type I interferons (IFNs) and the transcription of cytokines and chemokines. Recent evidence suggests that the production of type I IFNs by infected fibroblasts and other cell types is regulated by the adaptor protein CARDIF (CARD adaptor inducing IFNβ; also known as MAVS), which acts downstream of MDA5 and RIG-I. The inflammasome may also induce IL-1β production by infected cells. MYD88 also acts as an adaptor for interleukin-1β receptor (IL-1R), which could be activated by the secretion of IL-1β from infected cells and induced type I IFN in non-infected cells.

IRF, IFN regulatory factor; NF-κB, nuclear factor-κB; TIR, Toll/IL-1 receptor domain; TRAF, tumor necrosis factor receptor-associated factor; TRIF, TIR domain-containing adaptor protein inducing IFNβ.

**Figure 2.5 Innate immune control of Chikungunya virus.**
2.3.2 Adaptive immune control of Chikungunya virus:

The acute nature of CHIKV infection and disease pathogenesis, and the urgent need to tackle the spreading epidemic, there has so far been little effort afforded to understanding the sequellae of chronic infection with CHIKV and the role of the adaptive immune system in protection from subsequent re-infection. In fact, a deeper understanding of the humoral and cell-mediated immune response is important, as it is relevant for vaccine development and may impinge on our understanding of the chronic joint pain experienced by 30–40% of CHIKV-infected individuals.

One marked effect of CHIKV infection is acute lymphopenia. CHIKV infection experienced a decrease in the frequency of circulating B cells and T cells. Nearly half of those individuals had lymphocyte levels that were one-quarter of the lower limit for healthy individuals (Borgherini G et al., 2006). This was probably not a direct effect of the virus on lymphocytes, as CHIKV does not infect B cells and T cells. Instead, it is possible that type I IFNs induce cell death in lymphocytes, as they do in other acute infections. In addition, upregulation of stromal IFN-stimulated chemokines can trigger the migration of lymphocytes from the blood to the tissues, leading to lymphopenia (Simon F et al., 2007). In most CHIKV-infected individuals, repopulation of the circulating pool of lymphocytes occurs soon after resolution of infection.

2.4 Chikungunya Infection, a Two-Stage Disease

2.4.1 Acute Stage

Most patients infected with CHIKV develop acute symptoms, usually 2 to 6 days after the infective mosquito bite. In Reunion Island in 2005–2006, the prevalence of asymptomatic infections was about 5% to 10% (Josseran L et al., 2006; Queyriaux B et al., 2008). The first symptoms start abruptly and last for about a week before spontaneous improvement. The acute stage is defined as the first 10 days after disease onset (Simon F et al., 2007). The most frequent symptoms are high fever, arthralgias, back pain, and headache (World Health Organization 2008). Fever is usually high, and is poorly responsive to antipyretics. Illness is associated with intense fatigue, anorexia, myalgias, nausea, and vomiting in adults, and even transient confusion in elderly patients.). The acute bilateral and symmetrical rheumatism is typically extensive and progressive within a few days (Hochedez P et al., 2007; Simon F et al., 2007). Peripheral joints are frequently very painful and swollen, especially interphalangeal joints, wrists, and ankles. The axis and proximal joints can also become inflamed with large joint effusions in the knees and elbows. The intensity of the symptoms prompts most patients to search for efficient treatment. Unfortunately,
no antiviral drug has proved effective against human CHIKV infection (De Lamballerie X et al., 2008). Thus, the treatment of the acute stage is limited to painkillers and nonsteroidal anti-inflammatory drugs (NSAIDs). Their efficacy is not complete, and adverse effects are not rare as a result of excessive self-medication. Acetaminophen is the elective drug. Aspirin should be avoided because of the risk of bleeding. Although sometimes dramatically effective, systemic corticosteroids are not recommended because of the strong rebound effect after stopping. A transient maculopapular rash, sometimes edematous and/or pruritic, can also be observed on the face and the trunk of half the patients (Simon F et al., 2007). Miscellaneous cutaneous and mucosal changes have been reported during the acute stage of the disease: photosensitivity, stomatitis, mouth ulcers, exfoliate dermatitis, vesicles, bullae, and purpura (World Health Organization 2008) and Gastrointestinal symptoms are common (Borgherini G et al., 2007). Initial biological changes are transient leukopenia and lymphopenia, mild thrombocytopenia, low rises in C-reactive protein, and hepatic cytolysis (Simon F et al., 2007, Borgherini G et al., 2007, Hochedez P et al., 2006). Beside the typical fever-artrhalgias-rash association, some clinical features and complications were recently described. Atypical features of CHIKV infection leading to hospitalization and/or death were prospectively recruited in Reunion Island, reflecting their incidence, clinical aspects, and outcome (Renault P et al., 2007). The most frequent complications involved the CNS: convulsions, meningoencephalitis, and Guillain-Barré syndrome; the direct role of CHIKV is evident in these early manifestations (Tournebize P et al., 2009). Other severe acute complications have been reported since 2005: myocarditis (Simon F et al., 2006), fulminant hepatitis in patients with chronic liver diseases (Borgherini G et al., 2008), pancreatitis, acute endocrine disorders (Economopoulou A et al., 2009), extensive epidermolysis (Borgherini G et al., 2007), kidney failures, respiratory failures, and decompensation of cardiovascular diseases (Renault P et al., 2007). Although minor bleedings were observed (Borgherini G et al., 2007), CHIKV infection is no longer considered as a viral hemorrhagic fever. CHIKV infection in children resembles that of adults, but can occasionally be complicated with neuropsychological changes, including lethal meningoencephalitis, with myocarditis or extensive epidermolysis (Ernould S, et al., 2008).

2.4.2 Chronic Stage

High CHIKV viral loads in the acute stage are also associated with the persistence of symptoms (Hoarau JJ et al., 2010). Within the first 3 months, most patients experience a rebound of general discomfort, inflammation in the joints and tendons, and an increased handicap in daily life (Simon F et al., 2007; Sissoko D et al., 2009).
Disabilities in the extremities are from a severe polyarthritis involving most distal joints and multiple hypertrophic tenosynovitides that are sometimes responsible for carpal or tarsal tunnel syndromes. Difficulties in walking and handling objects can induce frequent and/or prolonged sick leave. Transient vascular disorders (eg, Raynaud syndrome) are present in one patient in six at that period (Simon F et al., 2007), possibly in relation with the concomitant presence of mixed cryoglobulinemia (Oliver M et al., 2009). Ocular changes may also develop a few weeks after disease onset: anterior uveitis, retinitis, episcleritis, and optic neuritis, sometimes leading to blindness (Mahendradas P et al., 2008). In Reunion Island, 80% to 90% of CHIKV-infected patients complained of persistent symptoms after the first 3 months (Sissoko D et al., 2009; Soumahoro MK et al., 2009). Whether this prevalence of nonrecovery is similar in other epidemic countries remains to be determined. However, CHIKV-induced rheumatism is the most frequent manifestation of the chronic stage. It consists of three clinical components, singly or in combination: 1) distal polyarthritis or monoarthritis mildly improved with NSAIDs, 2) frequent tenosynovitides in the hands, wrists, or ankles, highly sensitive to short-term systemic corticotherapy, and exacerbation of pain in previously injured joints and bones requiring painkillers (Simon F et al., 2007). 3) Most of the time, chronic peripheral polyarthritis is not associated with significant biological changes or the appearance of autoantibodies (Simon F et al., 2007), but mild mixed cryoglobulinemia is frequent (Oliver M et al., 2009). First radiographs of the joints involved usually do not reveal significant changes, even in disabled patients. Relapses are common during the chronic stage, often triggered after exposure to cold. They include mild fever, asthenia, increased inflammation in previously involved joints, and occasionally in new joints. Intensification of symptomatic treatment is often necessary and additional localized treatments and physiotherapy can be of benefit to some patients. The chronic stage can severely deteriorate the patient’s quality of life for months.

2.5 Pathogenesis

It is expected that once after inoculation, primary viral multiplication occurs in lymphoid and myeloid cells (Fig: 2.6). The arthropod vectors acquire the virus by sucking blood during this period. The virus then spreads to the targeted organs and immune system starts functioning at this stage leading to the activation of both humoral and cellular immunity. This response of the body leads to the clinical features of the disease. The convincing evidence and studies are not available. CHIKV infection may lead to an incapacitating infirmity, most often characterized by
fever, headache, fatigue, nausea, vomiting, muscle pain, rash, and joint pain. The incubation period (time from infection to illness) can be 2-12 days, but is usually 3-7 days. "Silent" CHIKV infections (infections without illness) do occur, but their frequency is yet uncertain. After incubation period fever rises abruptly, often reaching 39°C to 40°C and is accompanied by intermittent shaking chills. This acute phase lasts 2-3 days. The temperature may remit for 1-2 days, resulting in a “saddle-back” fever curve. The arthralgia is polyarticular, migratory, and predominantly affects the small joints of the hands, wrists, ankles and feet, with lesser involvement of the larger joints. Joint swelling occurs due to accumulation of fluid. Patients with milder articular manifestations are usually symptom-free within a few weeks, but more severe cases require months to resolve entirely. Generalized magi as well as back and shoulder pain are common. Cutaneous manifestations are typical with many patients presenting with a flush over the face and trunk. A rash generally described as maculopapular usually follows this. The trunks and limbs are commonly involved but face, palms and soles may also show lesions. Pruritis or irritation may accompany the eruption. During the acute disease, most patients suffer from headache. Photophobia and retro-orbital pain also occur in moderation. In infants and younger children, prominent flushing and early appearance of maculopapular or urticarial eruption may be a useful indicator and they also display neurological symptoms. CHIKV then replicates in the skin, in fibroblasts, and disseminates to the liver, muscle, joints, lymphoid tissue (lymph nodes and spleen) and brain.
Figure 2.6 Dissemination of Chikungunya virus in vertebrates
The target cells are indicated for each tissue. Following transmission by mosquito bite, infected individuals experience an acute onset of disease 2–4 days after infection (Fig: 2.7).

**Figure 2.7 Pathogenesis of Chikungunya virus**

Disease onset coincides with rising viral titer, which triggers the activation of an innate immune response, the hallmark of which is the production of type I interferon (IFNs). Patients successfully clear the virus approximately 1 week after infection, and only at this time is there evidence of CHIKV-specific adaptive immunity (i.e. T cell and antibody-mediated responses). Importantly, ~30% of individuals experience long-term sequelae that include arthralgia and, in some cases, arthritis.

**2.6 Epidemiology**

Since its discovery in Tanganyika, Africa in 1952, Chikungunya virus outbreaks have occurred occasionally in Africa, South Asia, and Southeast Asia, but recent outbreaks have spread the disease over a wider range (Fig 2.8). Chikungunya is a crippling pandemic disease in Africa, India, and South-East Asia. The history of this disease epidemic was known since 1952 with its first ravage in East Africa followed by numerous epidemics in Asia, including the Philippines (1954, 1956, and 1968), Thailand, Cambodia, Vietnam, India, Burma, and Sri Lanka. In India, the first Chikungunya outbreaks were recorded during 1963-65 and later in 1973. After more than 20 long years, the re-emergence of the disease was documented in Kinshasa (1999-2000) with an estimated infection of 50,000 persons. Since then, epidemics
were noticed in Java (2001-2003) and in the islands of the South Western Indian Ocean during the end of 2004. The recent outbreaks accounted to be from the Comoros islands during January- March 2005 with 5,000 cases and later, the virus was circulated in other islands of Indian ocean, i.e., Mayotte, Seychelles, Réunion, and Mauritius (nearly 3,500 islanders have been hit with this virus).

**Figure 2.8 Geographical distribution of Chikungunya virus**

The drastic intensification was a consequence of the rainy season in December 2005, which renewed the epidemics claiming several thousands of cases in a very short period of time. The map of the Chikungunya affected countries and the morbidity statistics of the latest Chikungunya disease were estimated by mathematical modeling. Of all the islands in the Indian Ocean, Reunion with a total population of 770,000 was the most infected with an estimated 258,000 cases by May 2006.

The infection was thought to be imported from the Comoros islands. The latest estimates of the Chikungunya cases in other Indian Ocean islands revealed the alarming statistics for 2006, as 6,346 cases in Mayotte (Jan –May), 10,812 in Mauritius (Feb- Apr), Seychelles with 5,461 cases (Jan – Mar) and 180,000 cases in the Indian subcontinent (Jan- Apr). According to the Euro surveillance 2006, imported cases from these countries are approximated to be nearly 307 in France, 17 in Germany, 9 in United Kingdom, 12 in Belgium and 1 each in Czech Republic and Norway.
Table 2.1 Major outbreaks of Chikungunya

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Cases</th>
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</thead>
<tbody>
<tr>
<td>1952</td>
<td>Tanzania</td>
<td>Few Cases</td>
</tr>
<tr>
<td>1999</td>
<td>Port King Malaysia</td>
<td>27</td>
</tr>
<tr>
<td>2005</td>
<td>French Reunion India</td>
<td>1722</td>
</tr>
<tr>
<td>2006</td>
<td>French Reunion India Malaysia</td>
<td>Many</td>
</tr>
<tr>
<td>2007</td>
<td>Ravenna Italy</td>
<td>130</td>
</tr>
<tr>
<td>2008</td>
<td>India</td>
<td>Many</td>
</tr>
<tr>
<td>2009</td>
<td>Thailand India</td>
<td>Many</td>
</tr>
<tr>
<td>2011</td>
<td>Republic of Congo</td>
<td>Many</td>
</tr>
</tbody>
</table>

2.6.1 Factors contributing to the Outbreak

The precise reasons for the recurrence of Chikungunya in the Indian subcontinent as well as the other small countries in the Southern Indian Ocean are an enigma. Although, it is well acknowledged fact that re-emergence of viral infections are due to array of social, environmental, behavioral and biological changes, which of these contributed to the re-emergence of Chikungunya virus would be interesting to untangle. Prevalent poverty, year-round tropical climate, environmental disturbance due to war or natural disaster, and lack of public health infrastructure are all factors that prop up uncontrolled mosquito breeding and are conducive to outbreaks of Chikungunya.

2.7 Chikungunya in India

There is a confirmed history of outbreaks during 1963–64 in Kolkata (Shah KV et al., 1964) and 1965 in Chennai (Jadhav M et al., 1965; Thiruvengadam KV et al., 1965) when more than 3,00,000 people were affected. Last epidemic in India was reported from Barsi, Maharashtra in 1973 (Padbidri et al., 1979) when a morbidity of 37.5% was reported for the whole town. The Kolkata outbreak in 1963 had started in July reaching a peak in November and then rapidly declining in December that year. Hospital record for that period suggests that children and elderly were the most severely affected group (Sarkar JK et al., 1964; National Institute of communicable diseases., 2006). The entry of Chikungunya virus in India is unknown although Calcutta Sea and air roots are believed to be the probable entry points in India. There were lakhs of cases during the 1963–64 outbreaks with haemorrhagic manifestations and deaths (Sarkar JK et al., 1964). Chikungunya virus had almost
disappeared from India after 1973 and since then, no case was reported till end of 2005 (Neogi DK et al., 1995; Mourya DT et al., 2005). Consequently, there has been no active or passive surveillance carried out in the country and therefore, it ‘seemed’ that the virus had ‘disappeared’ from the subcontinent.

The current outbreak in India started at the end of 2005 when cases of suspected fever were reported from coastal parts of Andhra Pradesh and Karnataka (WHO, 2006; National Institute of Communicable Diseases, 2006). Most of the initial reports were in the media and in newspapers (Times of India April 6, 2006) when hospitals were flooded with patients complaining of fever and joint pain. The outbreak was first investigated in February 2006 in Andhra Pradesh and then in March 2006 in Karnataka by health officials of the country (WHO, 2006) confirmed the occurrence of Chikungunya virus in the region. World Health Organization confirmed Chikungunya fever in India. The numbers of suspected cases reported have varied from different sources (WHO, 2006, Times of India April 6, 2006; Kandath R et al., 2006; Depoortere E et al., 2006) ranging up to a million. The current outbreak has an attack rate of 4–45%. Till August 4, 2006, the confirmed cases of Chikungunya fever have been reported from Andhra Pradesh, Karnataka, Maharashtra, Tamil Nadu, Madhya Pradesh and Gujarat states. Total 129 districts in 8 states have been affected by the virus. National Institute of Communicable Diseases, Delhi and National Institute of Virology, Pune had tested 10,809 samples and 1,015 were found to be positive. Total cases are in the range of 10,000.

**Table 2.2 Reported no. of Chikungunya fever cases in India in the year 2006**

<table>
<thead>
<tr>
<th>State</th>
<th>No. of district affected</th>
<th>Total fever cases/suspected Chikungunya fever cases</th>
<th>Total no. of samples</th>
<th>No. of confirmed cases</th>
<th>No. of death reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andhra Pradesh</td>
<td>20</td>
<td>1,10,618</td>
<td>1,224</td>
<td>150</td>
<td>0</td>
</tr>
<tr>
<td>Karnataka</td>
<td>27</td>
<td>6,70,438</td>
<td>4,376</td>
<td>266</td>
<td>0</td>
</tr>
<tr>
<td>Maharashtra</td>
<td>31</td>
<td>2,16,455</td>
<td>4,443</td>
<td>507</td>
<td>0</td>
</tr>
<tr>
<td>Tamil Nadu</td>
<td>34</td>
<td>43,580</td>
<td>413</td>
<td>59</td>
<td>0</td>
</tr>
<tr>
<td>Madhya Pradesh</td>
<td>412</td>
<td>44,966</td>
<td>36</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Gujarat</td>
<td>1</td>
<td>13</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>129</strong></td>
<td><strong>11,09,033</strong></td>
<td><strong>10,809</strong></td>
<td><strong>1,015</strong></td>
<td><strong>0</strong></td>
</tr>
</tbody>
</table>
However, recent reports of large scale outbreaks of fever caused by Chikungunya virus infection in several parts of Southern India have confirmed the re-emergence of this virus. More than 1, 80,000 cases have been reported to occur in India since December 2005. Andhra Pradesh (AP) was the first state to report this disease in October 2005, and one of the worst affected (over 80,000 suspected cases).

**Figure 2.9 Epidemiology of Chikungunya in India**

![Epidemiology of Chikungunya in India](image)

Suspected Chikungunya cases in different states of India (adapted from National Vector Borne Disease Control Program, Delhi, as on 12 December 2006).

Symbols: Karnataka (KA), Maharashtra (MH), Andhra Pradesh (AP), Gujarat (GJ), Kerala (KR), Tamil Nadu (TN), Madhya Pradesh (MP), Andaman and Nicobar Islands (AN), Pondicherry (PO), Delhi (DL), Goa (GA), Rajasthan (RJ).

**2.7.1 Current outbreak:**

Since the beginning of 2005, Chikungunya virus has emerged in the islands of the southwestern Indian Ocean. The outbreak was first reported in Comoros in the beginning of the 2005 (Schuffenecker I et al., 2006; Paquet C et al., 2006). Later in the same year, the virus had circulated to the other islands and countries Mayotte, Seychelles, Reunion and Mauritius, Madagascar and India. Since the end of 2005, the rainy season renewed the circulation and led to the intensification of the epidemic.
The most affected island is Reunion where almost 35% of the population (2,60,000 out of 7,70,000) has been affected by Chikungunya virus till mid of May 2006 (WHO, 2006; Paquet C et al., 2006).

The 2005–2006 epidemics in India and Indian Ocean Islands was the most devastating and had very complicated clinico-pathological manifestations associated with encephalopathy and hemorrhagic fever (Pialoux et al., 2007). Arthralgia persisted for months and years with excruciating pain in joints and ankles making the people prostrate. The most affected were the aged adults and adults suffering from diabetes, alcoholic hepatopathy and impaired renal functions (Couderc et al., 2008). The epidemic also witnessed the first ever CHIKV associated deaths and mother to child transmission (Pialoux et al., 2007; Mavalankar et al., 2008). In India, Ahmedabad city of Gujarat and Kerala state experienced large scale outbreaks with high morbidity and extensive incapacitation. Complications with involvement of the neurological and renal system leading to deaths were reported from hospitalized cases (Mavalankar et al., 2007; Solanki et al., 2007). Kerala state had the worst epidemic as the infection run through 2006 to 2008 affecting the whole state. Unique complications such as swollen limbs with painful arthralgia which persisted for long periods were witnessed among the patients. The most common and distinct disabilities during convalescence include acute difficulty in sitting, lying down, standing straight, and walking as well as general weakness of mind and body. Though several CHIK associated deaths were reported in Kerala by the print media, the official website of Kerala government has not confirmed any deaths (Mavalankar et al., 2008). Carried out systematic studies and observed high incidence of deaths during the Ophthalmic involvements, hypokalemic paralysis, sensorineural hearing loss, Guillain Barre Syndrome, and acute flaccid paralysis were some of the other complications observed in the affected people across the country (Lalitha et al., 2007; Mittal et al 2007; Rampal et al., 2007; Singh et al., 2007; Bhavana et al., 2008). Significant abnormalities in CSF and CT scan with raised levels of hepatic enzymes, altered renal function tests and decreased electrolyte levels were also noted in a large number of patients.

2.8 Diagnosis

Since no effective vaccines or therapeutics are available, early detection and proper diagnosis play the key role in the effective control of the infection. Serological techniques [haemagglutination, Haemagglutination Inhibition assay, complement fixation and neutralization test (NT)] were used effectively in the identification and characterization of viruses (Clarke and Casals, 1958; Pavri 1964). The development of immunoglobulin M antibody (IgM) capture enzyme linked immunoabsorbent assay (MAC-ELISA) has been a major achievement in serology as it provided a rapid and
reliable technique for the diagnosis of arboviruses (Gadkari and Sheikh 1984; Bodemann and Genton 2006). Indirect immunofluorescent antibody technique is another reliable technique for detection and identification of viral antigens from clinical samples (Kuberski and Rosen 1977; Yergolkar et al., 2006). In the event of a viral outbreak, the situation permits rapid detection and identification of the etiological agent. Therefore, molecular devices become accessible for the detection and characterization of Chikungunya virus. Reverse transcription polymerase chain reaction (RT-PCR) using primers designed for structural and non-structural domains have been found useful in the rapid diagnosis of CHIKV (Pastorino et al., 2005). The combination of RT-PCR/nested PCR has proved efficient for specific detection and genotyping of CHIKV (Pfeffer et al., 2002; Hasebe et al., 2002). Recently, real time RT-PCR, has revolutionized the field with its unique advantages i.e. rapidity, sensitivity, reproducibility and reduced risk of contamination and is being routinely used for detection and quantitation of viruses (Parida et al., 2008).

Diagnostic methods available for the detection of CHIKV includes viral isolation (Couderc T et al., 2008), RT-PCR (Smith DR et al., 2009), real time loop mediated isothermal PCR (Parida MM et al., 2007), antigen capture ELISA (Shukla J et al., 2009). These methods are highly sensitive but can be used only within the first week of disease onset. Classical serological assays like IgM detection assay (Gerardin P et al., 2008), IgG based immunolateral flow assay (Rampal et al., 2007) immunofluorescence (Litzba N et al., 2008; Thon-Hon VG et al., 2012), complement binding and haemagglutination inhibition method (Saluzzo JF et al., 1981; Clarke DH et al., 1958) have also been reported, but they lack sufficient specificity and need trained person.

The firm diagnosis can only be made by laboratory means, but CHIK should be suspected when epidemic disease occurs with the characteristic triad of fever, rash and rheumatic manifestations. Viraemia will be present in most patients during the first 48 hours of disease and may be detected as late as day 4 in some patients. Virus-specific IgM antibodies are readily detected by capture ELISA in patients recovering from CHIK infection and they persist in excess of 6 months. Haemagglutination inhibition (HI) antibodies appear to the cessation of viraemia. All patients will be positive by day 5 to 7 of the illness.

Three main laboratory tests are used for diagnosing Chikungunya fevers: virus isolation, serological tests and molecular technique of Polymerase Chain Reaction (PCR).
2.8.1 Virus isolation

Virus isolation provides the most definitive diagnosis. It is readily accomplished by inoculation of mosquito cell culture, mosquito, mammalian cell culture or suckling mice. The technique involves exposing specific cell lines to samples from whole blood and identifying Chikungunya virus-specific responses. Between 2-5 ml of whole blood is collected during the first week of illness in commercial heparinized tube in the laboratory. The CHIK virus produces cytopathic effects in a variety of cell lines including BHK-21, HeLa and Vero cells. The cytopathic effects must be confirmed by CHIK specific antiserum and the results can take between 1-2 weeks. Virus isolation must only be carried in BSL-3 laboratories to reduce the risk of viral transmission (WHO laboratory diagnosis of Chikungunya fever 2008). A positive virus culture supplemented with neutralization is taken as definitive proof for the presence of Chikungunya virus.

2.8.2 RT-PCR

Recently, a reverse transcriptase, RT- PCR technique for diagnosing CHIK virus has been developed using nested primer pairs amplifying specific components of three structural gene regions, Capsid (C ), Envelope E2 and part of Envelope E1. PCR results can be available from within 1-2 days. Polymerase chain reaction itself is the process used to amplify DNA samples, via a temperature-mediated DNA polymerase. Conventional PCR requires primers complementary to the termini of the target DNA. A commonly occurring problem is primers binding to incorrect regions of the DNA, giving unexpected products. Nested polymerase chain reaction is a modification of polymerase chain reaction to reduce the contamination in products due to the amplification of unexpected primer binding sites. Nested polymerase chain reaction involves two sets of primers, used in two successive runs of polymerase chain reaction. The target DNA undergoes the first run of polymerase chain reaction with the first set of primers, shown in green. The selection of alternative and similar primer binding sites gives a selection of products, only one containing the intended sequence. The product from the first reaction undergoes a second run with the second set of primers, shown in red. It is very unlikely that any of the unwanted PCR products contain binding sites for both the new primers, ensuring the product from the second PCR has little contamination from unwanted products of primer dimers, hairpins, and alternative primer target sequences.
2.8.3 Serological diagnosis

Serologic diagnosis can be made by demonstration of four fold increase in antibody in acute and convalescent sera or demonstrating IgM antibodies specific for CHIK virus. A commonly used test is the Immunoglobulin M Antibody (IgM) capture enzyme-linked immunosorbent assay (MAC-ELISA). Results of MAC-ELISA can be available within 2-3 days. Cross-reaction with other flavivirus antibodies such as o’nyong-nyong and Semliki Forest occur in the MAC-ELISA; however, the latter viruses are relatively rare in South East Asia but if further confirmation is required it can be done by neutralization tests and Haemagglutination Inhibition Assay (HIA). For serological diagnosis between 10-15 ml of whole blood sera is required; an acute phase serum must be collected immediately after clinical onset and a convalescent phase serum 10-14 after the disease onset. The blood specimen is transported at 4 degrees and not frozen to the laboratory immediately. If testing cannot be done immediately, the blood specimen is separated into sera that should be stored and shipped frozen. Sero-diagnosis tests on demonstrating a fourfold increase in CHIK IgG titer between the acute and convalescent phase sera. However, getting paired sera is usually not practical. Alternatively, the demonstration of IgM antibodies specific for Chikungunya virus in acute-phase sera is used in instances where paired sera cannot be collected. Serological diagnosis requires a larger amount of blood than the other methods, and uses an ELISA assay to measure Chikungunya-specific IgM levels. Results require two to three days, and false positives can occur with infection via other related viruses, such as o’nyong’nyong virus and Semliki Forest virus.

Serological techniques includes

2.8.3.1 Haemagglutination (or haemagglutination assay; HA):

Haemagglutination assay is a method of quantification for viruses or bacteria by hemagglutination. It is an easy, simple and rapid method which can be applied to large numbers of samples. Some viral families and many bacteria have an envelope or surface proteins which are able to agglutinate (stick to) human or animal red blood cells (RBC) and bind to N-acetylneuraminic acid. As each of the agglutinating molecule attaches to multiple RBCs, a lattice-structure will form.

2.8.3.2 Haemagglutination Inhibition assay (HIA).

The haemagglutination inhibition assay is a common variation of the HA assay used to measure flu-specific antibody levels in blood serum. In this variation, serum antibodies to the influenza virus will interfere with the virus attachment to red blood cells. Therefore haemagglutination is inhibited when antibodies are present at a
sufficient concentration. Antibodies against the viral protein responsible for haemagglutination can prevent haemagglutination; this is the basis behind the haemagglutination-inhibition test (HAI). The specificity of the HAI test varies with different viruses. With some viruses such as influenza A, the haemagglutination antigen is the same as the antigen responsible for virus adsorption and thus virus neutralization, and therefore the HAI test is highly specific for the different strains of the virus. With other viruses, the HAI test is less specific eg. flaviviruses, where HAI antibodies against one flavivirus may cross-react with other related flaviviruses. HAI tests are more sensitive than complement-fixation tests but are less sensitive than EIAs and RIAs. The advantages of HAI tests are that they are relatively easy and inexpensive to perform. The disadvantages are that HAI tests are not as sensitive as EIAs or RIAs, the actual reading of results is subjective and the reagents should be fresh or else abnormal agglutination patterns may arise which makes the reading and interpretation of the test very difficult.

2.8.3.3 Complement fixation and neutralization test (NT):

Complement-fixation reactions are serological tests based on the depletion of a fixed amount of complement in the presence of an antigen-antibody reaction. It is useful for detecting very small amounts of antibody, when the amount of antibody is too low to cause a precipitation or agglutination reaction. Complement fixation was once the basis of the Wasserman test, a test to diagnose syphilis. It is still used to diagnose some viral, fungal, and rickettsia diseases. There are two steps, the complement fixation step and the indicator step.

a) Complement Fixation Step:

In this step antigen and complement added to serum. If the serum contains antibodies against the antigen they will bind to the antigen and fix the complement. These ties up all the free complement so it can't participate in the next step, the indicator step.

b) Indicator Step:

In Indicator step sheep red blood cells and anti-sheep red blood cell antibodies added to the serum. Antibodies to the sheep red blood cells bind and can fix complement, if any is available. If complement is available it will be fixed by the sheep red blood cell antigen-antibody complex and the sheep red blood cells will be lysed. This indicates that the serum did not contain antibodies against the antigen added in the complement fixation step and complement remained free. If no complement is available the sheep red blood cells will not be lysed. This indicates there were antibodies against the
antigen added in the complement fixation step and all the complement was tied up when it was fixed by the original antigen-antibody complex.

2.3.3.4 Enzyme-linked Immunosorbent Assays (ELISAs)

ELISA combine the specificity of antibodies with the sensitivity of a simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. ELISAs can provide a useful measurement of antigen or antibody concentration. There are two main variations on this method: The ELISA can be used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen.

2.3.3.4.1 MAC ELISA (IgM antibody capture ELISA):

The Frontline test for Chikungunya virus diagnosis is the IgM-capture enzyme-linked immunosorbent assay (MAC-ELISA). The MAC-ELISA is the ideal test because it is both simple and sensitive (i.e., highly likely to find true-positives) and it can be used with both serum and cerebrospinal fluid (CSF) specimens. MAC-ELISA testing can be completed in 1 to 2 days from the time samples arrive at the laboratory. The CSF specimen is the preferred specimen for rapid diagnosis of human Chikungunya virus infection using MAC-ELISA. A second test useful in identifying possible Chikungunya virus infections is the IgG ELISA.

2.9 Treatment

There are no specific treatments for Chikungunya, and no vaccine is currently available. Treatment is symptomatic rest, fluids, and ibuprofen, naproxen, acetaminophen, or paracetamol may relieve symptoms of fever and aching. Aspirin should be avoided during the acute stages of the illness. Rest is advised during acute joint symptoms. Movement and mild exercise may improve stiffness and morning joint pains. However these are not conclusive studies. Infected persons should be protected from further mosquito exposure so that they can’t contribute to the transmission cycle.
A Phase II vaccine trial, sponsored by the US Government and published in the *American Journal of Tropical Medicine and Hygiene in 2000*, used a live, attenuated virus, developing 98% of viral resistance in those tested after 28 days and 85% still showed resistance after one year *(Edelman R et al., 2000)*. A serological test for Chikungunya is available from the University of Malaya in Kuala Lumpur. Chloroquine is gaining ground as a possible treatment for the symptoms associated with Chikungunya, and as an anti-inflammatory agent to combat the arthritis associated with the virus. A University of Malaya study founded that for arthritis-like symptoms not relieved by aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs), chloroquine phosphate (250 mg/day) has given promising results. Some studies have also shown that Chloroquine has some antiviral activity against this virus. Unpublished studies in cell culture and monkeys show no effect of chloroquine treatment on reduction of Chikungunya disease. The fact sheet *(Chikungunya Fact sheet, 2008)* on Chikungunya advises against using aspirin, ibuprofen, naproxen and other NSAIDs that are recommended for arthritic pain and fever.

**2.10 Vaccine**

**2.10.1** A virus-like particle-based vaccine has protected monkeys from Chikungunya virus infection, and passive immunization from these monkeys protected immunodeficient mice against exposure to a dose of virus that would otherwise be lethal, demonstrating the humoral response was highly protective *(Akahata W et al., 2010)*. Virus-like particles resemble viruses, but are non-infectious because they do not contain any viral genetic material. The expression of viral structural proteins, such as Envelope or Capsid, can result in the self-assembly of virus like particles (VLPs). VLPs derived from the Hepatitis B virus and composed of the small HBV derived surface antigen (HBsAg) were described over 40 years ago from patient sera *(Lahariya C, et al., 2006)*. More recently, VLPs have been produced from components of a wide variety of virus families including Parvoviridae (e.g. adeno-associated virus), Retroviridae (e.g. HIV), and Flaviridae (e.g. Hepatitis C virus). VLPs can be produced in a variety of cell culture systems including mammalian cell lines, insect cell lines, yeast, and plant cells *(Powers AM et al., 2007)*. VLPs contain repetitive high density displays of viral surface proteins which present conformational viral epitopes that can elicit strong T cell and B cell immune responses *(Sourisseau M, et al., 2007)*. Additionally, since VLPs lack genetic material, they provide a safer alternative to attenuated viruses. VLPs have already been used to develop FDA approved vaccines for Hepatitis B and human papillomavirus. More recently, VLPs have been used to develop a pre-clinical vaccine against Chikungunya virus.
2.10.2 A DNA vaccine candidate is also being tested. The vaccine cassette was designed based on CHIKV capsid and envelope specific consensus sequences with several modifications, including codon optimization, RNA optimization, the addition of a Kozak sequence, and a substituted immunoglobulin E leader sequence. These constructs induced humoral and cellular immune responses in mice (Muthumani K et al., 2008). DNA vaccines are third generation vaccines, and are made up of a small, circular piece of bacterial DNA (called a plasmid) that has been genetically engineered to produce one or two specific proteins (antigens) from a pathogen. The vaccine DNA is injected into the cells of the body, where the "inner machinery" of the host cells "reads" the DNA and uses it to synthesize the pathogen proteins. Because these proteins are recognized as foreign, when they are processed by the host cells and displayed on their surface, the immune system is alerted, which then triggers a range of immune responses (Lahariya C et al., 2006; Powers AM et al., 2007). These DNA vaccines developed from “failed” gene therapy experiments. The first demonstration of a plasmid-induced immune response was when mice inoculated with a plasmid expressing human growth hormone elicited antibodies instead of altering growth (Sourisseau M et al., 2007).

**Advantage:**

- Subunit vaccination with no risk for infection.
- Antigen presentation by both MHC class I and class II molecules
- Able to polarize T-cell help toward type 1 or type 2
- Immune response focused only on antigen of interest
- Ease of development and production
- Stability of vaccine for storage and shipping
- Cost-effectiveness
- Obviates need for peptide synthesis, expression and purification of recombinant proteins and the use of toxic adjuvants
- Long-term persistence of Immunogens
- In vivo expression ensures protein more closely resembles normal eukaryotic structure, with accompanying post-translational modifications

**Disadvantages:**

- Limited to protein immunogens (not useful for non-protein based antigens such as bacterial polysaccharides)
- Risk of affecting genes controlling cell growth
- Possibility of inducing antibody production against DNA
- Possibility of tolerance to the antigen (protein) produced
2.11 Vaccine development

The initiative to stimulate protective immunity as a strategy for preventing CHIKV infection in humans began in the early 1970s. Two formulations showed early promise: formalin fixation and ether extraction were both successful means of inactivating CHIKV while maintaining its ability to stimulate the production of haemagglutination-inhibiting, complement-fixing and neutralizing antibodies (Eckels KH et al., 1970; Harrison VR et al., 1971). These initial studies included human trials, with 16 army recruits receiving formalin-fixed CHIKV vaccine prepared in bank-frozen green monkey kidney tissue culture (Harrison VR et al., 1971). The US Army carried out a Phase II clinical trial examining the safety and immunogenicity of the use of live attenuated CHIKV vaccine in 2000 (Levitt NH et al., 1986; McClain DJ et al., 1998; Edeman R et al., 2000). A 1962 strain of CHIKV from an outbreak in Thailand was used in this case, and the vaccine was formulated as a lyophilized supernatant from human MRC-5 cells. Of the 58 study subjects that received the vaccine, all developed neutralizing antibodies, and 5 subjects experienced mild to moderate joint pain (Edeman R et al., 2000). One important issue that arose during these early studies is the potential interference arising from sequential administration of vaccines specific for heterologous alpha viruses. Specifically, individuals vaccinated against VEEV showed poor neutralizing antibody responses to the CHIKV vaccine (McClain DJ et al., 1998). Similarly, vaccination with CHIKV followed by VEEV resulted in reduced VEEV-specific responses (McClain DJ et al., 1998).

2.12 Adjuvants

An adjuvant is a pharmacological or immunological agent that modifies the effect of other agents, such as a drug or vaccine. In many cases, the antigen itself is very weakly immunogenic; therefore an adjuvant is needed to increase the immune response. Adjuvant can also be included in vaccine to guide the type of immune response generated. They are often included in vaccines to enhance the recipient's immune response to a supplied antigen, while keeping the injected foreign material to a minimum. The desired immune response to vaccines is the production of antibodies, and this is enhanced by adding certain substances to the vaccines. Adjuvants are crucial in affecting the specificity and Isotype of the necessary antibodies. They are thought to be able to potentiate the link between the innate and adaptive immune responses. Due to the diverse nature of substances that can potentially have this effect on the immune system, it is difficult to classify adjuvants into specific groups. In most circumstances they consist of easily identifiable components of micro-organisms that are recognised by the innate immune system cells. A large number of natural and
synthetic adjuvants have been identified throughout the history of vaccine development.

The chemical nature of adjuvants, their mode of action and their reactions (side effect) are highly variable. According to Gupta et al., (1993), some of the side effects can be ascribed to an unintentional stimulation of different mechanisms of the immune system whereas others may reflect general adverse pharmacological reactions which are more less expected. Chemically, the adjuvants are a highly heterogenous group of compounds with only one thing in common: their ability to enhance the immune response ie. their adjuvanticity.

The mode of action of adjuvants was described by Chedid (1985) as: the formation of a depot of antigen at the site of inoculation, with slow release; the presentation of antigen immunocompetent cells; and the production of various and different lymphokines (interleukins and tumour necrosis factor). The discovery of adjuvants dates back to 1925 and 1926 by Ramon (Gupta et al., 1993). In the conventional vaccines, adjuvants are used to elicit an early, high and long-lasting immune response. The newly developed purified subunit or synthetic vaccines using biosynthetic, recombinant and other modern technology are poor immunogens and require adjuvants to evoke the immune response. The use of adjuvants enables the use of fewer antigens to achieve the desired immune response, and this reduces vaccine production costs. There are several types of adjuvants. Today the most common adjuvants for human use are aluminium hydroxide, aluminium phosphate and calcium phosphate. However, there are a number of other adjuvants based on oil emulsions, products from bacteria (their synthetic derivatives as well as liposomes) or gram-negative bacteria, endotoxins, cholesterol, fatty acids, aliphatic amines, paraffinic and vegetable oils. Recently, monophosphoryl lipid A, ISCOMs with Quil-A, and Syntex adjuvant formulations (SAFs) containing the threonyl derivative or muramyl dipeptide have been under consideration for use in human vaccines.

2.12.1 Major Adjuvant Groups

2.12.1.1 CpG ODN: (CpG ligodeoxynuclotide):

The innate immune system recognizes bacterial DNA containing unmethylated CpG dinucleotides in the context of particular base sequences (CpG motifs) to secrete various cytokines. Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs (CpG-ODNs) mimic the direct immunostimulatory effects of native bacterial DNA, and activate multiple cell types including macrophages, dendritic cells, NK cells, and B lymphocytes. Immunostimulatory activities of CpG-ODNs have gained attention as potentially useful therapeutics for immune adjuvant, inflammatory and allergic
disease, and for immunoprotective agent. Choosing the optimal CpG ODN, three different classes of ODN are available viz. Class A, Class B, and Class C. These classes have different effects on immune cells and some also have species specificity.

a) **Class A CpG ODN:** The highest number of NK cell stimulations as well as IFN-α secretion by plasmacytoid DC was found to occur only with A class CpG ODN.

b) **Class B CpG ODN:** B class CpG ODN stimulates strong B cell and NK cell activation and cytokine production.

c) **Class C CpG ODN:** It combines the effect of A and B Class CpG ODN. C class CpG ODN strongly stimulates B cell or NK cell activation (Vollmer et al., 2004).

### 2.12.1.2 Oil Emulsions

In the 1960s, emulsified water-in-oil and water-in-vegetable-oil adjuvant preparations used experimentally showed special promise in providing exalted "immunity" of long duration (Hilleman, 1966). The development of Freund’s adjuvants emerged from studies of tuberculosis. Several researchers noticed that immunological responses in animals to various antigens were enhanced by introduction into the animal of living *Mycobacterium tuberculosis*. In the presence of *Mycobacterium*, the reaction obtained was of the delayed type, transferrable with leukocytes. Freund measured the effect of mineral oil in causing delayed-type hypersensitivity to killed mycobacteria. There was a remarkable increase in complement-fixing antibody response as well as in delayed hypersensitivity reaction. Freund’s adjuvant consists of a water-in-oil emulsion of aqueous antigen in paraffin (mineral) oil of low specific gravity and low viscosity. Drakeol 6VR and Arlacel A (mannide monooleate) are commonly used as emulsifiers. There are two Freund’s adjuvants: *incomplete* and *complete*. The *incomplete* Freund’s adjuvant consists of water-in-oil emulsion without added mycobacteria; the *complete* Freund’s adjuvant consists of the same components but with 5 mg of dried, heat-killed *Mycobacterium tuberculosis* or butyricum added. The mechanism of action of Freund’s adjuvants is associated with the following three phenomena:

A. The establishment of a portion of the antigen in a persistent form at the injection site, enabling a gradual and continuous release of antigen for stimulating the antibody;

B. The provision of a vehicle for transport of emulsified antigen throughout the lymphatic system to distant places, such as lymph nodes and spleen, where new foci of antibody formation can be established; and,

C. Formation and accumulation of cells of the mononuclear series which are appropriate to the production of antibody at the local and distal sites.
2.12.1.3 Mineral Compounds

Aluminium phosphate or aluminium hydroxide (alum) is the mineral compounds most commonly used as adjuvants in human vaccines. Calcium phosphate is another adjuvant that is used in many vaccines. Mineral salts of metals such as cerium nitrate, zinc sulphate, colloidal iron hydroxide and calcium chloride were observed to increase the antigenicity of the toxoids, but alum gave the best results.

2.12.1.4 Bacterial Products

Micro-organisms in bacterial infections and the administration of vaccines containing whole killed bacteria and some metabolic products and components of various micro-organisms have been known to elicit antibody response and act as immunostimulants. The most commonly used micro-organisms, whole or their parts, are Bordetella pertussis components, Corynbacterium derived P40 component, cholera toxin and mycobacteria.

2.12.1.5 Pertussis components

The killed Bordetella pertussis has a strong adjuvant effect on the diptheria and tetanus toxoids in the DPT vaccines. However, there are a number of admitted and well-describe reactions to it, such as convulsion, infantile spasms, epilepsy, sudden infant death syndrome (SIDS), Reye syndrome, Guilain-Barre syndrome, transverse myelitis and cerebral ataxia.

2.12.1.6 Corynebacterium-derived P40

P40 is a particulate fraction isolated from Corynebacterium granulosum, composed of the cell wall peptidoglycan associate with a glycoprotein. In animals, it displays a number of activities such as stimulation of the reticulo-endothelial system, enhancement of phagocytosis and activation of macrophages.

2.12.1.7 Lipopolysaccharide (LPS)

LPS is an adjuvant for both humoral and cell-mediated immunity. It augments the immune response to both protein and polysaccharide antigens. It is too toxic and pyrogenic, even in minute doses, to be used as an adjuvant in humans.

2.12.1.8 Mycobacterium and its components

Interestingly, Mycobacterium and its components, as originally formulated, were too toxic to be used as adjuvants in humans. However, the efforts to detoxify them resulted in the development of N-acetyl muramyl-L-alanyl-D-isoglutamine, or
muramyl dipeptide (MDP). When given without antigen, it increased nonspecific resistance against infections with bacteria, fungi, parasites, viruses, and even against certain tumours (McLaughlin et al., 1980). However, MDPs are potent pyrogens and their action is not completely understood; hence they are not acceptable for use in humans.

2.12.1.9 Cholera Toxin

A major drawback with cholera toxin as a mucosal adjuvant is its intrinsic toxicity.

2.12.1.10 Liposomes

Liposomes are particles made up of concentric lipid membranes containing phospholipids and other lipids in a bilayer configuration separated by aqueous compartments. Liposomes are synthetic spheres that can encapsulate antigens and act as both vaccine delivery vehicle and adjuvants. Liposomes have been used widely in experimental vaccine delivery system. The potency of liposome depends on the number of lipid layers, electric charge, composition and method of preparation. They enhance both humoral and cellular immunity to proteins and polysaccharide antigens (Nakanishi T., 1999). Liposomes help extend the half life of antigens in blood ensuring a higher antigen exposure to APCs after vaccination. Stability, manufacturing and quality assurance problems seem to have been major factors behind the fact that as yet no adjuvant based on liposome has been registered for human use. They have been used as carriers of biologically active substances (Gregoriadis, 1976) and considered safe.

2.12.1.11 Immunostimulating complexes (ISCOMs)

ISCOMs (DeVries et al., 1988; Morein et al., 1998; Lovgren et al., 1991) represent an interesting approach to stimulation of the humoral and cell-mediated immune response towards amphipathic antigens. It is a relatively stable but non-covalently-bound complex of saponin adjuvant Quil-A, cholesterol and amphipathic antigen in a molar ratio of approximately 1:1:1. The spectrum of viral capsid antigens and non-viral amphipathic antigens of relevance for human vaccination, incorporated into ISCOMs, comprises influenza, measles, rabies, gp340 from EB-virus, gp120 from HIV, Plasmodium falciparum and Trypanosoma cruzi.

2.12.1.12 Squalene

Squalene is an organic polymer with some antigenic epitopes which might be shared with other organic polymers acting as immunostimulators. It has been used in experimental vaccines since 1987 (Asa et al., 2000).
2.13 Vaccine delivery system

The role of delivery systems is primarily to direct the chosen adjuvant and antigen into target cells to attempt to increase the efficacy of the vaccine further, therefore acting synergistically with the adjuvant. There is increasing concern that the use of very potent adjuvants could precipitate autoimmune responses, making it imperative that the vaccine is focused on the target cells only. Specific delivery systems can reduce this risk by limiting the potential toxicity and systemic distribution of newly developed adjuvants.

To increase, accelerate or modify the development of an immune response to a vaccine candidate it is often necessary to combine the antigenic substance to be delivered with an adjuvant or specialized delivery system. An adjuvant is typically thought of as a substance used in combination with the antigen to produce a more substantial and robust immune response than that elicited by the antigen alone. This is achieved through three mechanisms: by affecting the antigen delivery and presentation, by inducing the production of immunomodulatory cytokines, and by affecting the antigen presenting cells (APC) (e.g. liposomes).

2.13.1 Microspheres

The potential of poly(DL-lactide-co-glycolide) (DL-PLG) microspheres as an oral vaccine delivery system (Eldridge JH et al., 1990; Eldridge JH et al., 1989). Several aspects of these microspheres make them attractive as an adjuvant for human vaccines. One important aspect is that DL-PLG is in the class of biodegradable and biocompatible copolymers from which resorbable sutures, resorbable surgical clips, and controlled-release implants are made (Redding TW et al., 1984). These copolymers are approved for, and have a history of safe use in, humans. When a vaccine is microencapsulated with DL-PLG, the vaccine is dispersed within the DL-PLG matrix of the microsphere in a dry state, thus providing extended shelf life without the need for stabilizers or a cold chain. After introduction into the body, DL-PLG induces only a minimal inflammatory response and biodegrades through the hydrolysis of its ester linkages to yield biocompatible lactic and glycolic acids (Tice TR and Cowsar DR, 1984).
Lacunae and Rationale
Group A: Malaria
3. Lacunae and Rationale

3.1 Lacunae and Rationale:

To avoid non target effects, to delay the advent of resistance, and to save cost on alternative drugs rational therapy of malaria is essential. In the many malaria endemic areas, where laboratory support is often out of reach clinical diagnosis is still the basis of therapeutic care for the majority of febrile patients. However, the overlapping of malaria symptoms with other tropical diseases impairs its specificity and therefore encourages the indiscriminate use of anti-malarials for managing febrile conditions in endemic areas. The accuracy of a clinical diagnosis varies with the level of endemicity, malaria season, and age group. Accurate diagnosis is the only way of effecting rational therapy. Microscopic examination of Giemsa-stained blood smears has subsequently become the gold standard for malaria diagnosis. In the past 50 years it is the widely used routine method for detection of malaria parasite. But microscopic examination is laborious and requires considerable expertise for its interpretation, particularly at low levels of parasitaemia. In addition, in patients with *Plasmodium falciparum* malaria, sometimes the parasites can be sequestered and are not present in peripheral blood. Thus a *P. falciparum* infection could be missed due to absence of the parasite in a blood film. However, sometimes it is a failure to differentiate *P. falciparum* from *P. vivax*, the two most common species, can be quite frequent in routine microscopy but is underreported.

Alternative methods became available e.g., detection of malaria antibodies by indirect immunofluorescence antibody assay [IFA] based on Acridine Orange (AO) and Benzothiocarboxypurine (BCP) but they have certain limits as AO is a very intense fluorescent stain, it is nonspecific and stains nucleic acid from all cell types and it is hazardous and require special disposal mechanism that makes it inappropriate for use in the field. Molecular methods, namely, DNA probes and polymerase chain reaction (PCR) were introduced in the 1980s–1990s but the use of these techniques are limited by the need for expensive, specialized equipment, trained technologist and high cost of enzymes and primers. Detection of malaria pigments by depolarized laser light and mass spectrometry showed limited success. All these techniques are time consuming and takes several hours to produce the result.

Malaria rapid diagnostic tests (RDTs), newer diagnostic modalities that identify circulating antigens of malaria parasites, may offer a reliable alternative for case management. Detection of malaria antigens, which forms the basis of commercial malaria RDTs available today has gained attention. Histidine-Rich Protein 2 (HRP-2)
is the most common malaria antigen targeted and is specific for *P. falciparum*. Some commercial tests carry both an assay for genus specific aldolase enzyme and an HRP-2 assay thus making it capable of distinguishing an infection with non-*P. falciparum* only from that due to *P. falciparum* (with/without nonfalciparum). Parasite lactate dehydrogenase (pLDH) enzymes are the other major group of targeted antigens. However, false positive RDT results occur in a few percent of tests. Cross-reactivity with rheumatoid factor in blood generates a false positive test line. Cross-reactivity with heterophile antibodies may also occur. Monoclonal antibodies against pLDH are commercially available for the detection of *Plasmodium* spp. (pan-malaria), *P. falciparum*, and *P. vivax*. But, Isolation of the whole protein from the parasite culture and production of monoclonal antibodies is a cumbersome and difficult process. Overhand the high cost of such tests limits its use in the resource poor countries.

### 3.2 Selection of protein and peptides:

On the basis of established potential in detection of *P. falciparum* two specific protein HRP-2 and pLDH has been selected for the evaluation of the study. PfHRP2 is a histidine- and alanine-rich protein with repetitive epitopes that is synthesized by both the asexual and early sexual stages of the parasite and, thereafter, is exported through the erythrocyte cytoplasm and the surface membrane to accumulate in the extracellular plasma (Makler et al., 1998). Although the amount of PfHRP2 released continues to increase throughout the erythrocytic cycle, most of it is released during schizont rupture. In *in-vitro* assays, the antigen can be detected in culture supernatants of synchronized parasites as early as 2 to 8 hours after ring development. PfHRP2 has a long half-life and persists in the circulation for up to 3 weeks, even after successful treatment.

To address the need for a diagnostic test for malaria, we have selected *Plasmodium* lactate dehydrogenase (pLDH) a soluble glycolytic enzyme that is expressed at high levels in the blood-stage parasite. It has reported that all four human malarial parasites produce a unique pLDH activity and that this activity follows the level of parasitemia in *in-vitro* cultures. The pLDH I peptide sequence is selected from the region which contains a unique five amino acid region DKEWN act as substrate site in the conserved mobile loop region important for the conserved catalytic residue R109. The active site of pLDH enzyme composed of two critical amino acid residues P246 and P250. We had selected second peptide pLDH-II from the active site of enzyme. The third peptide pLDH-III were selected from the N-terminal region of pLDH and contain amino acid different from other LDH including human LDH. pLDH activity in patient plasma samples can follow parasitemia measured by microscopy, indicating that pLDH may be a good marker for following active malarial infections.
3.3 **Advantage of synthetic peptides**: Synthetic peptide has following advantages.

a) Synthetic peptides are linear and functional epitopes of protein so they are more specific than native or conventional antigen in the detection of antigen and antibodies.

b) During the synthesis of peptide it may easier to eliminate the high proportion of unwanted epitopes represented in the crude antigen preparation which can sometimes give a false positive cross reaction.

c) Synthetic peptides have advantages of low cost production due to mass production of peptides of specific proteins, easier standardization and quality control. The chemical synthesis ensures reproducibility as well as batch to batch consistency.

3.4 **Delivery system: Microsphere**

Most conventional antigen requires multiple recall to sustain and optimal body response. New strategy to overcome this problem is a usage of a safe and potent antigen delivery system for developing antibodies. This polymer has following advantages eg. non toxic for animals, tissue compatible and degradable, adjustable degradation rate, available commercially in medical grade quality. Further, PLGA/PLA polymer microspheres have been shown to be able to induce both humoral and cell-mediated immunological responses to various immunogens including natural immunogen including natural protein and short synthetic peptide. Therefore, in the present study utilizing peptide epitopes of *P. falciparum*, we have tested the efficacy of PLGA microsphere in generating long lasting immune response against peptide immunogen with CpG ODN adjuvant.

3.4 **Adjuvant: CpG ODN**

To develop high titer antibodies along with choice of adjuvants is of crucial importance. CpG ODN is a novel adjuvant which has been shown to activate innate immune cells. It can trigger B-cell proliferation, polyclonal (Ig) IL-6 and IL-12 seaction and protect B-cell from apoptosis (*Yi et al. 1996*). Immunostimulatory DNA sequences containing unmethylated CpG dinucleotide in the context of particular base sequence (CpG motifs) exert a strong stimulatory influence on the immune system. Such sequences which are either found naturally in bacterial DNA or produced as synthetic ODNs directly activate human B cells and plasmacytoid dendritic cells viaTlr-9. Cpg oligos act as polyclonal activated which directly activate B cells to proliferate and differentiate into IgG producing cells. CpG oligos also indirectly activate other cells such as monocytes and macrophages to produce a variety of pro-inflammatory cytokines and in particular those associated with these stimulatory influences CpG ODNs were capable of enhancing Cd4, Cd8 cytotoxic...
antibody response to a wide variety of antigens. As a result of their strong
adjuvanticity and low reactogenicity CpG ODNs are currently considered as one of
the most promising adjuvants for the development of future vaccines against diverse
conditions including infectious diseases, allergies or cancer (Verthelji D., 2003).

3.5 Selection of animal model: Mice and Rabbit

Mice (H2d) and rabbits were chosen for the study because-

1. They are easy to produce, handle and maintain.
2. The breeding can be carried out on a large scale in a small interval of time.
3. Mice and rabbits have genomic similarity to humans and thus, provide the closest
model for correlating the study with humans.
4. The immune system and its regulation in mice, rabbits and humans are considered
to be remarkably similar.
Lacunae and Rationale
Group B: Chikungunya
3.1 Lacunae and Rationale:

Despite the effort engaged in control, Chikungunya remains a major public health problem in India, Indian Ocean Islands and African region. Accurate and confirmatory diagnosis before treatment initiation is the only way to control the disease. ELISA provides a reliable alternative for the management and control of the disease. To avoid non target effects, to delay the advent of resistance, and to save cost on alternative drug ratio. Chikungunya (CHIK) fever is a mosquito-borne viral infection which affects large populations in Asia and Africa. It shares common clinico-pathological symptoms with Dengue virus (DENV). In most of the cases, diagnosis of Chikungunya virus (CHIKV) is symptomatic but RT-PCR, virus Isolation and Immunological approaches are the existing methods of diagnosis of the disease. Some of these methods showed cross reactivity with other related alpha virus family too. Since both Chikungunya virus (CHIKV) and Dengue virus (DENV) cause febrile disease, and share the same vectors and geographical distributions. Therefore, it is difficult to distinguish clinical manifestations of Chikungunya with Dengue hemorrhagic fever. A co-infection of CHIKV and DENV is also reported in the same patient (Ummul et al., 2010; Yap G, Pok et al., 2010). The prognosis as well as diagnosis of DENV infection is much more predominant over CHIKV, thus there is a need of laboratory confirmation of suspected Chikungunya cases to control its epidemic and endemic outbreaks worldwide. The transmission of arthropod-borne virus (arbovirus) causes an acute infection associated with sudden onset of high fever, headache, skin rashes, joint and muscle pain. Generally, the symptoms associated with CHIKV appear after 4-7 days of mosquito bites and acute infection last up to 10 days (Marion et al., 2007; Peters et al., 1990; Pialoux et al., 2007). Despite the above disease burden the diagnosis is still based on leucopenia, anemia and elevation of serum aminotransferase enzymes. However, none of the above laboratory features are specific for the diagnosis. Detection of CHIK can be done by molecular methods such as RT-PCR, serological diagnosis and virus culture. Due to lack of proof reading, it is sometime difficult to design the primers for detection of virus RNA using RT-PCR. Additionally, the high cost of RT-PCR analysis is also another problem associated with small laboratories. Thus, there is an urgent need to develop a cheaper as well as a sensitive diagnostic reagent to diagnose CHIKV specific antibodies in the sera of clinical samples without any cross reactivity with other related alpha viruses. The commercial kits are based on detecting IgM antibodies using recombinant envelope proteins. However, use of peptide based ELISA in the diagnosis is very scanty in the literature. Peptides are highly accessible in terms of binding to its own antibodies than native antigen. They are functional and linear epitopes of protein and does not get distorted or undergo conformational changes
during the coating on microtiter plates, while native antigen are usually adsorbed on the surface in a disordered orientation, as a result the functional site of native antigen are sometimes inaccessible to its antibodies (Billsten et al., 1995). During the synthesis of peptide it is easy to eliminate the high proportion of unwanted epitopes represented in the crude antigen preparation which can sometimes give false positive cross reaction (Gartner et al., 2003). Sometimes peptides are more specific to react with its own antibodies than the native antigen (Mabel et al., 2001). Therefore, the peptide based diagnostic could provide a more specific and cost effective commercial diagnostic test for the diagnosis of CHIK infection without any cross reactivity with other related alpha viruses. In the present study high binding (Immunol 2HB) plates were used to evaluate the assay and peptides were directly adsorbed on the surface of the plate and we did not couple with any of the carriers, this is because after coupling with carrier it may change the orientation and hence immunoreactivity of peptides can vary with antibodies. IgM antibodies appear between 4-5 days after onset of fever. In convalescent sera, IgG starts appearing from the 10th day of clinical symptoms and can persist up to months (Gilles et al., 2007). In infected patients viraemia is present in serum that lasts up to 6 days whereas symptoms appear between 4-6 days. There are reports where IgM antibodies can be detected up to 10-12 days after CHIK fever (Taubitz et al., 2007).

3.2 Selection of protein and peptides:

On the basis of established potential in the detection of Chikungunya disease, E1 and E2 have now been considered as two major glycoproteins responsible for infection. The virion envelope consists of a lipid bilayer in which are embedded multiple copies of two virus encoded glycoproteins. The two glycoproteins, E1 and E2, each have a molecular mass of about 50 kDa and are anchored in the membrane by conventional membrane-spanning anchors in the C-terminal regions. E1 is 440 amino acids and E2 is a 404 amino acid long in Chikungunya virus S27 strain. Both E1 and E2 are glycosylated, but the number and position of the attached chains are not absolutely conserved among alpha viruses. E1 usually carries one or two chains, whereas E2 usually carries two or three chains. E2 and E1 form a stable heterodimer that remains intact upon dissociation of the virus with mild detergents. E2 is formed as a precursor called PE2 or p62 that is cleaved into E2 and a small glycoprotein called E3. The normal pathway of entry for alphaviruses is endocytosis in clathrin-coated vesicles followed by transfer to endosomes, where the low pH leads to a conformational reorganization of the E1-E2 heterodimer such that the fusion domain in E1 in exposed and the virus envelope fuses with the endosomal membrane. E1 glycoprotein is responsible for cell membrane fusion, while E2 is primarily involved in receptor
binding and cell entry as well as eliciting neutralizing antibodies. In the present study we targeted peptide based approach for the detection of CHIKV specific IgM/IgG antibodies in the diagnosis of infection.

All peptides were selected from E1, E2 and E3 envelope proteins of S27 strain of CHIKV. Earlier studies demonstrated the usefulness of peptides for the diagnosis of infectious diseases (Barbara et al. 1991; Deepak et al., 2006, Gokulan et al., 1999; Shweta et al., 2010; zhengi et al., 2002). In another study role of fusogenic peptides in the serological diagnosis of Dengue fever was reported (Pattanaik et al., 2006). Synthetic peptides offer the advantage of eliminating nonspecific reactions with enhanced specificity by averting the selection of cross reactive sequence from the designed peptides.

3.3 Advantage of synthetic peptides: Synthetic peptide has following advantages.

d) Synthetic peptides are linear and functional epitopes of protein so they are more specific than native or conventional antigen in the detection of antigen and antibodies.

e) During the synthesis of peptide it may easier to eliminate the high proportion of unwanted epitopes represented in the crude antigen preparation which can sometimes give a false positive cross reaction.

f) Synthetic peptides have advantages of low cost production due to mass production of peptides of specific proteins, easier standardization and quality control. The chemical synthesis ensures reproducibility as well as batch to batch consistency.

3.4 Study subject:

In the present study Chikungunya infected human samples were enrolled for evaluation of assay. A total of 195 samples were selected from different regions of India. 123 samples were confirmed CHIKV positive (clinical diagnosis, RT-PCR or IgM kit method), 33 sera were confirmed dengue virus (DENV) positive (IgM kit method), and 39 healthy individual samples were used as negative controls.
Aims and Objectives
Group A: Malaria
4. Aim and Objectives

4.1 Aim and Objectives

**Aim:** The aim of the present study is to develop an Enzyme Linked Immunosorbent assay for the detection of HRP-2 and pLDH antigen in malaria infected patients.

**Objectives:**

1. Selection of peptides from HRP-2 and pLDH protein using computer algorithm programs.
2. Solid phase peptide synthesis of selected peptides from HRP-2 and pLDH protein.
4. Physiochemical characterization of peptides by:
   a) N-terminal analysis
   b) C-terminal analysis
   c) HPLC analysis
   d) Amino acid analysis
5. Incorporation of peptides in microspheres along with adjuvant CpG-ODN and generation of high titer antibodies in mice and rabbits.
6. Purification of antibodies by affinity chromatography on a protein A column chromatography.
8. In vitro culture of *P.falciparum* for the production of culture supernatant and parasitized RBC.
9. Detection of HRP-2 and pLDH antigen in the infected/parasitized RBC’s and culture supernatant using Sandwich ELISA.
10. Detection of HRP-2 and pLDH antigen in the *P.falciparum* proven patients.
Aims and Objectives
Group B: Chikungunya
3.1 **Aim and Objectives:**

**Aim:** The aim of this study was to identify specific antibodies against peptides of E1, E2 and E3 protein for diagnosis of CHIK infection using *in-house* reagents.

**Objectives:**

1. Selection of peptides from E1, E2 and E3 envelope protein and Surface localization using computer algorithm programs.
2. Solid phase peptide synthesis of selected peptides.
3. Purification of peptides by gel permeation chromatography on Sephadex G-25 column.
4. Physiochemical characterization of peptides by:
   e) N-terminal analysis
   f) C-terminal analysis
   g) HPLC analysis
   h) Amino acid analysis.
5. Selection of study subject i.e Selection of CHIKV positive Patients.
6. Standardization of Assay
7. Detection of IgM and IgG antibody in patients' sera.
Material and Methods
Group A: Malaria
5. Material and Methods

Material and Methods

5.1 Computer algorithmic analysis of peptide: The entire protein sequence of Plasmodium Histidine rich protein (HRP-2) was analyzed by B-cell prediction program (EMTECH, Chandigarh) and the protein sequence for Lactate dehydrogenase (P/LDH) (PBD id PF3D7_1324900) and PvLDH (PVX_116630) were obtained from the PlasmoDB version 9.2 malaria database (www.plasmodb.org). DNAstar and Bcelpred software (www.IMTECH Bioinformatics) were used for the selection of peptides on the basis of physiochemical properties such as antigenicity, hydrophilicity, hydrophobicity, flexibility/mobility, exposed surface antigenicity and β turns. Structural data for epitope location was drawn by PYMOL [DeLano WL (2002) The PYMOL Molecular Graphics System on World Wide Web http://www.pymol.org]]. Alignments were performed by EMBOSS Stretcher alignment software.

5.2 Selection of *P. falciparum* peptide: All the five peptides of *P. falciparum* were selected from the conserved region of two specific protein viz. HRP-2 and pLDH because of their recognized prospective in specific detection of *P. falciparum* infection (Table 5.1).

Table: 5.1 Peptide sequence selected from HRP-2 and pLDH antigen

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfHRP-2 peptide I</td>
<td>AHH(AHHAAD)₄A</td>
</tr>
<tr>
<td>PfHRP-2 peptide II</td>
<td>(AHHA)₄</td>
</tr>
<tr>
<td>PfLDH peptide I</td>
<td>(LFDIVKNMPHKGALDTSHT)₂</td>
</tr>
<tr>
<td>PfLDH peptide II</td>
<td>(TNVMAYSNCKVSGNTYDDL)₂</td>
</tr>
<tr>
<td>PfLDH peptide III</td>
<td>(VVLGANGVEQVIELQLNSEEKA)₂</td>
</tr>
</tbody>
</table>

Hence the conserved regions are more valuable for development of diagnostic reagent compare to variable regions based on immune responses.

HRP-2 is a water soluble protein produced by asexual stage of parasite and expressed by both Knob (+) and Knob (-) RBC surface ([Rock et al., 1987](#)). Because of their
abundance in RBC these are the first antigen targeted for development in RDT (Rapid diagnostic Test).

Like HRP-2, pLDH is another protein used in RDT. The LDH peptide sequences are selected from the region which is very specific for *P. falciparum* and not showing any homology with human LDH and other vertebrate LDH. The key difference between the pLDH and other LDH is the five residue insertion within the substrate specificity loop which is highly conserved region. All peptide sequences are conserved in the different isolates of falciparum and LDH had no homology with the other vertebrate LDH including humans.

5.3 Chemical synthesis Peptide.

All peptides were synthesized using F-moc chemistry on C-terminal amino acid substituted phenylacetamidomethyl (PAM) resin. The Fast-moc chemistry, with double coupling throughout was used to synthesize both HRP-2 and pLDH peptides by automated peptide synthesizer (PS3, Protein Technologies, U.S.A.). All the amino acids were orthogonally protected. The glycine wang resin was used as a solid support for the growing amino acid chain. The Fmoc carbonate group was used for the protection of amino acids.

**Chemicals used:**

1. Fmoc protected L-amino acids derivatives
2. Glycine wang resin
3. 1-H-benzotrizolium hexaflouro phosphate 3-oxide (HBTU)

**Reagents used:**

1. N,N-dimethyl formamide (DMF)
2. N-methyl-morpholine (NMM)
3. Piperidine
4. Chloroform

All the protected amino acid derivatives were procured from Sigma chemicals Co. (St. Louis, USA) and Chem Impex (Technoconcept, Wood dale, IL, USA). The side-chain protecting groups were the following: Glu, Thr and Tyr, O-tButyl; Trp, 1-H-benzotrizolium hexaflouro phosphate 3-oxide (HBTU), coupling reagent, was procured from Peptide International. Trifluoroacetic acid (TFA), 2-Ethanedithiol, Anisole and Thioanisole were purchased from Sigma chemicals Co. (St. Louis, USA). Amino acid substituted Glycine wang resin was taken from Advanced Chemtech. All the reagents used were of analytic grade and obtained from SRL, India. Piperidine
was purchased from Spectrochem Pvt. Ltd. while N-methyl-morpholine (NMM) was taken from Merck, Germany.

5.5 Distillation of DMF:

The number of end products and moisture was formed due to the longtime of storage of DMF, which will slow down the coupling reaction. So, before starting peptide synthesis, distillation of DMF is required to remove impurities.

Reagents required:

1. Dimethylformamide (DMF)
2. Benzene
3. Sodium metal
4. Sodium hydroxide (NaOH)
5. N₂ gas

Dimethylformamide (DMF) is a colorless, high-boiling, mobile, polar liquid with a faint, characteristic odor. It does not decompose on distillation and is freely miscible with water, alcohols, ethers, ketones, esters, carbon disulfide and chlorinated and aromatic hydrocarbons. It is either immiscible or only partly miscible with aliphatic hydrocarbons. Even at elevated temperatures, aqueous solutions of DMF have very little tendency to hydrolyze. However, the addition of acids or bases accelerates hydrolysis to formic acid and 1, 3-dimethylamine. DMF is an aprotic solvent with a high dielectric constant. DMF was vigorously shaken with Sodium hydroxide (NaOH) pellets (20g/litre of DMF). It was decanted and mixed with equal volume of dry benzene. Benzene was used as an entrainer for separating a solution of DMF and moisture, the two being miscible in each other. The boiling point of DMF is 153°C and the water-benzene azeotrope boil at 69.25°C with 8.83% mass water. It was distilled using fractionating column. Pure DMF was collected at 153°C, flushed with N₂ gas and further stored in a brown bottle.

5.6 Silanization of glass vessel:

To neutralize the (-) ve charge of glass vessel, reaction vessel attached to automated peptide synthesizer was silanized with Chloroform (CHCl₃) and kept for 6-8 hrs on shaker at RT.

5.7 Swelling of Wang resin:

Amino acid attached to Wang resin was left suspended in the DMF overnight for swelling. Swelling will increase the surface area of the beads and making the reactions feasible.
5.8 Peptide Chain Elongation:

All the five peptides from HRP-2 and pLDH antigen were synthesized with Fmoc-Gly-wang resin using HBTU as a coupling agent.

Protocol:

1. After swelling of resin, DMF wash was given thrice to the resin (5ml, 3min each).
2. Deprotection of Fmoc group from N-terminus attached to resin was carried out in 20% piperidine twice in DMF (5ml, 3min each).
3. DMF wash was given thrice to the resin (5ml, 3min each).
4. For coupling, protected amino acid from N-terminus and side chains were mixed with HBTU, coupling agent in 5ml of 0.4M NMM and continuously stirred for 1hr.
5. DMF wash was given thrice (5ml) to the resin (3min each).

5.9 Ester bond formation:

Each amino acid was taken 2.5 times in excess of the actual amount of amino acid required, coupled to the resin to ensure 100% coupling. The coupling agent, HBTU, was also taken 2.5 times of required amount to increase the coupling efficiency for the synthesis. Protected amino acids and HBTU were dissolved in 0.4M NMM solution. Coupling time was kept more than 1hr. The coupling efficiency at every step was monitored by Kaiser’s Test.

5.10 Kaiser’s Test:

Ninhydrin (2,2-Dihydroxyindane-1,3-dione) is a chemical used to detect ammonia or primary and secondary amines. When reacting with these free amines, a deep blue or purple color known as Ruhemann's purple is produced. Ninhydrin is most commonly used to monitor deprotection in solid phase peptide synthesis as Kaiser Test. The chain is linked via its C-terminus to the solid support, with the N-terminus extending off it. When that nitrogen is deprotected, a ninhydrin test yields blue (Kaiser H., 1970). Amino-acid residues are attached to their N-terminus protected, so if the next residue has been successfully coupled onto the chain, the test gives a colorless or yellow result. Kaiser’ Test was used to monitor the coupling efficacy and if found positive, all the steps of peptide chain elongation were repeated till 100% coupling efficiency was achieved. It is to test completeness of coupling of each amino acid on the resin.
Reagents required:

1. Potassium cyanide (KCN)
2. Phenol
3. Ninhydrin

Protocol:

1. A small aliquot of peptide-resin was taken in a test tube and two drops of Potassium cyanide (KCN), Ninhydrin and Phenol were added.
2. Tubes were then placed in boiling water bath at 100°C for 5 min.
3. The tube containing plain amino acid substituted resin was taken as negative control.
4. The color of beads and solution was observed by holding them against white background.
5. The degree of coupling, the color of beads and solution was interpreted as follows:

<table>
<thead>
<tr>
<th>Color Percentage reacted</th>
<th>Bead Color</th>
<th>Solution color</th>
</tr>
</thead>
<tbody>
<tr>
<td>76.0</td>
<td>Dark blue</td>
<td>Dark blue</td>
</tr>
<tr>
<td>84.0</td>
<td>Dark blue</td>
<td>Moderate blue</td>
</tr>
<tr>
<td>94.0</td>
<td>Moderate blue</td>
<td>Light blue</td>
</tr>
<tr>
<td>99.5</td>
<td>Light blue</td>
<td>Trace blue</td>
</tr>
<tr>
<td>100</td>
<td>White</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

The color of beads with ninhydrin test varied substantially in intensity depending on the terminal residues or sequence. Aspartic acid, asparagine, glutamine and the secondary amino acids i.e. proline on resins gives a maroon or brown color.

5.11 Drying and Cleavage of peptides from resin:

Before acid cleavage of the peptidyl resin, the N-terminal Fmoc group on the N-terminus was deprotected in 10 min with a 20% solution of piperidine in dimethylformamide.

Reagents required:

Chemicals used:

1. Trifluoroacetic acid (TFA)
2. Dichloromethane (DCM)
3. Absolute ethanol


Scavengers used:

1. 1-2-Ethanedithiol
2. Anisole
3. Thioanisole

Protocol:

1. The peptidyl resin was taken out from resin and washed extensively with DMF, DCM and finally with absolute ethanol (10-15ml, 3 times each).
2. The peptide resin was dried in vacuum desiccators overnight.
3. Dried resin was taken in a small (50ml) beaker with magnetic bar.
4. The unprotected peptidyl resin was then treated with TFA in dichloromethane containing thioanisole, 1-2-Ethanedithiol and distilled water (1ml each) (10:1:1:1 v/v).
5. Mixture was kept for stirring at room temperature for 2hrs.
6. After completion of the reaction, the TFA peptide solution was separated from the resin using G-3 sintered glass funnel.
7. The resin in the sintered funnel was washed with 2-3ml of TFA and evaporated with the help of rotary vacuum evaporator at 30°C.
8. Peptides were directly precipitated using dry and ice-cold di-ethyl ether on ice bath.
9. Ether was then removed and the peptides were tritrated with fresh, dry and ice cold ether (2-3 times) to remove scavengers.
10. All the peptide were hydrophilic, the solubility of the peptides were then checked in increasing percentage of acetic acid (5-30%) and
11. The peptide solution was then lyophilized to obtain solid crude material.

5.12 Purification of peptides:

Purification of the crude peptides was carried out by gel permeation chromatography using Sephadex G-25 column. Sephadex G-25 was procured from Sigma Chemical Co. (St Louis, USA).

Material required:

1. Sephadex G-25
2. Whatmann filter paper
**Reagents required:**

1. Acetic acid
2. Methanol
3. N-Butanol
4. Ethyl acetate
5. Pyridine
6. 1-Fluoro-2,4-dinitrobenzene (FDNB)
7. Butanone

**Protocol:**

1. The sephadex G-25 (Cut off 1-5KDa) was swollen in double distilled water for 24 hrs.
2. After thorough wash, gel was packed in a glass column of 80 x 1cm dimensions.
3. The gel packed column was washed three bed volumes with double distilled water and finally equilibrated with two bed volume of 20% acetic acid.
4. The baseline of UV monitor was adjusted using the same percentage of acetic acid.
5. The crude peptide (100-200mg) was dissolved in 2.0ml of acetic acid and loaded onto the column.
6. The loaded peptides were eluted with respective % of acetic acid and the 2-2ml of fractions was collected in each tube kept in the attached fraction collector (LKB Pharmacia).
7. The mode of elution was being isocratic with the flow rate adjusted to 20ml/hr using peristaltic pump.
8. The peptide peak were monitored by reading absorbance at 254nm and 280nm using spectrophotometer.
9. The peak fractions showing identical Rf values were pooled and lyophilized.
10. The whole crude peptide was then subjected to a same protocol using same condition of gel chromatography.
11. The lyophilized purified peptide samples were stored in airtight vials at 4°C.
12. The reconfirmation of peaks at each stage of purification was done by pooling of fractions and spotting on Whatmann filter paper using different solvent systems and spotted on Whatmann filter paper.
13. The spots were visualized either by spraying with Ninhydrin spray or by characteristic amino acid spray i.e. Ehrlich spray (For Tryptophan and Pauly’s spray (for tyrosine and Histidine).
Material required:

For Ninhydrin spray:

1. Ninhydrin
2. Acetone

0.2mg of ninhydrin dissolved in 100ml of acetone

For Pauly’ spray:

1. Sulfanilic acid- 0.9g in 90ml of conc. HCl and 170ml of DDW
2. Sodium nitrite (5%) - 5g in 100ml of DDW
3. Sodium carbonate (10%) - 10g dissolved in 100ml of DDW

Reagent 1 and 2 were mixed (1:1 v/v) in cold and kept at 4°C for 5min. reagent 3 was then added twice the volume and the chromatogram was sprayed. Tyrosine containing peptides stains pink whereas histidine containing peptides stains orange.

Solvent system used for chromatography of various peptide antigens

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>Composition (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Ethyl acetate: pyridine: acetic acid: DDW</td>
<td>1:1:1:1</td>
</tr>
<tr>
<td>3. Pyridine: acetic acid: DDW</td>
<td>1:1:1</td>
</tr>
<tr>
<td>4. N-butanol: acetic acid: DDW</td>
<td>1:1:1</td>
</tr>
</tbody>
</table>

5.13. Physiochemical Characterization of the peptides:

5.13.1 Identification of C-terminal amino acid

Reagents/Chemicals required:

1. Carboxypeptidase A (1mg/ml in Tris-HCl buffer)
2. Tris HCl buffer (25mmol/lts, pH 7.5)
3. Peptide stock (5mg/ml in Tris HCl buffer)
4. 10% Trichloroacetic acid (TCA) in DDW
5. 0.2% ninhydrin in acetone

Protocol:

1. Carboxypeptidase A was mixed with equal volume of peptide solution.
2. After thoroughly mixing, the samples were kept at 37°C in water bath.
3. 200 μl of samples were removed at specific time intervals (0, 10, 20, 30 and 60 min) and immediately mixed with 0.2 ml of 10% TCA in another vial.
4. Samples were centrifuged at 5000 rpm for 5 min to settle down the precipitate.
5. Each sample was spotted on whatmann filter paper along with the respective amino acid standard and the unhydrolysed peptide in an appropriate solvent system.
6. The paper was dried and sprayed with 0.2% ninhydrin.
7. The developed colored spot on chromatogram was compared with the standard amino acid based on Rf values.

5.13.2 Identification of N-terminal amino acid:

Reagents required:
1. 1-fluoro-2,4 dinitrobenzene (FDNB) (5% v/v in ethanol)
2. Sodium carbonate (Na₂CO₃)
3. 0.2mM peptide stock (0.2μM)

Protocol:
1. 200μl of 0.2μM peptide antigens were mixed with equal amount of Sodium bicarbonate (Na₂CO₃) and dissolved in 2 ml DDW.
2. 100X excess molar ration of FDNB solution (20μM) was added and kept in continuous shaking for 2 hrs in an end to end shaker at RT.
3. The pH of the mixture was adjusted to 8.0 with 1 M Na₂CO₃.
4. The precipitate formed was washed number of times with dry ether to remove excess dinitrophenol (DNP) and FDNB.
5. The peptide was then hydrolyzed with 1 ml of 6N HCl in a sealed ampoule and kept at 110°C for 18 hrs.
6. The excess of HCl is then removed under vacuum and hydrolyzed peptide samples spotted on Whatmann filter paper along with the DNP- amino acid standards.
7. The chromatogram was developed in Acetic acid: N-butanol: DDW: ethyl acetate (1:1:1:1 v/v) and the Rf values of N-terminal tagged hydrolyzed peptide compared with the DNP-standard amino acids.

5.14 Reverse phase High performance Liquid chromatography (RP-HPLC):

The extent of purity of all the five HRP-2 and pLDH peptides was assessed using a Reverse phase high performance liquid chromatography (Ageilent Technologies, USA). For fulfilling this goal, peptides were subjected to RP-HPLC using a Zorbax 300 SB-C-8 column (250 x 9.4 nm inner diameter, 6.5μm particle size, 300Å pore size) with a linear gradient of acetonitrile and water in 0.1% TFA.
Analytic conditions and materials required:

Column: Zorbax 300 SB-C-8 column

(250 x 9.4 nm inner diameter; 6.5\(\mu\)m particle size, 300\(\AA\) pore size)

Buffer ‘A’ Millipored double distilled water with 0.1% TFA

Buffer ‘B’ HPLC grade Acetonitrile containing 0.1% TFA

Methanol: HPLC grade

Detection: 254nm/280nm

Flow rate: 1ml/min

Retention time: 5-45 min

Protocol:

1. Prior to sample injection, the column was washed thoroughly with HPLC grade methanol and equilibrated with 1:1 ration of buffer A: B.

2. 2mg of peptides dissolved in 0.2ml of Buffer ‘A’ was first clarified using clarification unit.

3. Once the baseline achieved, the peptide sample (0.1mg/100\(\mu\)l) was injected into the column.

4. The chromatogram was developed using a linear gradient of Buffer ‘A’ and Buffer ‘B’ (0-100%) at 1ml/hr flow rate.

5. This step was continued for purification of different peptides and the peptide peak was detected 254nm/280nm.

5.15 Amino acid analysis by RP-HPLC:

Amino acid analysis was also done after pre-derivatization with Phenylisothiocyanate (PITC) using PICO-TAG column.

Materials required:

1. RP-HPLC system
2. PICO-TAG column
3. Phenylisothiocyanate (PITC)
4. Standard amino acids
5. Speedvac
Reagents required:

1. Sodium acetate trihydrate
2. Conc. HCl
3. Phosphoric acid
4. Glacial acetic acid
5. Disodium hydrogen phosphate
6. Acetonitrile
7. Millopored double distilled water

5.15.1 Hydrolysis of the peptide:

Protocol:

1. The peptides (1mg) were first hydrolyzed in the hydrolysis vials with 6N HCl at 110°C for 18hrs.
2. The released amino acids from the samples and the amino acid standard solution (10μl) were dried under vacuum using Speedvac.

5.15.2 Step of Neutralization:

Protocol:

1. The dried hydrolyzed samples and the amino acid standards were re-dried in neutralization buffer using re-drying solution (water: ethanol: TEA:: 2:2:1)
2. The buffer was removed under vacuum.

5.15.3 Pre-column derivatization:

Protocol: The dried free amino acid mixture and the amino acid standards were then derivatized with Phenylthioisocyanate (PITC).

1. 200ul solution of ethanol: water: TEA: PITC at a ratio of 7: 1: 1: 1 was added to the dried samples and vortex and further incubated at room temperature for 20min.
2. After the reaction time over, PITC amino acid samples was again evaporated to dryness using Speedvac.

5.15.4 Analysis by RP-HPLC:

Protocol:

1. Derivatized samples and standards were reconstituted in 200μl of sample diluents
2. The PICO-TAG column was equilibrated with eluant ‘A’ (90%) and eluant ‘B’ (10%) till baseline was reached.
3. The standard samples were injected to get a representative chromatogram with elution timing of each amino acid.
4. This was followed by the injection of the test samples.
5. The amounts of individual amino acids were calculated from the peak area based on retention time of each derivative amino acid and by comparing with standard amino acid profile.

5.16 PLGA microspheres:

Microsphere based on poly (lactide) (PLA) or poly (lactide co glycolide) (PLGA) is probably the most promising of all the antigen delivery system studied. These are biodegradable, having less side effects and slowly release antigen that is entrapped inside it. The polymer degrades by non-enzymatic hydrolysis to the metabolites, lactic and glycolic acid (Random chain scission process) and the degradation rate depends in molecular weight and the ratio of lactide and glycolide.

5.17. PLGA microsphere preparation:

Microspheres with entrapped HRP-2 and pLDH peptides were prepared using poly (DL-lactide-co-glycolide; 50:50) by double solvent evaporation method (water-in-oil-in-water) (Blanco MD, 1997). All the materials required were purchased from Sigma Chemical Co. while the reagents used were procured from SRL, India. All the reagents were of analytical grade.

Material required:

1. Dicholomethane (DCM)
2. Poly (DL-lactide-co-glycolide; 50:50) (PLGA) (15%w/v): 190mg of PLGA was dissolved in 5ml of DCM.
3. Polyvinyl alcohol (PVA; 10%): 1.5g of PVA was dissolved in 15ml of cold DDW.
4. 0.01M PBS, pH 7.2
5. HRP-2 and pLDH peptides

Protocol:

1. The prepared 15% PLGA solution was emulsified together with the HRP-2 and LDH peptides (1mg of peptides in 100μl of 0.01M PBS, pH 7.2).
2. The mixture was homogenized in homogenizer (Silverson Machine Ltd.) for 3min to form primary water in oil (w1/o) emulsion.
3. The primary emulsion (w1/o) was slowly added to 15ml of 10% PVA (w2) solution.
4. Both together were homogenized for again 3min to form secondary (w1/o/w2) emulsion.
5. The resulting (w1/o/w2) emulsion was stirred overnight at RT to allow solvent evaporation to proceed.
6. The solution was centrifuged at 10,000rpm for 15min to get the final microsphere.
7. Washed three times with double distilled water to remove non entrapped peptide and excess PVA.
8. The final product was freeze-dried and stored in a desiccator at 25° C till use.

5.18 Disruption of PLGA microsphere by Double solvent extraction method:

**Reagents required:**
1. Acetonitrile
2. 0.01M PBS, pH 7.2
3. 0.01N NaOH
4. BCA assay kit

**Protocol:**
1. 1 ml of acetonitrile was added in the 10 mg of microsphere containing peptides.
2. The solution was centrifuged at 10,000 rpm for 5 minutes at RT.
3. The supernatant was discarded and the obtained pellet was air-dried.
4. To the air dried pellet, 1ml of 0.01M PBS, pH 7.2 was added and incubated at room temperature for 1 hr.
5. After incubation, solution was centrifuged at 10,000 rpm for 10 minutes at RT.
6. The PBS supernatant was preserved for peptide content estimation.
7. The pellet was again incubated for 1 hr with 1 ml of 0.1 NaOH to extract any undissolved antigen.
8. Followed by centrifugation at 10,000 rpm for 10 min.
9. The NaOH supernatant was again kept for peptide content estimation.
10. The PBS and NaOH supernatants were separately assayed for peptide content by Bicinchoninic Acid Assay method using BCA reagent.
11. From the standard curve (200-1000μg of the peptide of 30 amino acid length) obtained by plotting the peptide standard (1mg/ml of 30 amino acid length) concentrations against the O.D. at 562 nm, the amount of peptide present in the supernatant was calculated.
5.19 Percentage entrapment by BCA estimation:

The percentage entrapment for different peptides was determined by the bicinchoninic acid (BCA) protein assay (Sigma, IL, USA) (Smith P. K., 1987).

**Materials required:**

1. Protein stock (BSA) - 1 mg/ml
2. BCA reagent: Bicinchoninic acid and copper sulphate were taken in 50:1 ratio respectively
3. Peptide supernatants collected during the disruption of microspheres.

**Protocol:**

1. Put BSA standards and simultaneously add 10μl, 10 μl of preserved PBS and NaOH supernatants.
2. Add 200μl of BCA reagent in each well.
3. Incubate at 37°C for 1 hour.
4. Take absorbance at 562 nm.

5.20 Sizing of microsphere:

The prepared PLG microspheres were sized using Diffraction spectrophotometer (GALAI CIS 1).

**Materials required:**

1. PLG microspheres 1mg/200μl
2. PBS-Tween-20 (0.05%) 
3. Sonicator
4. Glass cuvette

**Protocol:**

1. 1mg/200μl of microsphere containing peptides were taken.
2. Resuspended it in PBS-Tween-20 (0.05%) 
3. Sonicate for 1min to get the homogenous mixture.
4. The microsphere suspension was taken in a cuvette and the size was determined using diffraction spectrophotometer.
5.20 **Morphological study of microspheres:**

Microsphere morphology was also studied by scanning electron microscopy (Phillips, CM 10) by amplifying signals with narrow electron beams and collected and feeding them to the cathode ray tube for the display.

**Reagents required:**
1. Glutaraldehyde (2.5%)
2. PBS (0.1M, pH 7.2)

**Protocol:**
1. 1mg of microsphere suspension was fixed with and without entrapped peptides in 2.5% glutaraldehyde for 2 hrs.
2. after centrifugation for 5min at 1500rpm, fixative was decanted and washed with PBS (0.1M, pH 7.2)
3. The pellet was smeared on glass slide.
4. After air drying, resuspended in DDW.
5. It was coated with silver or gold by sputtering at 300Å.
6. The PLG microspheres were viewed under Scanning electron microsphere (CM10).

5.21 **Animals Species:**

Female inbred Balbc (H-2d) mice with age group of 6-8 weeks and 2-3 month female rabbit (weight 2-3 kg) were used in the study. Each experimental group consisted of 6-8 mice and 4 rabbit. All the mice and rabbit were procured from the Experimental Animal Facility (E.A.F.), A.I.I.M.S., New Delhi. All animals were fed *ad libitum*. The experiments were conducted in accordance with the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, for care and use of laboratory animals), Ministry of Social Justice, Government of India and adopted by the Ethics Committee on animal experimentation, AIIMS, New Delhi

5.22 **Immunization doses along with Adjuvant Cpg ODN**

A group of six mice were immunized subcutaneously at the footpad with *Pf*HRP-2 peptide I and *Pf*LDH peptides (three regions I, II and III) respectively in microsphere containing Cpg ODN with primary dose of 30 g each on day 0 followed by a booster of 15 g on day 32 and 45. Likewise, four rabbits were also immunized with *Pf*HRP-2 peptide II with a primary dose of 100 g along with the Cpg ODN on day 0 followed by a booster of 50 g.
5.23 Microsphere immunization schedule and serum collection:
Mice and rabbits were bled from the retro-orbital plexus and the sera were collected on day 15, 28, 42, 60 and 90. The collected sera were separated by centrifugation at 10,000 rpm for 10 min at 4°C and kept at -70°C till further use.

5.24 ELISA (Enzyme Linked immunosorbent assay):
The following Buffers required in ELISA:

1. Coating buffer (0.05M Sodium carbonate-bicarbonate buffer, pH 9.6) 1X
   - Na₂CO₃ 1.59g
   - NaHCO₃ 2.93g
   - DDW 1000ml

2. Phosphate buffer saline (PBS) (0.1M, pH 7.25 ) 10X
   - NaCl 80g
   - KH₂PO₄ 2g
   - Na₂HPO₄·2H₂O 14.4g
   - KCl 2.2g
   - DDW 1000ml
   Solution diluted 10 times before use.

3. Phosphate buffer saline (PBS) Tween: 0.05%
   - PBS (0.1M, pH 7.25) 1000ml
   - Tween-20 0.5ml

4. Substrate buffer (0.015M Citrate phosphate buffer, pH 5.0) 1X
   - Solution ‘A’: Citric acid- 21.014g/ 100ml of DDW
   - Solution ‘B’: Na₃HPO₄- 28.392g/ 100ml of DDW
   Mixed Solution ‘A’ and ‘B’ in the ratio of 24.3:25.7ml. Each solution is diluted 10 times before use.

5. Substrate solution:
   - OPD·HCl 4mg
   - H₂O₂ 10 µl
   - Substrate buffer 10ml

6. Stop solution: 8N H₂SO₄
   - H₂SO₄ (Conc., 36N) 4ml
   - DDW 32ml
5.24.1 Estimation of peptide specific IgG in sera and end point titres:

The peptide specific antibody level and peak titres were estimated using standard ELISA protocol:

1. The 96 well immunol II plates were coated with 100ng/well of peptide antigens in coating buffer overnight at 4°C.
2. The nonspecific binding sites were blocked with 1% BSA or 5% milk powder for 2hrs at 37°C.
3. Wash thrice with PBS-Tween 20 (0.05%).
4. Mice and rabbit sera at 1:100 dilution were (for peptide specific IgG level) and twofold serially diluted (for measurement of peak antibody levels) were added for 2hr. at 37°C,
5. The plates were incubated at 37°C for 2hrs and later washed three times with PBS containing 0.5% Tween 20.
6. The peroxidase conjugated secondary antibody (Goat anti mouse IgG-HRPO/goat anti rabbit IgG-HRPO conjugated, Sigma) was added at the dilution of 1:1000.
7. The plates were incubated at 37°C for 1hr.
8. The color was developed using OPD (O-phenylene di-amine) as a chromogen, H₂O₂ as substrate in citrate buffer.
9. The reaction of color development was stopped finally using 8N H₂SO₄
10. The absorbance was read at 492nm.

The end point titres were expressed as reciprocal of the highest sera dilution which gave an absorbance = pre-immune sera +4SD.

5.25 Purification of antibodies by affinity chromatography on a protein A column

Materials:
Protein A sepharose CL-4B gel (Procured from M/S Bangalore Genei, Ltd, India)
Reagents required:
1. Tris buffered saline 50nM Tris HCl, pH 7.4, 150mM NaCl
2. Neutralization Buffer 1M Tris HCl, pH 8.0, 1.5 M NaCl, 1mM EDTA
3. Elution buffer 50mM Glycine HCl, pH 2.7

Protein A is isolated from *staphylococcus aureus* and bind to IgG via Fc (constant fragment) region of the IgG. The proteins A bind with antibodies by hydrophobic interactions and are disrupted by transient exposure to low pH.

The protein A column (bed volume 2ml) was initially equilibrate with 20ml of TBS. 500µl of sera diluted in 1:4 (2ml) in TBS was loaded on the column. The column was
run through TBS buffer (50mM Tris HCl, pH 7.4). To ensure the binding of the immunoglobulin to the protein A beads, elution profile was monitored by talking absorbance at 280nm. The bound immunoglobulin was eluted by using elution buffer (50mM glycine HCl, pH 7.2). 100 µl eluted fractions were collected and mixed with 100 µl of neutralization buffer (1M Tris HCl, pH 8.0, 1.5M NaCl, 1mM EDTA) in a microcentrifuge tubes. Absorbance was read at 280nm. All the fraction having IgG were pooled and dialyzed against PBS (pH 7.4) in a dialysis bag with cutoff value ≤ 100KD. After dialysis IgG were lyophilized and IgG concentration was calculated using BCA method.

5.26 Biotinylation of antibodies rose against Synthetic peptides of PfHRP-2 and PfLDH protein

Biotinylation of the purified antibodies was done using N-hydroxy succinimide ester of biotin (Sigma immunoprobe Biotinylation reagents) according to the manufacture’s protocol. Biotin reacts with the ω-amino group of the peptide and proteins to give stable bonds in the form of N-hydroxy succinimide ester. The extended spacer arm from the carboxylic acid greatly improves the interaction between Avidin and Biotinylated macromolecules by overcoming steric hindrance present at the binding site of Avidin. The derivatives are water soluble. Labeling of the antibodies was done by using biotinylation process at neutral pH values.

Reagent required

A. Biotinylation reagent: Biotinamidocarporate-N-hydroxy sulfoximide ester (BAC-Sulfo NHS) 5mg was dissolved in 30µl of DMSO and 0.1M of PBS (pH 7.4) was added to a total volume of 500 µl (stock 10mg/ml).

B. Avidin, affinity purified (10mg), was dissolved in 19.4 ml of 0.1M of PBS, pH 7.4.

C. HABA Reagents: The reagent was prepared by dissolving 10mM of 4'-Hydroyazobenzene-2-carboxylic-acid (HABA) in 1ml of 10mM NaOH. Reagent A was taken along with purified IgG (5mg each) in a molar ratio of (5:1) in a total volume of 250µl and incubated for overnight at room temperature on end shaker. Following labeling reaction, the reaction mixture was loaded on to a Sephadex G-25 column (bed volume 10ml) equilibrate with PBS1X and 1.0 ml of fractions were collected. The presence of antibodies in fraction was monitored by measuring at 254 nm or 280 nm. Again the Biotinylated antibodies (IgG) and the free biotin molecules were separated by dialysis in PBS1X buffer (pH 7.4).
5.26.1 Avidin–HABA assay:
The extent of biotinylation and ratio of biotin to antibody was determined by the avidin–HABA (4′-Hydroyazobenzene-2-carboxlic-acid) assay (Wilchek M et al, 1990). 0.1ml of reagent ‘C’ was added and gently mixed to 3.2ml of reagent ‘B’. The absorbance of the mixed solution was read at 500nm that is approximately 1.0 (1cm path length). In a 1 ml cuvette or pipet 900μl HABA/avidin reagent (reconstituted as above) were treated with 100μl sample, mix by inversion, and then read at 500nm. In some cases, as with a biotinylated protein, the absorbance may slowly decrease with time. If this occurs, a wait of 2 minutes before reading the absorbance is recommended. For colored samples, a blank must also be prepared as well. Dilute 100 μl samples with 900 μl water or diluent read at A500. The molar ratio of Biotin/IgG was calculated to give the average number of biotin molecule per each molecule of antibody.

Calculations

a) \[
\Delta A_{500} = 0.9(A_{\text{HABA/Avidin}}) - A_{\text{HABA/Avidin}} + \text{sample}
\]

\[
0.9 = \text{Dilution factor of HABA/Avidin upon addition of the sample}
\]

(For colored samples, the absorbance of the blank must be taken into consideration as well):

\[
\Delta A_{500} = 0.9(A_{\text{HABA/Avidin}}) + (A_{\text{sample blank}}) - (A_{\text{HABA/Avidin sample}})
\]

\[
0.9 = \text{Dilution factor of HABA/Avidin upon addition of sample}
\]

b) \[
\mu\text{mole biotin/ml} = (\Delta A_{500}/34)(10)
\]

34 = mM extinction coefficient at 500 nm 10 = dilution factor of sample into cuvette

c) Mole biotin/mole protein = \mu\text{mole biotin/ml sample}

\mu\text{mole protein/ml sample}

The absorbance of HABA/avidin complexes at 500nm (A_{500}) decreases proportionally with increased concentration of biotin as the HABA dye is displaced from avidin due to the high affinity of Avidin for biotin.

5.27 Cross Reactivity experiment to conform the specificity of the antibodies produced

To confirm the specificity of the antibodies produced, the antisera generated in the mice and rabbit against the synthesized peptides of HRP-2 and pLDH were reacted with different peptides synthesized from different stages of the life cycle of the parasite viz. CSP and RESA and cross species antigens like p24 antigen of HIV. In
brief, 100ng/100µl of the peptide (OCTA, CSP, HIV-1, LDH-1, and LDH-3) was coated per well in a 96 well Immunol-2Hb plate. The plate was kept overnight 4°C and blocked with 5% skimmed milk solution for 2hrs at 37°C. The wells were then washed thrice with PBS-T-20 (0.05%). Mice and rabbit antisera at 1:100 dilutions were added and incubate at 37°C for 2 hrs. After washing with PBS-T-20 secondary antibody, goat antimouse IgG HRPO/goat antirabbit IgG HRPO were added in each well at 1:1000 dilution and incubate at 37°C for 2 hrs. Again plate was washed thrice with PBS-T-20 and 100 µl of substrate was added to each well and color was developed after 5min. The reaction was stopped by adding 8N H₂SO₄. Absorbance was read at 492nm. Pre immune sera were used as negative control.

5.28 Binding affinity of the anti peptide antibodies (K<sub>d</sub>)

The affinity of antibodies raised against different peptides in mice was determined by measuring the dissociation constant (K<sub>d</sub>) (Friguet B et al. 1985). In brief, immunized sera from mice/rabbit at 1: 200 dilutions were incubated with different concentrations of the peptide (0.1-10 nM) for 15 h at 20°C so as to attain antigen-antibody equilibrium. The antigen-antibody complexes were transferred onto the wells of the microtitre plates previously coated with the respective peptide (500ng/well). The plates were incubated for 90 min at 37°C. After washing 3 times with PBS-T, goat anti-mouse/rabbit IgG HRPO (1:1000) conjugate was added (100 µl /well) and incubated for 1 h at 37°C. Color was developed as mentioned above. Dissociation constants were then calculated using regression analysis and a simplification of the mathematical equation of Scatchard and Klotz.

\[ \frac{A_0}{A_0 - A} = 1 + \frac{K_d}{a_0} \]

Where \( A_0 \) is the absorbance measured for the antibody in the absence of peptide, \( A \) is the absorbance measure for the antibody with peptide and \( a_0 \) is the total peptide concentration.

5.29 In-vitro culture of \( P. falciparum \)

Culture of \( P. falciparum \) (Indian isolates FDL-B, FDL-NG, FSH-4 and FSH-11) was done by using O<sup>+</sup> RBCs and AB<sup>+</sup> serum using the standard procedure (Trager W et al. 1976). This part of the study was done in collaboration with National Institute of Malaria Research (NMRC), New Delhi, India.

Reagents Required:

RPMI- 1640, L-glutamine, N-2 Hydroxyethyl piperazine N-2 ethane sulphonic acid (HEPES), Gentamycin sodium bicarbonate, Normal human serum (AB<sup>+</sup>), Acid citrate Dextrose solution (ACD), Normal human blood (A<sup>+</sup> or O<sup>+</sup>), P.falciprum infected human blood, Nutrient broth, JSB stain/ Giemsa Stain, Methanol.
In Brief, 3-5ml of blood in ACD (Acid citrate and Dextrose) solution was taken from clinically proven malaria patients having 1-2% parasitemia and 5ml of blood from a healthy person A+ and O+ group in ACD solution. Both infected and normal blood was centrifuged, at 1,500rpm for 10min, the plasma was aspirated and buffy coat was removed. RBC pellet was washed thrice with plain RPMI-1640 medium. 50% suspension from both RBC pellet was prepared with complete medium (RPMI-1640 + 15% human AB+ serum) and mix in 1:1 ratio. Then the thin smear was prepared and examined. Suspension of 8% was prepared by mixing RBC with complete medium and 1.2ml of 8% erythrocyte suspension was seeded per vial. Culture vials were arranged in dessicator with burning candle. The lid was placed on dessicator with the stopcock at the open position. Stopcock was closed as soon as the candle snuffed off. Dessicator was transferred in BOD incubator at 37°C and the medium was changed every 24 hrs. After 96 hrs the contents of the vials were pooled and centrifuged at 1,500 rpm for 10 min. Supernatant was taken and stored at -20°C.

5.30 Preparation of *P. falciparum* Lysate/

*P. falciparum* isolates (Indian isolates FDL-B, FDL-NG, FSH-4 and FSH-11) were maintained in routine in vitro culture by technique described above. Each batch of culture was monitored for parasitemia by microscopy, then parasites were harvested and culture supernatants were aspirated. Parasitized RBCs were washed thrice with PBS, and both the pellet and supernatant were stored at -20°C. Antigen was prepared from cultures enriched with late trophozoite and schizonts. Parasites were freed by saponin lysis and soluble extract was obtained after MSE Soniprep (sonication) at 14 μA for 90s.

5.31 Study Subjects

A cross-sectional survey was conducted in the villages of Baghpat district of Uttar Pradesh, in northern India, from August to November in the years 2010 to 2011. This area is endemic for malaria having seasonal transmission of both *P. vivax* and *P. falciparum*. Early and prolonged monsoons are responsible for intensive transmission for both the species. In this area, among the anopheline population, *Anopheles culicifacies* is most abundant vector followed by *An. annularis* and *An. subpictus*. A total of 2050 individuals reported with history of fever. Giemsa stained thick and thin peripheral blood smears of each patient were examined microscopically. In addition, finger-prick blood samples of malaria positive and healthy subjects were tested by Immunochromatographic based rapid diagnostic test (RDT) kit, FalciVax, rapid test for *Pv/Pf* (Zephyr Biomedicals, Verna, Goa, India). Parasite density was estimated by counting the number of parasites per 200 leucocytes and the counts were converted to
number of parasites/µl blood taking 8000 leucocytes/µl as a standard mean. More than 100 microscopic thick smear fields were checked before declaring a slide negative. Blood samples from 200 patients diagnosed by above test with uncomplicated *P. falciparum* were collected by finger-prick in heparinized tubes. In a similar way, blood samples were collected from 50 cases of *P. vivax* malaria and 50 healthy individuals as negative control. The patients diagnosed with malaria were treated with recommended antimalarial as per National Drug Policy. The samples were brought to the laboratory in cold chain.

5.32 Standardization of the HRP-2 and pLDH antigen detection in the culture supernatants/parasite lysate of *in-vitro* culture of *P. falciparum*.

In brief, each well of the microtiter plates were coated o/n at 4°C with 1µg/2.5µg of purified antibody against both the HRP-2 peptides (anti HRP-2 peptide I IgG and anti HRP-2 peptide II IgG respectively) and pLDH peptides (anti LDH peptide I IgG/ anti LDH peptide II IgG/ anti LDH peptide III IgG and a cocktail of anti LDH peptide- I,II IgG/ cocktail of anti LDH peptide- I,III/ cocktail of anti LDH peptide- II,III respectively) in coating buffer (carbonate buffer, pH 9.6). After incubation plates were washed thrice with PBS-T-20 and dried. Then the plate was blocked with 5% BSA and incubates for 2 hrs. at 37°C. Plate was washed three times with PBS-T-20. Then the 100µl of *P.f* culture supernatant and parasitized RBC lysate was added in each well and incubate at 37°C for 1 hr. The plate was washed three times with washing buffer and 1µg /2.5µg of biotinylated antibody against HRP-2 (anti HRP-2 peptide I IgG and anti HRP-2 peptide II IgG respectively, raised in mice and rabbit) and 1µg /2.5µg of biotinylated antibody against LDH peptide (anti LDH peptide I IgG/ anti LDH peptide II IgG/ anti LDH peptide III IgG and a cocktail of anti LDH peptide- I,II IgG/ cocktail of anti LDH peptide- I,III/ cocktail of anti LDH peptide-II,III respectively, raised in mice) were added and incubated at 37°C for 1 hr. after washing with washing buffer, 100µl of streptavidin-HRPO conjugate at 1:1000 dilution were added in each well and incubated for 1hr. at 37°C. After usual washing with PBS-T-20, 100µl of substrate solution were added in each well and the reaction was stopped by 8N H₂SO₄. At last absorbance was taken at 492 nm. Normal RBC lysate (NRBC Lysate) and culture supernatant of NRBC lysate were used as control.

5.33 Detection of HRP-2 and pLDH antigen in culture supernatants/parasite lysate *P.falciparum*.

In brief, each well of the microtiter plates were coated o/n at 4°C with 2.5µg of purified antibody against HRP-2 peptides I (anti HRP-2 peptide I IgG) and 2.5µg of pLDH peptides III (anti LDH peptide III IgG) in 100µl coating buffer (carbonate buffer, pH 9.6). After incubation plates were washed thrice with PBS-T-20 and dried. Then the plate was blocked with 5% BSA and incubates for 2 hrs. at 37°C. Plate was washed three times with washing buffer and 1µg /2.5µg of biotinylated antibody against HRP-2 (anti HRP-2 peptide I IgG and anti HRP-2 peptide II IgG respectively, raised in mice and rabbit) and 1µg /2.5µg of biotinylated antibody against LDH peptide (anti LDH peptide I IgG/ anti LDH peptide II IgG/ anti LDH peptide III IgG and a cocktail of anti LDH peptide- I,II IgG/ cocktail of anti LDH peptide- I,III/ cocktail of anti LDH peptide-II,III respectively, raised in mice) were added and incubated at 37°C for 1 hr. after washing with washing buffer, 100µl of streptavidin-HRPO conjugate at 1:1000 dilution were added in each well and incubated for 1hr. at 37°C. After usual washing with PBS-T-20, 100µl of substrate solution were added in each well and the reaction was stopped by 8N H₂SO₄. At last absorbance was taken at 492 nm. Normal RBC lysate (NRBC Lysate) and culture supernatant of NRBC lysate were used as control.
After incubation plates were washed thrice with PBS-T-20 and dried. Then the plate was blocked with 5% BSA and incubates for 2 hrs. at 37°C. Plate was washed three times with PBS-T-20. Then, the 100μl of P.f culture supernatant at different dilutions (neat, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64) and parasitized RBC lysate at parasitized RBC lysate at various parasitemia levels (ranging from 250,000-50 parasites/μl) was added in each well and incubate at 37°C for 1 hr. The plate was washed three times with washing buffer and 2.5μg of biotinylated antibody against HRP-2 peptide II (anti HRP-2 peptide II IgG respectively, raised in rabbit) and a cocktail of 2.5 μg of purified biotinylated anti-Pf/LDH peptide I and anti-Pf/LDH peptide II antibody were added and incubated at 37°C for 1 hr. after washing with washing buffer, 100μl of streptavidin-HRPO conjugate at 1:1000 dilution were added in each well and incubated for 1hr. at 37°C. After usual washing with PBS-T-20, 100μl of substrate solution were added in each well and the reaction was stopped by 8N H₂SO₄. At last absorbance was taken at 492 nm. Normal RBC lysate (NRBC Lysate) and culture supernatant of NRBC lysate were used as control.

5.34 Development of PfHRP2 and PfLDH antigen assay design

For detecting the PfHRP2 antigen levels in P. falciparum positive patients, an ELISA was designed in which the plates were coated overnight at 4°C with 2.5 μg/100μl of purified anti PfHRP2 peptide I antibody raised in mice. After blocking with 5% BSA, 100μl of RBC lysates of all the blood samples from P. falciparum positive patients were added at 1:100 dilutions in each well and incubated at 37°C for 1 h. Plates were washed with PBS-Tween-20 and then 2.5 μg/100μl of purified Biotinylated rabbit anti-PfHRP2 peptide II antibody was added and incubated at 37°C for 1h. For detecting parasite LDH antigen levels in the P. falciparum positive patients, an ELISA was designed in which plates were coated with 2.5 μg of purified anti-Pf/LDH peptide III antibody in coating buffer and kept overnight. After blocking with 5% BSA, 100μl of RBC lysates of all the blood samples from P. falciparum positive patients were added at 1:100 dilution in each well and incubated at 37°C for 1 h. Plates were washed with PBS-Tween-20 and then a cocktail of 2.5 μg of purified biotinylated anti-Pf/LDH peptide I and anti-Pf/LDH peptide II antibody was added and incubated at 37°C for 1h. The bound antigen-antibody complex was detected using streptavidin-HRPO (1:1000 dilutions) and color was developed using OPD as a chromogen and the absorbance was read at 492 nm. Similar ELISA was done with P.f culture supernatant at different dilutions (neat, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64) or parasitized RBC lysate at various parasitemia levels (ranging from 250,000-50 parasites/ l) to develop a standard curve. RBC lysate and culture supernatant from non-infected blood of normal volunteers were used as a control.
5.35 Statistical Analysis

All the analysis of data was done with the help of Stata/IC (version 12.1). ELISA result was analyzed by Rank-Sum test (non-parametric approach) to compare the values of HRP-2 and LDH antigen between cases and control and comparison between two sets of ELISA and microscopy was done by Spearman Rank correlation coefficient. The level of statistical significance was seen at p<0.05 (5%). Of total IgG and antibody titres from different bleeds were determined by the Friedman Test separately for each group and group variability was analyzed by Kruskal-Wallis (data not shown). The slope of the lines (KD Values) was calculated by regression analysis.
Material and Methods
Group B: Chikungunya
5. **Material and Methods**

5.1 **Computer algorithmic analysis of peptide:**

The entire protein sequence of Chikungunya E1, E2, and E3 envelope protein were analyzed by B-cell prediction program (EMTECH, Chandigarh) and DNASTAR software. Peptides were selected from E1, E2 and E3 envelope proteins of S27 strain of CHIKV on the basis of physiochemical properties such as antigenicity, hydrophilicity, hydrophobicity, flexibility/mobility, exposed surface antigenicity and \(\beta\) turns, using different Bcelpred and DNAstar software program.

Surface localization of all the peptides was done by PDB blast using PYMOL software (PYMOL molecular graphics system). The protein sequence for CHIKV S7 strain were obtained from the Gene Bank accession number AF369024 ((GenBank Accession AF369024). DNAstar and Bcelpred software (www. IMTECH Bioinformatics) were used for the selection of peptides on the basis of physiochemical properties such as antigenicity, hydrophilicity, hydrophobicity, flexibility/mobility, exposed surface antigenicity and \(\beta\) turns. Structural data for epitope location was drawn by PYMOL [DeLano WL (2002) The PYMOL Molecular Graphics System on World Wide Web](http://www.pymol.org)

5.2 **Selection of peptides:**

E1, E2 and E3 peptides from Chikungunya virus E1, E2 and E3 protein were synthesized by solid phase peptide synthesis using the F-moc chemistry on phenylacetamidomethyl (PAM) resin. A total of 7 peptides from E, 17 peptides from E2 and 1 peptide from E3 were synthesized (Table 1).

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Peptides</th>
<th>Sequence of selected peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E1P1</td>
<td>KCCGTAECDDKLPDYS</td>
</tr>
<tr>
<td>2</td>
<td>E1P2</td>
<td>DAENTQLSEAHVEKSESCKT</td>
</tr>
<tr>
<td>3</td>
<td>E1P3</td>
<td>FGIQSTPSKVDVYA</td>
</tr>
<tr>
<td>4</td>
<td>E1P4</td>
<td>AASKKGKCAVHSMTN</td>
</tr>
<tr>
<td>5</td>
<td>E1P5</td>
<td>DAPSVDMSCEVPACTHSSDF</td>
</tr>
<tr>
<td>6</td>
<td>E1P6</td>
<td>ASAYRAHTASASAK</td>
</tr>
<tr>
<td>7</td>
<td>E1P7</td>
<td>AYANGDHAVTVKDAKF</td>
</tr>
<tr>
<td>8</td>
<td>E2P1</td>
<td>HCPDCGEGHSCHSPV</td>
</tr>
<tr>
<td>9</td>
<td>E2P2</td>
<td>RIRNEATDGLKI</td>
</tr>
</tbody>
</table>
Hence the conserved regions are more valuable for development of diagnostic reagent compare to variable regions based on immune responses. E1 and E2 have now been considered as two major glycoproteins responsible for infection. E1 glycoprotein is responsible for cell membrane fusion, while E2 is primarily involved in receptor binding and cell entry as well as eliciting neutralizing antibodies. After BLAST analysis all the conserved regions were selected from the envelope protein of CHIKV and these selected peptides were not showing any sequence similarity with other related alpha viruses.

### 5.3 Chemical synthesis Peptide.

All peptides were synthesized using F-moc chemistry on C-terminal amino acid substituted phenylacetamidomethyl (PAM) resin. The Fast-moc chemistry, with double coupling throughout was used to synthesize all the selected peptides from E1, E2 and E3 envelope protein by automated peptide synthesizer (PS3, Protein Technologies, U.S.A.). All the amino acids were orthogonally protected. The glycine wang resin was used as a solid support for the growing amino acid chain. The Fmoc carbonate group was used for the protection of amino acids.
Chemicals used:

1. Fmoc protected L-amino acid derivatives
2. Glycine Wang resin
3. 1-H-benzotrizolium hexafluoro phosphate 3-oxide (HBTU)

Reagents used:

1. N,N-dimethyl formamide (DMF)
2. N-methyl-morpholine (NMM)
3. Piperidine
4. Chloroform

All the protected amino acid derivatives were procured from Sigma chemicals Co. (St. Louis, USA) and Chem Impex (Technoconcept, Wood dale, IL, USA). The side-chain protecting groups were the following: Glu, Thr and Tyr, O-tButyl; Trp, 1-H-benzotrizolium hexafluoro phosphate 3-oxide (HBTU), coupling reagent, was procured from Peptide International. Trifluoroacetic acid (TFA), 2-Ethanedithiol, Anisole and Thioanisole were purchased from Sigma chemicals Co. (St. Louis, USA). Amino acid substituted Glycine Wang resin was taken from Advanced Chemtech. All the reagents used were of analytic grade and obtained from SRL, India. Piperidine was purchased from Spectrochem Pvt. Ltd. while N-methyl-morpholine (NMM) was taken from Merck, Germany.

5.4 Distillation of DMF:

The number of end products and moisture was formed due to the longtime of storage of DMF, which will slow down the coupling reaction. So, before starting peptide synthesis, distillation of DMF is required to remove impurities.

Reagents required:

1. Dimethylformamide (DMF)
2. Benzene
3. Sodium metal
4. Sodium hydroxide (NaOH)
5. N₂ gas

Dimethylformamide (DMF) is a colorless, highly-boiling, mobile, polar liquid with a faint, characteristic odor. It does not decompose on distillation and is freely miscible with water, alcohols, ethers, ketones, esters, carbon disulfide and chlorinated and aromatic hydrocarbons. It is either immiscible or only partly miscible with aliphatic hydrocarbons. Even at elevated temperatures, aqueous solutions of DMF have very
little tendency to hydrolyze. However, the addition of acids or bases accelerates
hydrolysis to formic acid and 1, 3-dimethylamine. DMF is an operatic solvent with a
high dielectric constant. DMF was vigorously shaken with Sodium hydroxide (NaOH)
pellets (20g/liter of DMF). It was decanted and mixed with equal volume of dry
benzene. Benzene was used as an entrainer for separating a solution of DMF and
moisture, the two being miscible in each other. The boiling point of DMF is 153°C
and the water-benzene azeotrope boil at 69.25°C with 8.83% mass water. It was
distilled using fractionating column. Pure DMF was collected at 153°C, flushed with
N₂ gas and further stored in a brown bottle.

5.5 Silanization of glass vessel:

To neutralize the (-) ve charge of the glass vessel, reaction vessel attached to
automated peptide synthesizer was silanized with Chloroform (CHCl₃) and kept for 6-
8 hrs on a shaker at RT.

5.6 Swelling of Wang resin:

Amino acid attached to Wang resin was left suspended in the DMF overnight for
swelling. Swelling will increase the surface area of the beads and making the
reactions feasible.

5.7 Peptide Chain Elongation:

All the selected peptides from E1, E2 and E3 protein were synthesized with Fmoc-
Gly-wang resin using HBTU as a coupling agent.

Protocol:

1. After swelling of resin, DMF wash was given thrice to the resin (5ml, 3min each).
2. Deprotection of Fmoc group from N-terminus attached to resin was carried out in
20% piperidine twice in DMF (5ml, 3min each).
3. DMF wash was given thrice to the resin (5ml, 3min each).
4. For coupling, protected amino acid from N-terminus and side chains were mixed
with HBTU, coupling agent in 5ml of 0.4M NMM and continuously stirred for
1hr.
5. DMF wash was given thrice (5ml) to the resin (3min each).

5.8 Ester bond formation:

Each amino acid was taken 2.5 times in excess of the actual amount of amino acid
required, coupled to the resin to ensure 100% coupling. The coupling agent, HBTU,
was also taken 2.5 times of required amount to increase the coupling efficiency for the
synthesis. Protected amino acids and HBTU were dissolved in0.4M NMM solution.
Coupling time was kept more than 1hr. The coupling efficiency at every step was monitored by Kaiser’s Test.

5.9 Kaiser’s Test:

Ninhydrin (2,2-Dihydroxyindane-1,3-dione) is a chemical used to detect ammonia or primary and secondary amines. When reacting with these free amines, a deep blue or purple color known as Ruhemann's purple is produced. Ninhydrin is most commonly used to monitor deprotection in solid phase peptide synthesis as Kaiser Test. The chain is linked via its C-terminus to the solid support, with the N-terminus extending off it. When that nitrogen is deprotected, a ninhydrin test yields blue (Kaiser H., 1970). Amino-acid residues are attached to their N-terminus protected, so if the next residue has been successfully coupled onto the chain, the test gives a colorless or yellow result. Kaiser’ Test was used to monitor the coupling efficacy and if found positive, all the steps of peptide chain elongation were repeated till 100% coupling efficiency was achieved. It is to test completeness of coupling of each amino acid on the resin.

Reagents required:

1. Potassium cyanide (KCN)
2. Phenol
3. Ninhydrin

Protocol:

1. A small aliquot of peptide-resin was taken in a test tube and two drops of Potassium cyanide (KCN), Ninhydrin and Phenol were added.
2. Tubes were then placed in boiling water bath at 100°C for 5min.
3. The tube containing plain amino acid substituted resin was taken as negative control.
4. The color of the beads and the solution was observed by holding them against a white background.
5. The degree of coupling, the color of beads and the solution was interpreted as follows:
<table>
<thead>
<tr>
<th>Color Percentage reacted</th>
<th>Bead Color</th>
<th>Solution color</th>
</tr>
</thead>
<tbody>
<tr>
<td>76.0</td>
<td>Dark blue</td>
<td>Dark blue</td>
</tr>
<tr>
<td>84.0</td>
<td>Dark blue</td>
<td>Moderate blue</td>
</tr>
<tr>
<td>94.0</td>
<td>Moderate blue</td>
<td>Light blue</td>
</tr>
<tr>
<td>99.5</td>
<td>Light blue</td>
<td>Trace blue</td>
</tr>
<tr>
<td>100</td>
<td>White</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

The color of beads with ninhydrin test varied substantially in intensity depending on the terminal residues or sequence. Aspartic acid, asparagine, glutamine and the secondary amino acids i.e. proline on resins gives a maroon or brown color.

**5.10 Drying and Cleavage of peptides from resin:**

Before acid cleavage of the peptidyl resin, the N-terminal Fmoc group on the N-terminus was deprotected in 10 min with a 20% solution of piperidine in dimethylformamide.

**Reagents required:**

**Chemicals used:**

1. Trifluoroacetic acid (TFA)
2. Dichloromethane (DCM)
3. Absolute ethanol

**Scavengers used:**

1. 1-2-Ethanedithiol
2. Anisole
3. Thioanisole

**Protocol:**

1. The peptidyl resin was taken out from resin and washed extensively with DMF, DCM and finally with absolute ethanol (10-15ml, 3times each).
2. The peptide resin was dried in vacuum desiccators overnight.
3. Dried resin was taken in a small (50ml) beaker with magnetic bar.
4. The unprotected peptidyl resin was then treated with TFA in dichloromethane containing thioanisole, 1-2-Ethanedithiol and distilled water (1ml each) (10:1:1:1 v/v).
5. Mixture was kept for stirring at room temperature for 2hrs.
6. After completion of the reaction, the TFA peptide solution was separated from the resin using G-3 sintered glass funnel.
7. The resin in the sintered funnel was washed with 2-3ml of TFA and evaporated with the help of rotary vacuum evaporator at 30°C.
8. Peptides were directly precipitated using dry and ice-cold di-ethyl ether on ice bath.
9. Ether was then removed and the peptides were tritrated with fresh, dry and ice cold ether (2-3 times) to remove scavengers.
10. All the peptide were hydrophilic, the solubility of the peptides was then checked in an increasing percentage of acetic acid (5-30%) and
11. The peptide solution was then lyophilized to obtain solid crude material.

5.11 Purification of peptides:
Purification of the crude peptides was carried out by gel permeation chromatography using Sephadex G-25 columns. Sephadex G-25 was procured from Sigma Chemical Co. (St Louis,USA).

Material required:
1. Sephadex G-25
2. Whatmann filter paper

Reagents required:
1. Acetic acid
2. Methanol
3. N-Butanol
4. Ethyl acetate
5. Pyridine
6. 1-Fluoro-2,4-dinitrobenzene (FDNB)
7. Butanole

Protocol:
1. The sephadex G-25 (Cut off 1-5KDa) was swollen in double distilled water for 24 hrs.
2. After thorough wash, gel was packed in a glass column of 80 x 1cm dimensions.
3. The gel packed column was washed three bed volumes with double distilled water and finally equilibrated with two bed volume of 20% acetic acid.

4. The baseline of UV monitor was adjusted using the same percentage of acetic acid.

5. The crude peptide (100-200mg) was dissolved in 2.0ml of acetic acid and loaded onto the column.

6. The loaded peptides were eluted with respective % of acetic acid and the 2-2ml of fractions was collected in each tube kept in the attached fraction collector (LKB Pharmacia).

7. The mode of elution was being isocratic with the flow rate adjusted to 20ml/hr using peristaltic pump.

8. The peptide peak were monitored by reading absorbance at 254nm and 280nm using spectrophotometer.

9. The peak fractions showing identical Rf values were pooled and lyophilized.

10. The whole crude peptide was then subjected to a same protocol using same condition of gel chromatography.

11. The lyophilized purified peptide samples were stored in airtight vials at 4°C.

12. The reconfirmation of peaks at each stage of purification was done by pooling of fractions and spotting on Whatmann filter paper using different solvent systems and spotted on Whatmann filter paper.

13. The spots were visualized either by spraying with Ninhydrin spray or by characteristic amino acid spray i.e. Ehrlich spray (For Tryptophan and Pauly’s spray (for tyrosine and Histidine).

**Material required:**

**For Ninhydrin spray:**

1. Ninhydrin
2. Acetone

0.2mg of ninhydrin dissolved in 100ml of acetone

**For Pauly’ spray:**

1. Sulfanilic acid- 0.9g in 90ml of conc. HCl and 170ml of DDW
2. Sodium nitrite (5%)- 5g in 100ml of DDW
3. Sodium carbonate (10%)- 10g dissolved in 100ml of DDW

Reagent 1 and 2 were mixed (1:1 v/v) in cold and kept at 4°C for 5min. Reagent 3 was then added twice the volume and the chromatogram was sprayed. Tyrosine containing peptides stains pink whereas histidine containing peptides stains orange.
Solvent system used for chromatography of various peptide antigens

| 2. (Universal Solvent)                      |
| 4. Pyridine: acetic acid: DDW               | 1:1:1 (v/v)   |

5.12. Physiochemical Characterization of the peptides:

5.12.1 Identification of C-terminal amino acid

Reagents/Chemicals required:

1. Carboxypeptidase A (1mg/ml in Tris-HCl buffer)
2. Tris HCl buffer (25mmol/lts, pH 7.5)
3. Peptide stock (5mg/ml in Tris HCl buffer)
4. 10% Trichloroacetic acid (TCA) in DDW
5. 0.2% ninhydrin in acetone

Protocol:

1. Carboxypeptidase A was mixed with equal volume of peptide solution.
2. After thoroughly mixing, the samples were kept at 37°C in water bath.
3. 200μl of samples were removed at specific time intervals (0, 10, 20, 30 and 60min) and immediately mixed with 0.2ml of 10% TCA in another vial.
4. Samples were centrifuged at 5000rpm for 5min to settle down the precipitate.
5. Each sample was spotted on whatmann filter paper along with the respective amino acid standard and the unhydrolysed peptide in an appropriate solvent system.
6. The paper was dried and sprayed with 0.2% ninhydrin.
7. The developed colored spot on chromatogram was compared with the standard amino acid based on Rf values.

4.12.2 Identification of N-terminal amino acid:

Reagents required:

1. 1-fluoro-2,4 dinitrobenzene (FDNB) (5% v/v in ethanol)
2. Sodium carbonate (Na₂CO₃)
3. 0.2mM peptide stock (0.2μM)
**Protocol:**

1. 200μl of 0.2μM peptide antigens were mixed with equal amount of Sodium bicarbonate (Na₂CO₃) and dissolved in 2ml DDW.
2. 100X excess molar ration of FDNB solution (20μM) was added and kept for continuous shaking for 2 hrs in an end to end shaker at RT.
3. The pH of the mixture was adjusted to 8.0 with 1M Na₂CO₃.
4. The precipitate formed was washed number of times with dry ether to remove excess dinitrophenol (DNP) and FDNB.
5. The peptide was then hydrolyzed with 1ml of 6N HCl in a sealed ampoule and kept at 110°C for 18 hrs.
6. The excess of HCl is then removed under vacuum and hydrolyzed peptide samples spotted on Whatmann filter paper along with the DNP- amino acid standards.
7. The chromatogram was developed in Acetic acid: N-butanol: DDW: ethyl acetate (1:1:1:1 v/v) and the Rf values of N-terminal tagged hydrolyzed peptide compared with the DNP-standard amino acids.

4.13 **Reverse phase High performance Liquid chromatography (RP-HPLC):**

The extent of purity of all the seven peptides from E1, seventeen peptides from E2 and one peptide from E3 were assessed using a Reverse phase high performance liquid chromatography (Agilent Technologies, USA). For fulfilling this goal, peptides were subjected to RP-HPLC using a Zorbax 300 SB-C₈ column (250 x 9.4 nm inner diameter; 6.5μm particle size, 300Å pore size) with a linear gradient of acetonitrile and water in 0.1% TFA.

**Analytic conditions and materials required:**

<table>
<thead>
<tr>
<th>Column:</th>
<th>Zorbax 300 SB-C-8 column (250 x 9.4 nm inner diameter; 6.5μm particle size, 300Å pore size)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer ‘A’</td>
<td>Millipored double distilled water with 0.1% TFA</td>
</tr>
<tr>
<td>Buffer ‘B’</td>
<td>HPLC grade Acetonitrile containing 0.1% TFA</td>
</tr>
<tr>
<td>Methanol:</td>
<td>HPLC grade</td>
</tr>
<tr>
<td>Detection:</td>
<td>254nm/280nm</td>
</tr>
<tr>
<td>Flow rate:</td>
<td>1ml/min</td>
</tr>
<tr>
<td>Retention time:</td>
<td>5-45 min</td>
</tr>
</tbody>
</table>
Protocol:

1. Prior to sample injection, the column was washed thoroughly with HPLC grade methanol and equilibrated with 1:1 ratio of buffer A: B.
2. 2mg of peptides dissolved in 0.2ml of Buffer ‘A’ was first clarified using clarification unit.
3. Once the baseline achieved, the peptide sample (0.1mg/100µl) was injected into the column.
4. The chromatogram was developed using a linear gradient of Buffer ‘A’ and Buffer ‘B’ (0-100%) at 1ml/hr flow rate.
5. This step was continued for purification of different peptides and the peptide peak was detected 254nm/280nm.

5.14. Amino acid analysis by RP-HPLC:

Amino acid analysis was also done after pre-derivatization with Phenylisothiocyanate (PITC) using PICO-TAG column.

Materials required:

1. RP-HPLC system
2. PICO-TAG column
3. Phenylisothiocyanate (PITC)
4. Standard amino acids
5. Speedvac

Reagents required:

1. Sodium acetate trihydrate
2. Conc. HCl
3. Phosphoric acid
4. Glacial acetic acid
5. Disodium hydrogen phosphate
6. Acetonitrile
7. Millopored double distilled water

5.15 Hydrolysis of the peptide:

Protocol:

1. The peptides (1mg) were first hydrolyzed in the hydrolysis vials with 6N HCl at 110°C for 18hrs.
2. The released amino acids from the samples and the amino acid standard solution (10µl) were dried under vacuum using Speed vac.

5.15.1 Step of Neutralization:

Protocol:

The dried hydrolyzed samples and the amino acid standards were re-dried in neutralization buffer using re-drying solution (water: ethanol: TEA:: 2:2:1). The buffer was removed under vacuum.

5.15.2 Pre-column derivatization:

Protocol: The dried free amino acid mixture and the amino acid standards were then derivatized with Phenylisthiocyanate (PITC).

1. 200ul solution of ethanol: water: TEA: PITC at a ratio of 7: 1: 1: 1 was added to the dried samples and vortex and further incubated at room temperature for 20min.
2. After the reaction time over, PITC amino acid samples was again evaporated to dryness using Speedvac.

5.15.3 Analysis by RP-HPLC:

Protocol:

1. Derivatized samples and standards were reconstituted in 200µl of sample diluents
2. The PICO-TAG column was equilibrated with eluant ‘A’ (90%) and eluant ‘B’ (10%) till baseline was reached.
3. The standard samples were injected to get a representative chromatogram with elution timing of each amino acid.
4. This was followed by the injection of the test samples.
5. The amounts of individual amino acids were calculated from the peak area based on retention time of each derivative amino acid and by comparing with the standard amino acid profile.

5.16 Study Subjects

In the present study a total of 155 samples were selected from different regions of India. 123 samples were confirmed CHIKV positive (clinical diagnosis, RT-PCR or IgM kit method), 33 sera were confirmed dengue virus (DENV) positive (IgM kit method), and 39 healthy individual samples were used as controls. All the blood samples were collected from the department of Microbiology, AllMS, New Delhi, Vector Control Research Centre (VCRC), Pondicherry, Department of microbiology,
Nizam Institute of Medical Sciences, Hyderabad and virology department, NIMHANS, Bangalore, India from July 2010 to September 2011. This study was approved by the human ethics committee, AIIMS, New Delhi.

5.17 Selection of CHIKV positive Patients

The sera of CHIKV-infected patients were collected between 5-15 days of infection. The parallel detection of IgG antibody in the same sera was done for comparison purpose whether the patients are able to generate detectable IgG antibodies or not with the same peptides.

6.15 Reagents

E1 & E2 recombinant proteins, and purified virus lysate of the virus were used as a positive control to compare the degree of seroreactivity with individual peptides. These were provided by Virology Department, DRDE, Gwalior.

7.15 Standardization of Assay (peptide antigen)

Immunol 2 HB flat-bottomed microplates (high binding capacity) were used for all assays. E1, E2 and E3 peptides at different concentration (25ng, 50ng, 75ng and 100ng) in 100μl coating buffer (carbonate-bicarbonate, pH 9.6) were coated in each microwell and incubated overnight at 4°C. After washing with PBS-T, Plates were blocked with 5% milk solution and incubated at 37°C for 2 h. Afterwards, the plates were washed with PBS-T and then100μl of pooled sera (1:100 dilutions) from twenty five confirmed CHIK positive patients were added to each well and incubated at 37°C for 2 h. Subsequently, the plates were washed again and 100μl of donkey anti human IgM Fc5μ HRP (1:1000 dilution) (Jackson Immuno Research Europe Ltd) was then added in each microwell. After 1h incubation at 37°C and washing with PBS-T, the color was developed using OPD as a chromogen in substrate buffer/H2O2. The reaction was finally stopped by adding 8N H2SO4 and absorbance was read at 492 nm.

8.15 Detection of IgM and IgG antibody in patients sera

100ng/100μl of peptides of E2 protein were coated in each microwell (Immunol 2Hb, Flat bottom) and incubated overnight at 4°C. After washing with PBS-T, wells were blocked with 5% milk solution and incubated for 2 h at 37°C. The wells were then washed with PBS-T and 100μl of serum of confirmed Chikungunya positive patients, healthy individuals, confirmed dengue patients (1:100 dilution) were added in duplicate wells. After subsequent washings, 100μl of donkey anti human IgM and IgG HRP conjugate (1:1000 dilutions) was added in each well and incubated for 1h at
37°C. To examine the antigen-antibody complexes, color was developed by OPD (orthphenyldiamine) as chromogen in substrate buffer/H₂O₂. After 10 min of incubation with substrate, 8N H₂SO₄ was added to stop the reaction and absorbance was measured at 492nm. The patient’s sera was considered positive if the absorbance value was found to be > mean of negative sera + 2 SD.

8.16 Sensitivity and Specificity of Selected peptides

The sensitivity (true-positive rate) for the test was calculated as the [number of samples in the CHIKV-infected groups with an absorbance of greater than or equal to (Mean+2SD) of the absorbance for the healthy group divided by the total number positive patient samples for CHIKV-infected group] X 100. The specificity (true negative rate) for the test was calculated as [the number in the sample less than (Mean+2SD) the total absorbance for the healthy group divided by the total number of samples for the healthy group] X 100.

8.17 Statistical analysis:

The sensitivity and specificity of the peptide based IgM/IgG detection in CHIKV patients were calculated and results were expressed in absorbance together with the range. Comparisons of the proportion of positive results were made between the different tests and subgroups using the t-test non parametric analysis of variance. P value of less than 0.05 was considered significant and cut-off value for each peptide was calculated using receiver operating characteristic (ROC) curve (Prism version: 5).
Results
Group A: Malaria
6. Results

3.1 Selection of peptides for generating high titer antibody in animal models:

Three regions from PLDH were identified that showed amino acid residues differed between \( P. falciparum \) and \( P. vivax \) (Fig 6.1). All three selected regions showing four to six amino acid difference were thought to be sufficient to generate specific antibodies against each selected LDH peptide and also able to differentiate one malaria LDH from another malaria species (Mateu MG et al., 1990).

3.2 Computer algorithmic analysis of peptide:

Before synthesis, the peptides were analyzed by various computer algorithm software ie. Bcelpred and DNASTAR for prediction of protein characteristic such as hydrophilicity, hydrophobicity, antigenicity and secondary structure regions such as \( \alpha \)-helix, \( \beta \) -sheets, and \( \beta \)-turns for the selection of potentially exposed and immunodominant region sequences for antibody generation. After analysis of various parameters of the computer algorithm program we had selected two peptide sequence from HRP region ie HRP-2 peptide I (Fig: 6.2a), HRP-2 peptide II (Fig: 6.2b) and three from LDH region ie LDH I (Fig: 6.2c), LDH II (Fig: 6.2d), LDH III (Fig: 6.2e).

For possible secondary structure viz \( \alpha \)-helix, \( \beta \)-sheet, \( \beta \)-turn and coil regions, the computer aided algorithm prediction by Eisenberg, Garnier-Robson and Chou-Fasman parameters were used. According to Eisenberg analysis, \( \alpha \)-helix, regions were detected towards the N-terminal of LDH peptide I while LDH peptide II showed \( \alpha \)-helix, regions t both N terminal and C-terminal. However, \( \alpha \)-helix, regions were detected throughout the peptide I and peptide II of HRP-2 protein. The Garnier Robson analysis also detected the \( \alpha \)-helix, regions throughout the HRP-2 peptide I and peptide II and towards the N-terminal and C-terminal of LDH peptide II while LDH peptide I did not show any \( \alpha \)-helix, regions. However, LDH III showed \( \alpha \)-helix, regions towards the C-terminal. According to Garnier Robson analysis the \( \beta \)-sheet and \( \beta \)-turns is suggestive of putative B-cell epitope property. The \( \beta \)- sheet were present in C-terminal of LDH peptide I and peptide III, while no \( \beta \) regions were present in LDH peptide II, HRP-2 peptide I and HRP-2 peptide II. According to Chou-Fasman analysis two \( \beta \)-regions were present in LDH peptide III. The surface accessibility of all the peptides in the study was analyzed by the hydrophilicity index by Kyte-Doolittle that showed high surface accessibility of HRP-2 peptide I and HRP-2 peptide II while LDH peptide II and LDH peptide III has less surface accessibility as compared to LDH peptide I. According to Karplus Shultz plot LDH peptide I and LDH peptide III have central flexible regions.
Figure 6.1 Selection of High Titer antibody peptides from LDH protein

Figure Ligand 6.1: EMBOSS Stretcher alignment of Plasmodium Lactate Dehydrogenase amino acid sequences. The unique plasmodial epitope differentiating PfLDH and PvLDH appear below the solid line. Accession numbers are PfLDH: PlasmoDBid: PF3D7_1324900, PVLDH: PlasmoDBid: PVX_116630.

Figure Ligands 6.2a: DNASTAR for prediction of protein characteristic such as hydrophilicity, hydrophobicity, antigenicity and secondary structure regions such as α-helix, β-sheets, a and β-turns for the selection of potentially exposed region.
Figure 6.2b: DNASTAR for prediction of protein characteristic such as hydrophilicity, hydrophobicity, antigenicity and secondary structure regions such as α-helix, β-sheets, α and β-turns for the selection of potentially exposed region.

Figure 6.2c: DNASTAR for prediction of protein characteristic such as hydrophilicity, hydrophobicity, antigenicity and secondary structure regions such as α-helix, β-sheets, α and β-turns for the selection of potentially exposed region.
Figure 6.2 d-e: DNASTAR for prediction of protein characteristic such as hydrophilicity, hydrophobicity, antigenicity and secondary structure regions such as α-helix, β-sheets, α and β-turns for the selection of potentially exposed region.
Eisenberg plot the presence of amphipathic regions randomly in all the five peptides is also suggestive of B and T cell epitope property. The James-wolf antigenicity scale analysis correlates surface accessibility with the antigenic index. According to James-wolf antigenicity scale and Emini surface accessibility plot HRP-2 peptide I and LDH peptide I showed a very high antigenic index as compared to HRP-2 peptide II and LDH peptide II and LDH peptide III. After analyzing all the above peptide sequences of Pf LDH by BLAST, no sequence similarity with the other vertebrate LDH including humans were observed.

3.3 Surface localization of all selected peptides from HRP-2 and LDH protein:

Surface localization of all the three selected peptides was necessary to evaluate the accessibility of raising antipeptide antibody for native antigen (PfLDH protein). Surface localization of the peptides was by a PYMOL software program (Fig 6.3) illustrated that all the peptide was found to be located on the surface of the native protein.

Figure 6.3: Surface Localization of LDH peptides

Figure 6.3 a-b: Van der Waals Surface representation of pLDH protein at (A) 0° rotation and (B) 180° rotation along the y axis (PDB Id: 2A94). Surface localization of specific pf-peptide in magenta. The figure has been drawn by PyMOL (DeLano WL (2002) The PyMOL Molecular Graphics System on World Wide Web http://www.pymol.org)
3.4 Characteristic of Peptide sequences:
The characteristic properties such as total no. of amino acids, molecular weight of the peptide, net charges, isoelectric point and solubility in acetic acid of all the peptide selected for the study from HRP-2 and LDH region are summarized in Table 6.1

Table 6.1 Peptide characteristics and solubility in (%) acetic acid

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Peptide</th>
<th>Amino acid residues</th>
<th>Mol. Wt. (kDa)</th>
<th>Solubility in acetic acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HRP-2 peptide I</td>
<td>27 a</td>
<td>2.97 kDa</td>
<td>20%</td>
</tr>
<tr>
<td>2</td>
<td>HRP-2 peptide II</td>
<td>20 aa</td>
<td>2.20 kDa</td>
<td>20%</td>
</tr>
<tr>
<td>3</td>
<td>LDH peptide I</td>
<td>38 aa</td>
<td>4.18 kDa</td>
<td>25%</td>
</tr>
<tr>
<td>4</td>
<td>LDH peptide II</td>
<td>42 aa</td>
<td>4.62 kDa</td>
<td>25%</td>
</tr>
<tr>
<td>5</td>
<td>LDH peptide III</td>
<td>44 aa</td>
<td>4.84 kDa</td>
<td>25%</td>
</tr>
</tbody>
</table>

3.5 Peptide Synthesis and chain elongation:
All the selected HRP-2 and LDH sequences were synthesized by solid phase peptide synthesis (SPPS) using Glycine wang resin and suitably protected Fmoc amino acid. Peptides were assembled on appropriate amino acid substituted Wang resin. The amount of each amino acid, HBTU and resin were calculated according to the substitution factor. The Fmoc group on the N-terminus was deprotected with the solution of 20% piperidine in dimethylformamide (DMF). The [N-bis (Dimethylamino methylene) 1-H-Benzotriazolium Hexafluorophosphate 3-oxide] (HBTU) was used as an activator/ coupling reagent for growing chain. The HBTU, coupling reagent was used to elongate the peptide chain using suitably protected amino acid. The coupling efficiency was monitored after each step of elongation was monitored with Kaiser Test. The test was complete only if coupling was 100% complete and then the reaction was continued for the next step. The characteristic of the respective amminoacylated Wang resin for all the five peptides are given in (Table: 6.2).

Table 6.2 Aminoacylated resin characteristics

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Peptide</th>
<th>Fmoc-AA-wang resin</th>
<th>Initial amount of resin taken (gm)</th>
<th>Substitution factor (meq/g) of resin</th>
<th>Total meq/g of resin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HRP-2 peptide I</td>
<td>F-moc-Gly-Wang resin</td>
<td>0.2</td>
<td>0.5</td>
<td>0.12</td>
</tr>
<tr>
<td>2</td>
<td>HRP-2 peptide II</td>
<td>F-moc-Gly-Wang resin</td>
<td>0.2</td>
<td>0.5</td>
<td>0.12</td>
</tr>
<tr>
<td>3</td>
<td>LDH peptide I</td>
<td>F-moc-Gly-Wang resin</td>
<td>0.2</td>
<td>0.5</td>
<td>0.12</td>
</tr>
<tr>
<td>4</td>
<td>LDH peptide II</td>
<td>F-moc-Gly-Wang resin</td>
<td>0.2</td>
<td>0.5</td>
<td>0.12</td>
</tr>
<tr>
<td>5</td>
<td>LDH peptide III</td>
<td>F-moc-Gly-Wang resin</td>
<td>0.2</td>
<td>0.5</td>
<td>0.12</td>
</tr>
</tbody>
</table>
All the linear peptides are of 20-44 amino acids in length and synthesized using F-moc chemistry by assembling on Gly-wang resin. Some difficulty was observed due to its longer length of sequences. On the other hand, during LDH peptide sequence synthesis, we have observed some difficulty in coupling as well as in the deprotection of Fmoc group. This difficulty might be explained due to bigger length in the sequence and due to the presence of Glutamine (Q) and Glutamic acid (E) in the LDH III sequences. To overcome the problem of the coupling, we have increasing number of coupling reaction (every time with fresh solution). Then the peptide synthesis was carried out with the same protocol.

3.6 Drying of the peptidyl resin and Cleavage of the peptide from resin:

Following synthesis, all the peptidyl resins were extensively washed thrice with chilled absolute ethanol followed by DMF and DCM. After washing, resin was kept for drying in desiccator overnight with P₂O₅ and CaCl₂. Once the constant dry weight of the peptidyl resin was observed, the dry weight of the resin was noted and then the peptides were cleaved from resin using TFA. According to the dry weight of resin, the respective volume of scavengers (1, 2 Ethanedithiol Thioanisole and Anisole) and TFA were calculated and water were used for selective removal of labile side chain protection group of amino acids. Almost all the side chain protecting groups were cleaved from the peptide with TFA for 2 hours constant stirring at room temperature. The excess of all the deprotecting, cleavage reagents and scavengers were removed using rota-evaporation under vacuum to get a viscous solution. The peptides were precipitated out using dry and cold absolute ether. The precipitated peptides were washed three times with dry and cold ether to remove last traces of scavengers. After evaporating ether, crude peptides were dissolved in 20-35% of acetic acid. After lyophilization, the peptides were finally obtained as white fluffy powder.

3.7 Purification of crude peptides:

Both the HRP-2 and LDH crude peptides were purified after cleavage using Gel exclusion chromatography. On the basis of peptides molecular weight, Sephadex G-25 column was used as stationary phase. While on the other hand, on the basis of peptide solubility, 20-35% of acetic acid was used as mobile phase to elute all the peptides. The sample volume was calculated from the void volume. Total 50 mg of crude peptides was dissolved in 250µl of an appropriate percentage of acetic acid. The 1ml-1ml fractions were collected and the flow rate of 20ml/hour was maintained throughout the purification. The void volume was calculated and collected in a separate container before elution of the sample volume. The peak elution profile was monitored at 254nm and 280nm using UV spectrometer. The fractions of each peak were identified based on the spectrophotometric analysis and based on TLC Rf values in two different solvent systems. All the peptides were showing more than one peaks. The fractions which showed single spot with identical Rf values after spraying with
ninhydrin and spraying for characteristic amino acid were pooled during each chromatogram development. The sample fraction peaks were pooled separately and lyophilized.

**Figure 6.4** Gel profiles of selected peptides from HRP-2 antigen
Figure 6.4 Gel profiles of selected peptides from LDH antigen
After first purification, the peak I of all the peptides was re-chromatographed on the same Sephadex G-25 column under identical conditions to get purified peptide. While the rest of the other peaks of scavengers and of deletion peptides were discarded. After second peptides’ purification, a single peak was obtained for all the HRP and LDH peptides (Figures 6.4a-6.4e). The purified peptides’ peak was pooled and lyophilized. The dry purified peptides were stored at -20°C till use. (Table: 6.3).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amount of resin taken (gm)</th>
<th>Dry wt. of resin (gm)</th>
<th>Expected amt. of peptide (gm)</th>
<th>Crude peptide (% yield)</th>
<th>Peak no.</th>
<th>Purified peptide yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP-Z peptide I</td>
<td>0.2</td>
<td>0.45</td>
<td>0.27</td>
<td>0.20 (74.1%)</td>
<td>19-33</td>
<td>0.17 (63%)</td>
</tr>
<tr>
<td>HRP-Z peptide I</td>
<td>0.2</td>
<td>0.37</td>
<td>0.18</td>
<td>0.16 (88.8%)</td>
<td>19-35</td>
<td>0.15 (83.3%)</td>
</tr>
<tr>
<td>LDH peptide I</td>
<td>0.2</td>
<td>0.53</td>
<td>0.29</td>
<td>0.25 (86.0%)</td>
<td>9-17</td>
<td>0.23 (79.9%)</td>
</tr>
<tr>
<td>LDH peptide II</td>
<td>0.2</td>
<td>0.50</td>
<td>0.24</td>
<td>0.19 (79.0%)</td>
<td>7-22</td>
<td>0.17 (71%)</td>
</tr>
<tr>
<td>LDH peptide III</td>
<td>0.2</td>
<td>0.54</td>
<td>0.31</td>
<td>0.26 (83.8%)</td>
<td>6-19</td>
<td>0.22 (71%)</td>
</tr>
</tbody>
</table>

3.8 Characterization of the peptides:

3.8.1 N-terminal analysis:

The N-terminal analysis was done by using Fluoro-2, 4, dinitrobenzene, hydrolyzed with HCl and spotted onto Whatman paper. The Rf value of samples were compared with Rf value of standard DNP amino acids. When the FDNB labeled peptides and the appropriate standard amino acids were lyophilized with HCl then the resultant hydrolysate were spotted onto Whatman filter paper 1 to compare the Rf value of the derivatized N-terminal amino acid of peptide with the appropriate derivatized standard amino acid. The Rf values of the hydrolyzed Dinitrophenyl (DNP) amino acid was found to be in agreement with the Rf values of standard Dinitrophenyl (DNP) amino acid. The chromatogram was developed in the appropriate solvent system depending on the resolution of the peptides. The results of all the peptides showed that the observed and expected Rf values are comparable and describing that the synthesis of desired sequences. (Table 6.4).

5.6.2 C-terminal analysis:

The C-terminal analysis was done by using the method of Bradshaw et al. Peptides were cleaved with CarboxypeptidaseA. The C-terminal amino acid was verified by
running the sample on TLC plates and the Rf values were compared with that of the standard DNP amino acids. The peptide was mixed with Carboxypeptidase A and then the reaction was stopped by adding 10% TCA at different time intervals. The maximal time of 15 minutes was found to be the optimal incubation time for the release of C-terminal amino acid from each of the respective peptides. The respective C-terminal amino acids released were verified by running the samples on TLC plates and the Rf values were compared with that of the standard amino acids. The observed Rf values of the sample amino acid was comparable with the expected Rf values of standard amino acids. It shows that the peptides had the expected amino acids at the C-terminals. (Table 6.5).

**Table 6.4 N-terminal analysis HRP-2 and LDH Peptides**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Amino Acid</th>
<th>Standard Rf value*</th>
<th>Sample Rf value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP-2 Peptide-I</td>
<td>Alanine</td>
<td>0.54</td>
<td>0.52</td>
</tr>
<tr>
<td>HRP-2 Peptide-II</td>
<td>Alanine</td>
<td>0.54</td>
<td>0.55</td>
</tr>
<tr>
<td>LDH Peptide-I</td>
<td>Leucine</td>
<td>0.69</td>
<td>0.70</td>
</tr>
<tr>
<td>LDH Peptide-II</td>
<td>Threonine</td>
<td>0.50</td>
<td>0.47</td>
</tr>
<tr>
<td>LDH Peptide-III</td>
<td>Valine</td>
<td>0.60</td>
<td>0.59</td>
</tr>
</tbody>
</table>

*Where Rf value = Distance covered by analyte / Distance covered by solvent

Dried and sprayed with 2% Ninhydrin.

**Table 6.5 C-terminal analysis HRP-2 and LDH Peptides**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Amino Acid</th>
<th>Standard Rf value*</th>
<th>Sample Rf value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP-2 Peptide-I</td>
<td>Aspartate</td>
<td>0.62</td>
<td>0.64</td>
</tr>
<tr>
<td>HRP-2 Peptide-II</td>
<td>Alanine</td>
<td>0.54</td>
<td>0.56</td>
</tr>
<tr>
<td>LDH Peptide-I</td>
<td>Threonine</td>
<td>0.50</td>
<td>0.48</td>
</tr>
<tr>
<td>LDH Peptide-II</td>
<td>Alanine</td>
<td>0.54</td>
<td>0.57</td>
</tr>
<tr>
<td>LDH Peptide-III</td>
<td>Alanine</td>
<td>0.54</td>
<td>0.53</td>
</tr>
</tbody>
</table>

*Where Rf value = Distance covered by analyte / Distance covered by solvent

Dried and sprayed with 2% Ninhydrin.
3.9 High Performance Liquid Chromatography (HPLC) analysis

High Performance Liquid Chromatography (HPLC) analysis of all the peptides was done to check the purity of all the peptides. All the peptides were run with a linear gradient of 0-100% Acetonitrile with 0.1% TFA against milli Q water with 0.1% TFA. Peptides peaks were monitored at 254nm with the flow rate adjusted to 1.5ml/minute for 30 minutes. All the purified peptides showed a single peak with a purity greater than 90% thereby confirming its homogeneity. (Table 6.6) (Figures 6.5a-6.5e).

Table: 6.6 Percentage purity of all HRP-2 and pLDH peptides after HPLC analysis

<table>
<thead>
<tr>
<th>Conditions for HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
</tr>
<tr>
<td>Gradient system</td>
</tr>
<tr>
<td>Solvent 'A'</td>
</tr>
<tr>
<td>Solvent 'B'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Retention time</th>
<th>Percentage purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP-2 peptide 1</td>
<td>3.431</td>
<td>97.52</td>
</tr>
<tr>
<td>HRP-2 peptide I</td>
<td>13.006</td>
<td>95.81</td>
</tr>
<tr>
<td>LDH peptide 1</td>
<td>2.627</td>
<td>95.87</td>
</tr>
<tr>
<td>LDH peptide -II</td>
<td>22.108</td>
<td>97.12</td>
</tr>
<tr>
<td>LDH peptide III</td>
<td>12.044</td>
<td>95.41</td>
</tr>
</tbody>
</table>
Figure 6.5 a-e: HPLC Profile of HRP-2 and LDH peptides
3.10 Amino Acid Analysis:

Amino acid analysis was done with the help of HPLC systems. The peptides were first hydrolyzed with 6N HCl at 110°C for 18 hrs. All the amino acids were released from the sample. The hydrolyzed sample and standard amino acid solutions were dried and again re-dried in the neutralization buffer using re-drying solution (water: ethanol: TEA: 2:2:1). The dried free amino acid mixture and the amino acid standards were derivatized with Phenylisothiocyanate (PITC). First standard samples and then free amino acids were injected into the PICO-TAG column. The amount of individual amino acid was calculated from the peak area based on the retention time of each derivatized amino acid after comparing with standard amino acids. Some amino acid like tryptophan, histidine and methionine are destroyed during amino acid hydrolysis hence their observed values were not detected. (Table 6.7).

3.11 PLGA microsphere preparation:

Microspheres with entrapped HRP-2 and LDH peptides were prepared using poly (DL-lactide-co-glycolide; 50:50) by double solvent evaporation method (water-in-oil-in-water). The PLGA encapsulation of antigens had number of advantages

a) It prolongs the release of antigen formulations in-vivo.

b) Reducing the need for boosting at regular intervals.

c) It protects the antigens from harsh environment.

d) It converts the soluble antigens into particulate antigens, improving uptake by phagocytic cells.

6.10 Characterization of microsphere (microparticles).

6.10.1 The percentage efficiency of entrapped peptides:

The efficiency of entrapped peptides was estimated by double solvent evaporation method. In this method, microspheres were disrupted by the treatment of 0.1M PBS and 0.1N NaOH. The total amount of antigens in PBS and NaOH supernatant was determined by Bicinchoninic acid method using known peptides as a standard protein. The percentage entrapment efficiency was calculated from the standard curve (Fig 6.6c).
### Table 6.7  Amino acid analysis of HRP-2 and LDH peptides

<table>
<thead>
<tr>
<th></th>
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<th></th>
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>obs. Value</td>
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<td>obs. Value</td>
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<td>obs. Value</td>
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<td>Aspartic acid</td>
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<tr>
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<tr>
<td>Isoleucine</td>
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<td>2.0</td>
<td>1.76</td>
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<td>Leucine</td>
<td>1.0</td>
<td>0.94</td>
<td>1.0</td>
<td>0.96</td>
<td>1.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.0</td>
<td>2.74</td>
<td>3.0</td>
<td>2.98</td>
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<tr>
<td>Methionine</td>
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<td>2.84</td>
<td>3.08</td>
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<tr>
<td>Phenylalanine</td>
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<tr>
<td>Proline</td>
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<td>Serine</td>
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<tr>
<td>Threonine</td>
<td>2.0</td>
<td>1.92</td>
<td>2.0</td>
<td>1.95</td>
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</tr>
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<td>Tryptophan</td>
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<td></td>
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<tr>
<td>Tyrosine</td>
<td></td>
<td></td>
<td></td>
<td>2.0</td>
<td>2.08</td>
</tr>
<tr>
<td>Valine</td>
<td>2.0</td>
<td>1.33</td>
<td>2.0</td>
<td>2.01</td>
<td>2.0</td>
</tr>
</tbody>
</table>
6.10.1.1 The percentage entrapment of entrapped HRP-2 peptides:
The percentage entrapment for different HRP-2 peptides was in the range of 80 - 85% as determined by bicinchoninic acid (BCA) protein assay (Sigma, IL, USA).

6.10.1.2 The percentage entrapment of entrapped LDH peptides:
The percentage entrapment for different LDH peptides was in the range of 70 - 72% as determined by bicinchoninic acid (BCA) protein assay (Sigma, IL, USA).

6.10.2 Sizing of PLGA microsphere:
The size distribution of the microsphere was determined by laser diffraction (Malvern Instrument, UK). The size was between 1 - 5µm for all the HRP-2 and LDH peptides (Figure 6.6b).

6.10.3 Morphology of microspheres: PLG microsphere morphology was also studied by scanning electron microscopy (Phillips, CM 10). The microspheres were found to be spherical in shape as shown in the photograph of typical microsphere preparation. (Figure 6.6a).

6.11 Standardization of route, dose:
We have studied the standardization of route and dose with adjuvants for HRP-2 and LDH peptides. Mice were immunized with HPR-2 peptide I and LDH peptide I, LDH peptide II and LDH peptide III. Three rabbit were immunized with HRP-2 peptide II to avoid cross reactivity with HRP-2 peptide I. Mice and Rabbits were immunized with four different doses as follows-

6.11.1 10µg, 30µg, 50µg of HRP-2 peptide I and LDH peptide with CpG-ODN alone in mice (subcutaneously at the foot-pad region).

When the peak antibody titer was quantitated, the titer of 12,800 was observed in the 1st bleed (day 15) and reached maximum of 1, 02,400 in the 4th bleed (day 60) and further declined to 25,600 in 5th bleed (day 90) in the formulation of 30µg/mice and two booster of 15µg/mice at day 32 and 45 through subcutaneously route at the foot-pad region of immunization. We have observed that the dose of 30µg/mice through subcutaneously at the foot-pad region evoked highest humoral immune response (peak titer 1, 02,000) and this titer was maintained to 25,600 till day 90.
6.11.2 25μg, 50μg, 100μg, 150μg of HRP-2 peptide II with CpG-ODN in rabbit (subcutaneously at multiple sites).

When the peak antibody titer was quantitated, the titer of 25,600 was observed in the 1st bleed (day 15) and reached maximum of 1, 02,400 in the 4th bleed (day 60) and further persist to 1, 02,400 in 5th bleed (day 90) in the formulation of 100μg/Rabbit and two booster of 50μg/rabbit at 32 and 45 days through subcutaneous route at multiple site of immunization. We have observed that the dose of 100μg/Rabbit through intramuscular route evoked highest humoral immune response (peak titer 1, 02,000) and this titer was maintained to 25,600 till day 90 than the other formulations.

6.12 Humoral immune response: estimation of fixed dilution (1:100) specific IgG antibody response in individual HRP-2 and LDH peptide I, II, III

The IgG response in sera was estimated by single dilution at a standard dilution of 1:100 and by serial dilution to get an antibody peak titer. When outbred strains of mice were immunized intramuscularly with HRP-2 and LDH peptides in PLGA microspheres then serum anti HRP-2 and LDH peptide specific IgG response was detectable in the bleed 1st (day 15) in all the strains of mice which was enhanced by the second bleed (day 28). This titer was reached maximum on day 60 (bleed 4th), further declined in the bleed 5th (day 90) (Fig 6.7a-6.11a). Two boosters were given at 32 and 45 days with CpG ODN adjuvant.

6.13 Serum antipeptide peak antibody titer humoral response in microparticle formulation of *P. falciparum* LDH and HRP-2 antigens

The antibody responses to all the five peptides rose in mice and rabbits were measured as a proportion of peak antibody titres. Peptides entrapped in PLGA microspheres with the CpG ODN generated peptide specific high antibody levels in all the five bleeds and antibody levels were maintained till 90 days post-immunization with all the peptides of *PfHRP-2* and *PfLDH* antigens. IgG peak titer for PfHRP-2 peptide I ranged 51,200 - 1, 02,400 on days 28 and 42 (Fig 6.7b). Mice/ rabbits immunized with PfLDH peptide I also showed peak antibody levels 51,200 on day 28 and 42 and the titres fell to 25,600 on day 90 (Fig 6.9b) and PfHRP-2 peptide II showed peak antibody levels 51,200 on day 28 and 42 and the titres fell to 25,600 on day 90 (Fig 6.8b). Similarly for PfLDH peptide II antisera, IgG peak titres were in the range of 51,200 – 1, 02,400 on days 28 and 42 and gradually declined by day 90 to 25,600 (Fig 6.10b). On the other hand for PfLDH peptide III antisera, IgG peak titres were in the range 25,600 - 51,200 on days 28 and 42 which persisted up to day 90 (Fig 6.11b) (Table 6.8).
Table 6.8: End point titres (in thousands) of antibodies rose against the peptides of PfHRP2 and PfLDH antigens.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Day 15</th>
<th>Day 28</th>
<th>Day 42</th>
<th>Day 60</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfHRP2 Peptide I</td>
<td>25,600</td>
<td>51,200</td>
<td>1,02,400</td>
<td>1,02,400</td>
<td>51,200</td>
</tr>
<tr>
<td>PfHRP2 Peptide II</td>
<td>25,600</td>
<td>51,200</td>
<td>1,02,400</td>
<td>1,02,400</td>
<td>1,02,400</td>
</tr>
<tr>
<td>PfLDH Peptide I</td>
<td>25,600</td>
<td>51,200</td>
<td>51,200</td>
<td>25,600</td>
<td>25,600</td>
</tr>
<tr>
<td>PfLDH Peptide II</td>
<td>25,600</td>
<td>51,200</td>
<td>1,02,400</td>
<td>1,02,400</td>
<td>25,600</td>
</tr>
<tr>
<td>PfLDH Peptide III</td>
<td>12,800</td>
<td>25,600</td>
<td>51,200</td>
<td>51,200</td>
<td>25,600</td>
</tr>
</tbody>
</table>

Figure 6.7a-b Peptide specific titres for HRP-2 peptide- I in sera of mice immunized Via subcutaneous at foot-pad region.
Figure 6.8a-b Peptide specific tires for HRP-2 peptide-II in sera of rabbit immunized Via subcutaneous at various region.

Figure 6.9a-b Peptide specific tires for LDH peptide-I in sera of mice immunized via subcutaneous at foot pad region.
Figure 6.10 a-b Peptide specific titres for LDH peptide-II in sera of mice immunized Via subcutaneous at foot pad region.

Figure 6.11 a-b Peptide specific titres for LDH peptide-III in sera of mice immunized Via subcutaneous at foot pad region.
6.14. Immuno Cross reactivity of antibodies to confirm the specificity

The antipeptide sera generated in mice and rabbit against the synthesized peptides of HRP-2 and LDH antigen were reacted with different peptides synthesized from different stages of the life cycle of the malaria parasite viz. Circumsporozoite protein (CSP), \((\text{Octa})_2\) Ring erythrocyte surface antigen (RESA) and cross species antigen like core protein of HIV. It was observed that the antibodies generated against the synthetic peptides of HRP-2 and LDH antigen were highly specific for the antigen against which they have been synthesized. No cross reactivity of the antipeptide sera was seen with the peptides of the CSP and RESA. Also, no cross species reactivity was observed with the peptides of the core antigen of HIV, thus confirming the specificity of the antibodies generated using microspheres as the delivery system (Fig 6.12 a, 6.12 b, 6.12 c).

6.15 Purification of antibodies

Initially albumin was removed from sera by ammonium sulfate \((40-50\% \text{ sol}^n)\) precipitation or “salting out” procedure (Grodzki, A.C. et al., 2010) and subsequently, the antibodies were purified by affinity chromatography on a protein A column. The pooled fractions were dialyzed against 0.01 M PBS (pH 7.4). The dialysate was concentrated and the IgG amount was determined using the BCA method (Smith PK et al., 1985). The concentration of the purified IgG was found to be 30mg/ml as quantified by BCA method.

6.16 Biotinylation of the purified antibodies

Biotinylation of the purified antibodies was done using N-hydroxysuccinimide ester of biotin (Sigma Immuno Probe Biotinylation reagents) according to the manufacture’s protocol. Further, biotinylated antibodies were separated from the free antibodies and unreacted reagent by a fast gel chromatography (G-25 column) and the fractions obtaining within peak 1 containing the biotinylated IgG were pooled and lyophilized (Fig 6.13a-e). The extent of biotinylation and the ratio of biotin to antibody were determined by the avidin-HABA (4’-Hydroyazobenzene-2-
Figure 6.12a Cross reactivity experiment to show the specificity of the antibodies raised against the HRP and LDH peptides

![Graph](image)

Figure legends 6.12a: The antipeptide sera generated in mice and rabbit against the synthesized peptides of HRP-2 and LDH antigen were not reacted with different peptides synthesized from different stages of the life cycle of the malaria parasite viz. Circumsporozoite protein (CSP), (Octa)$_2$, Ring erythrocyte surface antigen (RESA) and cross species antigen like core protein of HIV.

Figure 6.12b and 6.12c Cross reactivity experiment to show the specificity of the antibodies raised against the HRP and LDH peptides.

![Graph](image)

Figure legends 6.12 b-c: The antipeptide sera generated in mice and rabbit against the synthesized peptide of HRP-2 and LDH antigen were specifically reacted with their peptides synthesized from HRP-2 and LDH antigen.
Figure 6.13 a-e Gel profile of biotinylated antibody
6.17 Dissociation constant (KD) of PfHRP-2 peptides antisera and PfLDH peptides antisera

The dissociation constant of antibodies with different peptides were studied in the sera of day 42. The KD value of anti-PfHRP-2 peptide I antisera was lower than anti-PfHRP-2 peptide II antisera and the KD value of anti-PfLDH peptide II antisera was also lower as compared to the anti-PfLDH peptide I and III antisera (Table 6.9). Thus all the peptides generated high titer and high affinity antibodies.

Table 6.9 Dissociation constant of Peptide (KD) Antisera

<table>
<thead>
<tr>
<th>S. No</th>
<th>Peptides</th>
<th>KD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PfHRP2 Peptide I</td>
<td>0.73</td>
</tr>
<tr>
<td>2</td>
<td>PfHRP2 Peptide II</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>PfLDH Peptide I</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>PfLDH Peptide II</td>
<td>1.4</td>
</tr>
<tr>
<td>5</td>
<td>PfLDH Peptide III</td>
<td>3.0</td>
</tr>
</tbody>
</table>

6.18 Standardization for HRP-2 antigen detection in the *P. falciparum* culture supernatant and parasitized RBC lysates by sandwich ELISA

Various immunological parameters for HRP-2 antigen detection by sandwich ELISA were standardization using chequer-board titration of the coating (capture) antibody concentration and detecting antibody concentration. The various permutation and combination used for standardization were:

(i) Coating with 1μg /2.5μg of purified anti HRP-2 peptide I IgG antibody and detection with 1μg /2.5μg of biotinylated anti-HRP peptide –II IgG.

(ii) Coating with 1μg /2.5μg of purified anti HRP-2 peptide II IgG antibody and detection with 1μg /2.5μg of biotinylated anti-HRP peptide –I IgG.

It was observed that coating concentration of 2.5 μg/ well of anti HRP peptide–I IgG and detection with 2.5 μg/ well of the biotinylated anti HRP peptide–II IgG was found to be optimum for the detection of HRP-2 antigen in culture supernatant/ parasitized RBC lysate by sandwich ELISA (Table 6.10a and b).

This concentration of the capture antibody and the detection antibody were used for the detection of the HRP-2 antigen in the blood sample of clinically proven *P. falciparum* patients by antigen capture assay.
Table 6.9 a

Standardization for detection of HRP-2 antigen in culture supernatant of *in vitro* culture of *P. falciparum*

<table>
<thead>
<tr>
<th>Coating (C) = HRP peptide I IgG</th>
<th>Detection (D) = Biotinylated HRP peptide II IgG</th>
</tr>
</thead>
</table>
| C → 1μg  
D → 1μg  
A 492nm = 0.53 | C → 1μg  
D → 1μg  
A 492nm = 0.62 |
| C → 2.5μg  
D → 1μg  
A 492nm = 0.97 | C → 2.5μg  
D → 1μg  
A 492nm = 0.83 |
| C → 2.5μg  
D → 2.5μg  
A 492nm = 1.93 | C → 2.5μg  
D → 2.5μg  
A 492nm = 1.13 |

Table 6.9 b

Standardization for detection of HRP-2 antigen in infected RBC lysates of *in vitro* culture of *P. falciparum*

<table>
<thead>
<tr>
<th>Coating (C) = HRP peptide I IgG</th>
<th>Detection (D) = Biotinylated HRP peptide II IgG</th>
</tr>
</thead>
</table>
| C → 1μg  
D → 1μg  
A 492nm = 0.76 | C → 2.5μg  
D → 2.5μg  
A 492nm = 0.93 |
| C → 2.5μg  
D → 1μg  
A 492nm = 1.12 | C → 2.5μg  
D → 2.5μg  
A 492nm = 2.01 |
| C → 1μg  
D → 1μg  
A 492nm = 0.84 | C → 2.5μg  
D → 2.5μg  
A 492nm = 1.23 |

Controls: Pre Immune Sera (mice)-A 492nm = 0.13, Pre Immune Sera (Rabbit)-A 492nm = 0.14
Normal RBC culture supernatant A 492nm = 0.13, Normal RBC Lysate A 492nm = 0.13
Standardization for LDH antigen detection in the *P.falciparum* culture supernatant or parasitized RBC lysates by sandwich ELISA

The various immunological parameters for LDH antigen detection by sandwich ELISA were standardization using chequer-board titration of the coating (capture) antibody concentration and detecting antibody concentration. The various permutation and combination used for standardization were:

(i) Coating with 1μg/2.5μg of purified anti-LDH peptide-I IgG antibody and detection individually with 1μg/2.5μg of biotinylated anti-LDH peptide-II IgG, anti-LDH peptide–III IgG and a cocktail of anti LDH peptide-II IgG and anti-LDH peptide–III IgG.

(ii) Coating with 1μg/2.5μg of purified anti LDH peptide-II IgG antibody and detection individually with 1μg/2.5μg of biotinylated anti-LDH peptide-I IgG, anti-LDH peptide–III IgG and a cocktail of anti LDH peptide-I IgG and anti-LDH peptide–III IgG.

(iii) Coating with 1μg/2.5μg of purified anti LDH peptide-III IgG antibody and detection individually with 1μg/2.5μg of biotinylated anti-LDH peptide-I IgG, anti-LDH peptide–II IgG and a cocktail of anti LDH peptide-I IgG and anti-LDH peptide–II IgG.

(iv) Coating with 1μg/2.5μg of (a) cocktail of anti LDH peptide-I and anti LDH peptide-II IgG antibody and detection with 1μg/2.5μg of biotinylated anti-LDH peptide-III IgG, (b) cocktail of anti LDH peptide-I and anti LDH peptide-III IgG antibody and detection with 1μg/2.5μg of biotinylated anti-LDH peptide-II, (c) cocktail of anti LDH peptide-II and anti LDH peptide-III IgG antibody and detection with 1μg/2.5μg of biotinylated anti-LDH peptide-I IgG.

It was observed that coating with 2.5μg/ well of anti-LDH peptide-III IgG and detection with 2.5μg cocktail of anti LDH peptide-I IgG and anti-LDH peptide–II IgG was found to be optimum for the detection of LDH antigen in the *P.falciparum* infected RBC (IRBC) lysate by sandwich ELISA (*Table 6.11*).

This concentration of the capture antibody and the detection antibody were used for the detection of the LDH antigen in the blood sample of clinically proven *P.falciparum* patients by antigen capture assay.
Table 6.10  Standardization for detection of LDH antigen in culture supernatant of *in vitro* culture of *P. falciparum*

<table>
<thead>
<tr>
<th>Coating → L-I 1µg</th>
<th>Coating → L-I 2.5 µg</th>
<th>Coating → L-II 1µg</th>
<th>Coating → L-II 2.5µg</th>
<th>Coating → L-III 1µg</th>
<th>Coating → L-III 2.5µg</th>
<th>Coating → L-I, L-II, L-III 1µg</th>
<th>Coating → L-I, L-II, L-III 2.5µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>D → L-II 1µg A492nm=0.62</td>
<td>D → L-II 1µg A492nm=0.116</td>
<td>D → L-I 1µg A492nm=1.2</td>
<td>D → L-I 1µg A492nm=0.92</td>
<td>D → L-I 1µg A492nm=0.55</td>
<td>D → L-I 1µg A492nm=0.92</td>
<td>D → L-I 1µg A492nm=1.43</td>
<td>D → L-I 1µg A492nm=1.26</td>
</tr>
<tr>
<td>D → L-II 2.5µg A492nm=0.65</td>
<td>D → L-II 2.5µg A492nm=0.69</td>
<td>D → L-I 2.5µg A492nm=0.76</td>
<td>D → L-I 2.5µg A492nm=0.82</td>
<td>D → L-I 2.5µg A492nm=0.42</td>
<td>D → L-I 2.5µg A492nm=0.42</td>
<td>D → L-I 2.5µg A492nm=0.61</td>
<td>D → L-I 2.5µg A492nm=0.56</td>
</tr>
<tr>
<td>D → L-III 1µg A492nm=0.62</td>
<td>D → L-III 1µg A492nm=0.92</td>
<td>D → L-III 1µg A492nm=0.52</td>
<td>D → L-III 1µg A492nm=0.69</td>
<td>D → L-III 1µg A492nm=0.54</td>
<td>D → L-III 2.5µg A492nm=0.72</td>
<td>D → L-II, L-III 2.5µg</td>
<td></td>
</tr>
<tr>
<td>D → L-III 2.5µg A492nm=0.37</td>
<td>D → L-III 2.5µg A492nm=0.82</td>
<td>D → L-III 2.5µg A492nm=0.35</td>
<td>D → L-III 2.5µg A492nm=0.72</td>
<td>D → L-III 2.5µg A492nm=0.39</td>
<td>D → L-III 2.5µg A492nm=0.82</td>
<td>D → L-III 1µg A492nm=0.97</td>
<td>D → L-III 1µg A492nm=0.85</td>
</tr>
<tr>
<td>D → L-II, L-III 1µg A492nm=0.75</td>
<td>D → L-II, L-III 1µg A492nm=0.65</td>
<td>D → L-II, L-III 1µg A492nm=0.54</td>
<td>D → L-II, L-III 1µg A492nm=0.66</td>
<td>D → L-II, L-III 1µg A492nm=0.75</td>
<td>D → L-II, L-III 1µg A492nm=0.71</td>
<td>D → L-II, L-III 1µg A492nm=0.62</td>
<td>D → L-II, L-III 2.5µg A492nm=1.6</td>
</tr>
<tr>
<td>D → L-II, L-III 2.5µg A492nm=0.78</td>
<td>D → L-II, L-III 2.5µg A492nm=0.94</td>
<td>D → L-II, L-III 2.5µg A492nm=0.68</td>
<td>D → L-II, L-III 2.5µg A492nm=0.62</td>
<td>D → L-II, L-III 2.5µg A492nm=0.83</td>
<td>D → L-II, L-III 2.5µg A492nm=2.24</td>
<td>Control NRBC Lysate A492nm=0.16</td>
<td>Control PISC A492nm=0.62</td>
</tr>
</tbody>
</table>
6.18 Quantification of PfHRP-2 antigen in *P.falciparum* culture supernatant or parasitized RBC lysates using PfHRP-2-based ELISA

The PfHRP2 assay allowed the detection of PfHRP-2 antigen in the culture supernatant in all the four isolates of *P. falciparum* at different dilutions (neat, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64) of culture supernatant (Fig 6.14) and in the parasitized RBC lysate at parasitemia levels ranging 2,50,000-100 parasites/µl (Fig. 6.15a). The lower limit of PfHRP-2 antigen detection (50 parasites /µl) in parasitized RBC lysate is better than the detection limit by light microscopy which gave 100% sensitivity when the number of parasites/ µl of blood are > 50. The association between parasitemia and PfHRP-2 antigenaemia was found positively correlated in the culture supernatant (r =0.863, p<0.001) and in the infected RBC (IRBC) lysates (r = 0.839, p<0.001). The assay was repeated thrice for reproducibility.

**Figure 6.14 Quantification of PfHRP-2 antigens in culture supernatant of four different isolates of *P.falciparum*.**
Figure 6.15a
Quantification of PHRP2 antigen in RBC Lysate of four different isolates *P. falciparum*

Figure 6.15b
Quantification of pHLDH antigen in RBC Lysate of four different isolates *P. falciparum*
6.19 Quantification of LDH antigen in *P. falciparum* Culture supernatant or IRBC lysates using PfLDH-based ELISA

The assay allowed the detection of PfLDH antigen in the parasitized RBC lysate at parasitemia levels ranging 2, 50,000-100 parasites/ μl (Fig. 6.15b). The lower limit of PfLDH antigen detection (50 parasites/ μl) in parasitized RBC lysate is better than the detection limit by light microscopy which gave 100% sensitivity when the number of parasites/ μl of blood are > 50. The association between parasitemia and PfLDH antigenaemia was found positively correlated with the infected RBC lysates (r =0.831, p<0.001).

6.20 Detection of *Pf* HRP-2 antigen in blood of malaria patients by *Pf*HRP-2 assay

Two hundred malaria positive sera confirmed by microscopy and RDT were studied to evaluate the newly developed antigen capture assay for *Pf*HRP-2 and *Pf*LDH. The results are presented in Table 6.13. In this study, at 95% CI the specificity of *Pf*HRP-2 assays for diagnosis of *P. falciparum* parasites were 100% (91.1%-100%) and sensitivity was in the range 96% (77.7%-99.8%) to 100% (96.4- 100%) when the parasitemia was 0.0015% to 0.015% respectively. The positive predictive value % (PPV %) were found to be 74.7% to 100%, the negative predictive value % (NPV %) were found to be 91.1 to 100% and area under curve (AUC) was found to be 1.00 when parasite /μl >10,000-150. However PPV% was calculated to be 82.8%-100%, NPV% was 98.04% (88.2%- 99.2%) when parasite/μl >75-150 and AUC was found to be 0.98. This assay was able to detect the *Pf*HRP-2 antigen in the blood of all the subjects with parasitemia level ranging 75 to 250, 000 parasites/ l blood which corresponds to 0.0015% to 5% parasitemia (Fig. 6.16a). The number of parasites/ l blood were found to be positively correlated with *Pf*HRP2 antigenemia (r = 0.862, p<0.001) (Fig. 6.16b). Similarly the association between mean parasitemia levels in all the nine groups and the corresponding mean *Pf*HRP-2 antigenemia was also positively correlated (r = 0.979, p<0.001). At the parasitemia level increased, the *Pf*HRP-2 concentration was found to increase (Fig.6.16b). The indigenously developed antibodies were able to detect *Pf*HRP-2 in these blood samples at parasitemia of approximately 75 parasites / l (Table 6.12). 50 cases of each *P. vivax* positive and healthy smear negative cases served as control had undetectable levels of *Pf*HRP antigen. Cutoff for positivity was calculated as ≥Mean + 4SD of negative control. A sample is given an absorbance (Cutoff) 0.20 was considered positive for *Pf*HRP-2 antigen.
6.21 Detection of PfLDH antigen in blood of malaria patients by PfLDH assay

The same blood samples from two hundred patients were also examined for Pf/LDH and compared with microscopy and RDT. All the blood samples used in the study was *P. falciparum* positive by microscopic examination of thick and thin film. The results are presented in Table 6.12. In this study, the specificity of Pf/LDH assays for diagnosis of *P. falciparum* parasites were 100% (91.1% -100%) and sensitivity was 100% (83.4- 100%) at 95% CI. The positive predictive value % (PPV %) were found to be 83.4 % -100%, the negative predictive value % (NPV %) were found to be 91.1 to 100% and area under curve (AUC) was found to be 1.00 when parasite/μl >75 which correspond to >0.0015% parasitemia. The developed in-house assay, detected Pf/LDH antigen in the blood of all the subjects with parasitemia level ranging from 75 to 250,000 parasites/ 1 blood, which corresponds to 0.0015% to 5% parasitemia. The number of parasites/μl blood were found to be positively correlated with PfLDH antigenaemia (r = 0.878, p < 0.001) (Fig. 6.16b). The associations between mean parasitemia levels in all the nine groups and the corresponding mean Pf/LDH antigenaemia was also found positively correlated (r = 0.972, p<0.001). At the parasitemia level increased, the Pf/LDH concentration in the blood was also found to be increased in these patients (Fig. 6.16b). Thus, the indigenously developed antibodies detected PfLDH in these patient samples at parasitemia of approximately 75 parasites/ 1 of blood (Table 6.12). A sample is given an absorbance (Cutoff) 0.13 was considered positive for PfLDH antigen detection. The developed reagents were also tested with 50 samples of *P. vivax* positive sera. It did not show any reactivity with the *P. vivax* samples. The assay showed undetectable levels of Pf/LDH antigen in 50 healthy and smear negative cases as a control.
Figure: 6.16 a

Association between Mean Parasitemia and parasites count of all the eight groups of *P. falciparum* positive patients.

Figure: 6.16 b

Relationship between Parasite count and mean PfHRP2 (O) or mean PfLDH antigen concentration (Δ) in *P. falciparum* positive patients.
Table 6.12  Performance of *PfHRP2*- and *PfLDH*- based ELISA on *P. falciparum* samples from Baghpat district of Uttar Pradesh

<table>
<thead>
<tr>
<th>Blood Film % Parasitemia</th>
<th>Parasites/µL</th>
<th>Total</th>
<th>PfHRP2 assay</th>
<th>Sensitivity</th>
<th>PfLDH assay</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 0.2</td>
<td>&gt; 10,000</td>
<td>128</td>
<td>128</td>
<td>100%</td>
<td>128</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>0.1-0.015</td>
<td>5000-750</td>
<td>17</td>
<td>17</td>
<td>100%</td>
<td>17</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>0.015-0.008</td>
<td>750-390</td>
<td>15</td>
<td>15</td>
<td>100%</td>
<td>15</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>&gt;0.003</td>
<td>390-150</td>
<td>15</td>
<td>15</td>
<td>100%</td>
<td>15</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>&gt;0.0015</td>
<td>150-75</td>
<td>25</td>
<td>24</td>
<td>96%</td>
<td>25</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><em>P. Vivax</em> &gt;0.015</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
Results
Group B: Chikungunya
6.1 Computer algorithmic analysis of peptide:

The protein sequence for CHIKV S7 strain were obtained from the Gene Bank accession number AF369024 (GenBank Accession AF369024) (Fig 6.1). Before synthesis, the peptides were analyzed by various computer algorithm software ie. Bcelpred and DNASTAR for prediction of protein characteristic such as hydrophilicity, hydrophobicity, antigenicity and secondary structure regions such as α-helix, β-sheets, α and β-turns to the selection of potentially exposed and immunodominant region sequences for antibody generation. All the peptides of E1, E2 and E3 envelope proteins selected using Bcelpred (Protein id “AA033341.1”, db_xref “GI: 28193964”).

After analysis of various parameters of the computer algorithm program we had selected seven peptides from E1, seventeen peptides from E2 (Table 6.1) and one peptide sequence from E3 region.

Before synthesis, these peptides were analyzed by Pepscan program to measure the hydrophobicity, secondary structure (s), antigenicity etc. The protean program of the DNASTAR software program was used for the analysis of the peptide sequences of Chikungunya E1, E2, and E3 envelope protein. Proteins of CHIKV for possible secondary structures viz. α-helix, β-sheet, β-turn and coil regions, the computer aided algorithm prediction by Eisenberg; Garnier-Robson and Chou-Fasman parameters were used (Fig 6.2a-j).

According to Eisenberg analysis, α-helix, regions were detected in the interior of C-terminal portion of E2P3, E2P5, E2P13 and E2P17 peptides and towards the N-terminal of peptide E2P5, E2P10, E2P11, E2P13, E2P16 and E2P17, while in peptide E2P3, E2P5, E2P7, E2P9, E2P10, E211, E2P13, E2P15 and E2P16 were detected throughout the peptide of E2 protein. The Garnier Robson analysis also detected the α-helix, regions throughout the E2P9 and E2P15 peptide. E2P11 peptide showed α-helix, regions towards the N-terminal, while peptide in E2P5, E and E2P11 showed α-helix, regions towards the C-terminal.

According to Garnier Robson analysis the β-sheet and β-turns is suggestive of putative B-cell epitope property. The β-sheet was present in the N-terminal of E2P7 peptide and towards C-terminal E2P7, E2P9 and E2P15 peptide, while β-regions were present in E2P9 peptide. According to Chou-Fasman analysis two β-regions were present in E2P15 and E2P16 peptide. The surface accessibility of all the peptides in the study was analyzed by the hydrophilicity index by Kyte- Doolittle, which showed high surface accessibility of at terminal in E2P13 and E2P15 peptides while E2P10 peptide showed at hydrophilicity plot at N-terminal and between C and N termina. Peptides E2P3, E2P5, E2P7, E2P9, E2P11, E2P15, E2P16 and E2P17 showed high
Location of primers in the CHIKV genome. Positions are numbered with respect to S27 sequence (GenBank Accession AF369024).

6.2 Epitope localized on the surface of CHIKV protein

The surface localization of all selected peptides of E2 protein was necessary so that antibodies raised against the Chikungunya antigen would be reacting to individual peptide. The Surface representation of the selected ten E2 peptides showing significant high absorbance with CHIKV sera was analyzed for structural studies. Surface representation of E2 protein was retrieved from PDB (Id: 2XFC, Chain B) and visualization of all the selected peptides were done using software PYMOL [Delano WL (2002) The PYMOL Molecular Graphics System on World Wide Web http://www.pymol.org]. The orientation of the E2 domains relative to the viral membrane are based on previously described data (Voss et al., 2010). All the ten peptides that showed sero reactivity was found to be exposed on the surface of the E2 protein (Fig: 6.3a-b).

Figure 6.3 a and b: Surface representation of E2 peptides

Fig a

Fig b

Figure Ligand 6.3: Surface representation of E2 protein at (A) 0° rotation and (B) 180° rotation along the y axis (PBD Id: 2XFC, Chain B). The peptide localization of the E2 protein has been indicated in green, The figure has been drawn by PYMOL (DeLano WL (2000). The PYMOL Molecular Graphic System on World Wide Web http://www.pymol.org).
6.3 Peptide Synthesis and chain elongation:

All the selected E1, E2, E3 peptide sequences were synthesized by solid phase peptide synthesis (SPPS) using Glycine wang resin and suitably protected Fmoc amino acid. Peptides were assembled on appropriate amino acid substituted Wang resin. The amount of each amino acid, HBTU and resin were calculated according to the substitution factor. The Fmoc group on the N-terminus was deprotected with the solution of 20% piperidine in dimethylformamide (DMF). The [N-bis (Dimethylamino methylene) 1-H-Benzotriazolium Hexafluorophosphate 3-oxide] (HBTU) was used as an activator/ coupling reagent for the growing chain. The HBTU, coupling reagent was used to elongate the peptide chain using suitably protected amino acid. The coupling efficiency was monitored after each step of elongation was monitored with Kaiser test. The test was completely only if coupling was 100% complete and then the reaction was continued to the next step. The characterizations of the respective amminoacylated Wang resin for all the E2 peptides are given in Table: 6.2. All the linear peptides are of 10-20 amino acids in length and synthesized using F-moc chemistry by assembling on Gly-wang resin. Some difficulty was observed due to its longer length of sequences and due to the presence of Glutamine (Q) and Glutamic acid (E) in the sequences. We have observed some difficulty in coupling as well as in the deprotection of Fmoc group. This difficulty might be explained due to bigger length and the presence of cysteine residues in some sequence. To overcome the problem of the coupling, we have an increasing number of the coupling reaction (every time with fresh solution). Then the peptide synthesis was carried out with the same protocol.
Table 6.2: Characteristic features of all E2 envelope peptides

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Amino acid residues</th>
<th>Molecular weight (in KD)</th>
<th>Solubility in acetic acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2P1</td>
<td>15 aa</td>
<td>1.60</td>
<td>20%</td>
</tr>
<tr>
<td>E2P2</td>
<td>13 aa</td>
<td>1.43</td>
<td>20%</td>
</tr>
<tr>
<td>E2P3</td>
<td>14 aa</td>
<td>1.54</td>
<td>20%</td>
</tr>
<tr>
<td>E2P4</td>
<td>18 aa</td>
<td>1.98</td>
<td>20%</td>
</tr>
<tr>
<td>E2P5</td>
<td>16 aa</td>
<td>1.76</td>
<td>25%</td>
</tr>
<tr>
<td>E2P6</td>
<td>16 aa</td>
<td>1.76</td>
<td>25%</td>
</tr>
<tr>
<td>E2P7</td>
<td>19 aa</td>
<td>2.09</td>
<td>25%</td>
</tr>
<tr>
<td>E2P8</td>
<td>20 aa</td>
<td>2.20</td>
<td>25%</td>
</tr>
<tr>
<td>E2P9</td>
<td>19 aa</td>
<td>2.09</td>
<td>20%</td>
</tr>
<tr>
<td>E2P10</td>
<td>18 aa</td>
<td>1.98</td>
<td>20%</td>
</tr>
<tr>
<td>E2P11</td>
<td>20 aa</td>
<td>2.20</td>
<td>20%</td>
</tr>
<tr>
<td>E2P12</td>
<td>16 aa</td>
<td>1.76</td>
<td>25%</td>
</tr>
<tr>
<td>E2P13</td>
<td>13 aa</td>
<td>1.43</td>
<td>20%</td>
</tr>
<tr>
<td>E2P14</td>
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<td>25%</td>
</tr>
<tr>
<td>E2P15</td>
<td>17 aa</td>
<td>1.87</td>
<td>30%</td>
</tr>
<tr>
<td>E2P16</td>
<td>16 aa</td>
<td>1.76</td>
<td>30%</td>
</tr>
<tr>
<td>E2P17</td>
<td>11 aa</td>
<td>1.21</td>
<td>20%</td>
</tr>
</tbody>
</table>
6.4 Drying of the peptidyl resin and Cleavage of the peptide from resin:

Following synthesis, all the peptidyl resins were extensively washed thrice with chilled absolute ethanol followed by DMF and DCM. After washing, resin was kept for drying in desiccator overnight with P₂O₅ and CaCl₂. Once the constant dry weight of the peptidyl resin was observed, the dry weight of the resin was noted and then the peptides were cleaved from resin using TFA. According to the dry weight of resin, the respective volume of scavengers (1, 2 Ethanedithiol Thioanisole and Anisole) and TFA were calculated and water was used for selective removal of labile side chain protection group of amino acids. Almost all the side chain protecting groups were cleaved from the peptide with TFA for 2 hours constant stirring at room temperature. The excess of all the deprotecting, cleavage reagents and scavengers were removed using rota-evaporation under vacuum to get a viscous solution. The peptides were precipitated out using dry and cold absolute ether. The precipitated peptides were washed three times with dry and cold ether to remove last traces of scavengers. After evaporating ether, crude peptides were dissolved in 20-25% of acetic acid. After lyophilization, the peptides were finally obtained as white fluffy powder.

6.5 Purification of crude peptides:

All synthesized E1, E2, and E3 crude peptides were purified after cleavage using Gel exclusion chromatography. On the basis of peptides’ molecular weight, the Sephadex G-25 column was used as the stationary phase. While on the other hand, on the basis of peptide solubility, 20-30% of acetic acid was used as mobile phase to elute all the peptides. The sample volume was calculated from the void volume. Total 50 mg of crude peptides was dissolved in 250μl of an appropriate percentage of acetic acid. The 1ml-1ml fractions were collected and the flow rate of 20ml/hour was maintained throughout the purification. The void volume was calculated and collected in a separate container before elution of the sample volume. The peak elution profile was monitored at 254nm and 280nm using UV spectrometer. The fractions of each peak were identified based on the spectrophotometric analysis and based on TLC Rf values in two different solvent systems. All the peptides were showing more than one peaks. The fractions which showed single spot with identical Rf values after spraying with ninhydrin and spraying for characteristic amino acid were pooled during each chromatogram development. The sample fraction peaks were pooled separately and lyophilized. After first purification, the peak I of all the peptides was re-chromatographed on the same Sephadex G-25 column under identical conditions to get purified peptide. While rest of the other peaks of scavengers and of deletion peptides were discarded. After second peptides’ purification, a single peak was
obtained for all the E1, E2, and E3 (Figures 6.4a-6.6q, 6.5a-g and 6.6a). The purified 
peptides’ peak was pooled and lyophilized. The dry purified peptides were stored at 
-20°C till use. (Table: 6.3a and 6.3b).

Table 6.3a Obtained amount of crude peptides of E1 and E3 protein after cleavage

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Amount of Substitution</th>
<th>Total Substitution factor</th>
<th>Expected Amount of Peptide</th>
<th>Obtained Amount of Peptide</th>
<th>% Yield of Crude Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>ElP1</td>
<td>300 mg 0.6 meq/gm</td>
<td>0.18 meq</td>
<td>336.6 mg</td>
<td>246.4 mg</td>
<td>73.2</td>
</tr>
<tr>
<td>ElP2</td>
<td>300 mg 0.6 meq/gm</td>
<td>0.18 meq</td>
<td>396.0 mg</td>
<td>238.0 mg</td>
<td>60</td>
</tr>
<tr>
<td>ElP3</td>
<td>300 mg 0.6 meq/gm</td>
<td>0.18 meq</td>
<td>316.8 mg</td>
<td>226.8 mg</td>
<td>71.5</td>
</tr>
<tr>
<td>ElP4</td>
<td>300 mg 0.6 meq/gm</td>
<td>0.18 meq</td>
<td>297.0 mg</td>
<td>176.0 mg</td>
<td>59.25</td>
</tr>
<tr>
<td>ElP5</td>
<td>300 mg 0.6 meq/gm</td>
<td>0.18 meq</td>
<td>435.6 mg</td>
<td>289.2 mg</td>
<td>66.4</td>
</tr>
<tr>
<td>ElP6</td>
<td>300 mg 0.6 meq/gm</td>
<td>0.18 meq</td>
<td>277.2 mg</td>
<td>182 mg</td>
<td>65.6</td>
</tr>
<tr>
<td>ElP7</td>
<td>300 mg 0.6 meq/gm</td>
<td>0.18 meq</td>
<td>316.8 mg</td>
<td>201.2 mg</td>
<td>63.5</td>
</tr>
<tr>
<td>ElP1</td>
<td>300 mg 0.6 meq/gm</td>
<td>0.18 meq</td>
<td>277.2 mg</td>
<td>182.4 mg</td>
<td>65.8</td>
</tr>
</tbody>
</table>
Table: 6.3b  Obtained amount of crude peptides of E2 protein after cleavage

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Amt of Resin</th>
<th>Substitution factor of resin</th>
<th>Total substitution factor</th>
<th>Expected amt of peptide</th>
<th>Obtained amt of peptide</th>
<th>% yield of crude peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2P1</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>336.6 mg</td>
<td>246.4 mg</td>
<td>73.2</td>
</tr>
<tr>
<td>E2P2</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>396.0 mg</td>
<td>238.0 mg</td>
<td>60</td>
</tr>
<tr>
<td>E2P3</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>316.8 mg</td>
<td>226.8 mg</td>
<td>71.5</td>
</tr>
<tr>
<td>E2P4</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>297.0 mg</td>
<td>176.0 mg</td>
<td>59.25</td>
</tr>
<tr>
<td>E2P5</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>435.6 mg</td>
<td>289.2 mg</td>
<td>66.4</td>
</tr>
<tr>
<td>E2P6</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>277.2 mg</td>
<td>182 mg</td>
<td>65.6</td>
</tr>
<tr>
<td>E2P7</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>316.8 mg</td>
<td>201.2 mg</td>
<td>63.5</td>
</tr>
<tr>
<td>E2P8</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>356.4 mg</td>
<td>198.2 mg</td>
<td>55.6</td>
</tr>
<tr>
<td>E2P9</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>229.5 mg</td>
<td>229.5 mg</td>
<td>64.4</td>
</tr>
<tr>
<td>E2P10</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>336.6 mg</td>
<td>222.8 mg</td>
<td>66.2</td>
</tr>
<tr>
<td>E2P11</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>396.0 mg</td>
<td>235.6 mg</td>
<td>59.5</td>
</tr>
<tr>
<td>E2P12</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>277.2 mg</td>
<td>182.4 mg</td>
<td>65.8</td>
</tr>
<tr>
<td>E2P13</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>396.2 mg</td>
<td>213.84 mg</td>
<td>54.0</td>
</tr>
<tr>
<td>E2P14</td>
<td>300 mg</td>
<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>316.8 mg</td>
<td>187.3 mg</td>
<td>59.2</td>
</tr>
<tr>
<td>E2P15</td>
<td>300 mg</td>
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<td>0.18meq</td>
<td>356.4 mg</td>
<td>222.03 mg</td>
<td>62.3</td>
</tr>
<tr>
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<td>0.18meq</td>
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<td>203.38 mg</td>
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</tr>
<tr>
<td>E2P17</td>
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<td>0.6 meq/gm</td>
<td>0.18meq</td>
<td>237.6 mg</td>
<td>133.05</td>
<td>56.0</td>
</tr>
</tbody>
</table>
6.6 Characterization of the peptides:

6.6.1 N-terminal analysis:

The N-terminal analysis was done by using Fluoro-2, 4, dinitrobenzene, hydrolyzed with HCl and spotted onto Whatman paper. The Rf value of samples were compared with Rf value of standard DNP amino acids. When the FDNB labeled peptides and the appropriate standard amino acids were lyophilized with HCl then the resultant hydrolysate were spotted onto Whatman filter paper 1 to compare the Rf value of the derivatized N-terminal amino acid of peptide with the appropriate derivatized standard amino acid. The Rf values of the hydrolyzed Dinitrophenyl (DNP) amino acid was found to be in agreement with the Rf values of standard Dinitrophenyl (DNP) amino acid. The chromatogram was developed in the appropriate solvent system depending on the resolution of the peptides. The results of all the peptides showed that the observed and expected Rf values are comparable and describing that the synthesis of desired sequences. (Table 6.4a).

6.6.2 C-terminal analysis:

The C-terminal analysis was done by using the method of Bradshaw et al. Peptides were cleaved with CarboxypeptidaseA. The C-terminal amino acid was verified by running the sample on TLC plates and the Rf values were compared with that of the standard DNP amino acids. The peptide was mixed with Carboxypeptidase A and then the reaction was stopped by adding 10% TCA at different time intervals. The maximal time of 15minute was found to be the optimal incubation time for the release of C-terminal amino acid from each of the respective peptides. The respective C-
terminal amino acids released were verified by running the samples on TLC plates and the Rf values were compared with that of the standard amino acids. The observed Rf values of the sample amino acid were compared with the expected Rf values of standard amino acids. It shows that the peptides had the expected amino acids at the C-terminals. (Table 6.4b).

6.7 High Performance Liquid Chromatography (HPLC) analysis

High Performance Liquid Chromatography (HPLC) analysis of all the peptides was done to check the purity of all the peptides. All the peptides were run with a linear gradient of 0-100% Acetonitrile with 0.1% TFA against milliQ water with 0.1% TFA. Peptides peaks were monitored at 254nm with the flow rate adjusted to 1.5ml/minute for 30 minute. All the purified peptides showed a single peak with purity greater than 90% thereby confirming its homogeneity. (Fig: 6.7 a-g, Fig: 6.9 a-q and Fig:6.8a)

Table 6.5 a and 6.5b

6.8 Amino Acid Analysis:

Amino acid analysis was done with the help of HPLC systems. The peptides were first hydrolyzed with 6N HCl at 110°C for 18 hrs. All the amino acids were released from the sample. The hydrolyzed sample and standard amino acid solutions were dried and again re-dried in the neutralization buffer using re-drying solution (water: ethanol: TEA: 2:2:1). The dried free amino acid mixture and the amino acid standards were derivatized with Phenylisothiocyanate (PITC). First standard samples and then free amino acids were injected into the PICO-TAG column. The amount of individual amino acid was calculated from the peak area based on the retention time of each derivatized amino acid after comparing with standard amino acids. Some amino acid like tryptophan, histidine and methionine are destroyed during amino acid hydrolysis hence their observed values were not detected (Table: 6.6a-6.6b, Table 6.7a-d).

6.9 in-silico selection of CHIKV specific peptide

All the selected peptides of E1, E2 and E3 envelope proteins using Bcelpred (Protein id “AA033341.1”, db_xref “GI: 28193964”) and DNAsat software program were screened with 123 confirmed Chikungunya positive sera. These said peptides were found to be conserved in BLAST analysis using NCBI protein database with various strains of Chikungunya and homology was tested with other alpha viruses (e.g. Dengue, Simmiki forest, sindbis and ross rever virus).
### Table 6.4a

N-terminal analysis of amino acid of peptides of E2 protein by TLC

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<th>Peptide</th>
<th>Amino acid</th>
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<td>0.58</td>
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<td>0.58</td>
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### Table 6.4b

C-terminal analysis of amino acid of peptides of E2 protein by TLC

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Solvent system - Acetic acid:butanol:water:Ethyl acetate(1:1:1:1) v/v
Table 6.5a  Percentage purity of all E1 and E3 peptides after HPLC analysis

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Figure 5.9f: HPLC Profile of EP6
- Retention time:
- Absorbance at 254 nm

Figure 5.9h: HPLC Profile of EP8
- Retention time:
- Absorbance at 254 nm

Figure 5.9e: HPLC Profile of EP5
- Retention time:
- Absorbance at 254 nm

Figure 5.9g: HPLC Profile of EP7
- Retention time:
- Absorbance at 254 nm
Figure 6.9q  HPLC Profile of E2P17

On X-axis = Retention time
On Y-axis = Absorbance at 254nm
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Table 6.6a: Amino Acid Analysis of E1 Peptides
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Table 6.6b: Amino Acid Analysis of E1 and E3 Peptides
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Table 6.7: Amino Acid Analysis of E2 Peptides
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Table 6.7.d: Amino Acid Analysis of E2 Peptides
6.10 Standardization of peptides for antibody detection in infected patients.

Various Immunological parameters for IgM/IgG antibody detection by Indirect ELISA were standardized using Chequer-board titration of the peptide concentration. The various permutations and peptide concentration used for standardization were 25ng, 50ng, 75ng, 100ng. It was observed that the coating concentration of 100ng/well of the E1, E2 and E3 peptides was found to be optimum for the detection of IgM/IgG antibody in CHIKV infected patients by Indirect ELISA (Table 6.8a and 6.8b).

6.11 Detection of CHIKV specific IgM/IgG antibody with E1, E2 and E3 Peptide.

The selected E1, E2 and E3 peptides of CHIKV envelope protein were initially screened with pooled serum of confirmed CHIK positive patients in order to select the dominant sequences for further screening and validation of the assay. Peptides belonging to E1 and E3 envelope proteins showed poor seroreactivity with pooled sera as compared to E2 peptides and hence these peptides were not considered for further study (Figure: 6.10a). As ten peptides, out of seventeen, of E2 exhibited comparatively higher seroreactivity with pooled sera (Figure 6.10b), therefore, further screening of sera was attempting to develop in-house assay.
<table>
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<th>Peptides</th>
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Table 6.8 a Standardization of E1 peptide conc./100µl for evaluation of assay.

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Table 6.8 a Standardization of E2 peptide conc./100µl for evaluation of assay.
Figure 6.10 a and b: Bar graph showing IgM reactivity of twenty-five pooled sera of CHIKV infected patients with E2 peptides, E2 protein and virus lysate. Reactivity was compared with ten pooled sera of CHIKV healthy negative control sera.
The selected peptides of E2 protein were also screened individually with 123 confirmed CHIKV patients’ sera, 33 confirmed dengue positive sera and 39 healthy individual sera. All peptides showing absorbance value higher than the mean absorbance value of all healthy individuals plus 2SD were considered as positive. No sero-reactivity was observed with dengue positive and healthy individual sera with any of the peptides. The synthetic peptides showed wide range in the sero positivity by CHIKF patient’s sera (Fig 6.11). Those peptides showing sensitivity equal to or higher than 70% can be considered as antigenic peptide. The results of IgM based detection assay for 123 confirmed CHIK patients were expressed as absorbance value. Amongst ten selected peptides of E2 protein, E2P3, E2P5, E2P7, E2P9, E2P16 and E2P17 have shown very high seroreactivity with CHIKV patients’ sera. The sensitivity and specificity of these peptides for IgM assay was found to be in the range of 86.2- 93.5% and 85.7 to 100%, respectively (Table: 6.9). In the same way, the sensitivity and specificity of E2P10, E2P11, E2P13 and E2P15 peptides with the same number of CHIKV sera was found to be 77.2-84.6% and 74.3 to 85.7% (Table: 6.9). In all above cases sensitivity was found to be significantly higher ($P<0.0001$) as compared to the mean absorbance value of negative sera (healthy control) i.e. 0.195 (cut off). Surprisingly the peptides E2P8 and E2P12 showed poor IgM seroreactivity.

In light of above analysis, we can state here that a sample is considered positive when absorbance was found to be $\geq 0.195$ (cutoff). A cutoff value of ELISA for all the peptides was determined by ROC analysis. When absorbance values obtained from E2 protein and viral lysate were compared with E2 peptides a strong correlation was observed with positive sera, though there was minimal variation in absorbance value between peptide to peptide with Chikungunya positive sera, thus all the ten peptides can be considered as diagnostic antigens.

### 6.13 Detection of CHIKV specific IgG antibody with E2 Peptide

The above peptides were also screened with the same 123 CHIKV patients’ sera for IgG seroreactivity. The recognition pattern for peptides is not same for all patients, but still broader sero positivity was seen with IgG as compared to IgM (Fig 6.12). Interestingly a positive correlation was noticed in the recognition pattern of IgG and IgM antibodies with different peptides. In all the cases studied peptides showing IgM positivity had also showed IgG positivity but the reverse was not true. The result for IgG seroreactivity reveals E2P11, E2P13, E2P16 and E2P17 as most dominating peptides with a sensitivity of 95.1%, 94.3%, 93.5 and 95.9% respectively. Peptides E2P3, E2P15 was also founded to be 91.9%. Other peptides viz. E2P5, E2P7, E2P8,
E2P9, E2P10 and E2P12 has also showed above 70% seroreactivity (Table: 6.10). Surprisingly the peptides E2P8 and E2P12 showed poor IgM seroreactivity and specificity of all peptides were found to be 82.1-95% respectively (Table: 6.10). The mean absorbance range of patients with all above selected peptides was 1.0 with absorbance ranging 0.06 to 2.2 and showed a significant difference with mean absorbance 0.185 of healthy control groups. Herein, a sample was considered positive when absorbance was found to be ≥0.185 (cut-off). The mean absorbance of all positive sera was found to be significantly higher than negative sera ($P<0.0001$).
Figure 6.11  IgM Immunoreactivity of E2 Peptides, E2 Protein and virus lysate

Fig: 6.11 Graph showing IgM antibody reactivity of E2 peptides, E2 Protein and virus lysate with CHIKV positive sera (N=123), Healthy control (N=39). *P<0.0001, Solid Line showed mean value of control ≥0.15 and dotted line showed cutoff for positivity ≥ 0.195
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<th>NPV(95%CI)</th>
<th>Cut-off</th>
<th>Sensitivity (%)</th>
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<td>66.0%</td>
<td>0.166</td>
<td>86.2%</td>
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</tr>
<tr>
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<td>DTPDRTLSSQSGNVKIT</td>
<td>98/25</td>
<td>91.6%</td>
<td>51.0%</td>
<td>0.168</td>
<td>79.7%</td>
<td>74.3%</td>
<td>86%</td>
</tr>
<tr>
<td>E2P11</td>
<td>DKVTNNCKVDQCHAAVTNHK</td>
<td>104/19</td>
<td>95.4%</td>
<td>61.2%</td>
<td>0.161</td>
<td>84.6%</td>
<td>85.7%</td>
<td>91%</td>
</tr>
<tr>
<td>E2P13</td>
<td>LSANGTAHGHPHE</td>
<td>96/27</td>
<td>95.0%</td>
<td>52.6%</td>
<td>0.175</td>
<td>78.0%</td>
<td>85.7%</td>
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</tr>
<tr>
<td>E2P15</td>
<td>NSPLVPRNAELGDRKKG</td>
<td>95/28</td>
<td>92.2%</td>
<td>49.1%</td>
<td>0.170</td>
<td>77.2%</td>
<td>77.1%</td>
<td>84%</td>
</tr>
<tr>
<td>E2P16</td>
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<td>110/13</td>
<td>95.7%</td>
<td>69.8%</td>
<td>0.195</td>
<td>89.4%</td>
<td>85.7%</td>
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</tr>
<tr>
<td>E2P17</td>
<td>HEILYLYELYP</td>
<td>109/14</td>
<td>97.3%</td>
<td>69.6%</td>
<td>0.185</td>
<td>88.6%</td>
<td>91.4%</td>
<td>92%</td>
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</table>
Figure 6.12 IgG Immunoreactivity of E2 Peptides, E2 Protein and virus lysate.

Figure 6.12 Graph showing IgG antibody reactivity of E2 peptides, E2 Protein and virus lysate with CHIKV positive sera (N=123), Healthy control (N=39). *P<0.001, Solid Line showed mean value of control ≥ 0.14 and dotted line showed cutoff for positivity ≥ 0.185.
<table>
<thead>
<tr>
<th>Peptides</th>
<th>Amino acid Sequence</th>
<th>Total no. of positive/negative sample (n=123)</th>
<th>PPV (95%CI)</th>
<th>NPV (95% CI)</th>
<th>Cut-off</th>
<th>Sensitivity(%)</th>
<th>Specificity(%)</th>
<th>AUC (%)</th>
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<tr>
<td>E2P3</td>
<td>IGTDDSHDWTKLRY</td>
<td>113/10</td>
<td>98.3%</td>
<td>78.7%</td>
<td>0.160</td>
<td>91.9%</td>
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<td>85.7%</td>
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<td>92.3%</td>
<td>53.4%</td>
<td>0.160</td>
<td>78.0%</td>
<td>79.5%</td>
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<td>117/6</td>
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<td>95.9%</td>
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Discussion, Summary & Conclusions
Group A: Malaria
7. Discussion, Summary and Conclusion

The use of the core characteristics of Plasmodium HRP-2 and LDH antigens for detection of malaria, for estimation of parasite biomass (Dondorp et al., 2005), in rapid diagnostic test (Piper et al., 1999; Moody et al., 2002), in antigen and antibody assay has been reported (Dodderer et al., 2007; Kifude et al., 2008).

Histidine Rich Proteins

Three histidine are synthesized by Plasmodium falciparum. These are usually known as HRP-1, HRP-2, HRP-3. HRP-2 antigen is expressed by both knob positive (K+) and knob negative (K-) infected erythrocytes. This characteristic is the basis for development of diagnostic tests. Plasmodium falciparum HRP-1 is associated phenotypically with expression of knob-like protrusion on the surface membrane of infected erythrocytes (IRBCs) and hence expressed by knob positive erythrocytes only. This protein is formed of numerous tandem repeats, mostly of Histidine (34%), alanine (37%), and aspartic acid (10%), its molecular weights ranges from 65-85 kDa (Howard et al., 1986; Panton et al., 1989). However, HRP-1 is much more abundant than HRP-3. Pf HRP-1 appears to be associated with the membrane, particularly the cytoskeleton (Leech et al., 1984).

The Histidine rich protein 2 (HRP-2) is a Histidine and alanine rich water soluble protein, which is localized in several cell compartments including the parasite cytoplasm. The antigen is expressed only by P. falciparum trophozoites (Iqbal J et al., 2000; Beadle C et al., 1994). It circulates in the blood of a human host up to 14 days post infection and its C-terminal half induces a partially protective response (Parra et al., 1991). The parasite P. falciparum secretes a substantial amount of HRP-2 into the host blood stream and it can be detected in erythrocytes, serum, plasma, cerebrospinal fluids and even urine as a secreted water soluble protein (Rock EP et al., 1987). In culture of P. falciparum, HRP-2 can be detected in the supernatant medium only 2-8 hr after merozoites invasion (following the development of ring forms), including the active secretion from infected erythrocytes (Howard et al., 1986). After schizonts rupture in vitro, the amount of HRP-2 in the culture supernatant increases (Howard et al., 1986).

HRP-2 also used as vaccine component in several trials in splenectomized Aotus monkeys; immunization with a recombinant fusion protein containing a 165 amino acid sequence of HRP-2 in a lecithin-saponin adjuvant resulted in delayed onset of parasitemia after challenge and markedly depressed peak parasitemia (Knapp et al., 1992). HRP-2 have a number of crucial life functions in the parasite, has several
effects on the host immune system and can be successfully used as the antigen in the antigen detection tests for RDTs of *P. falciparum* infection (Shiff et al., 1993; Beadle et al., 1994; Makler et al., 1998). Although the sensitivity and specificities of these tests are generally high, samples that are known to be blood smear and/or PCR positive for *P. falciparum* are found to be RDT negative (Marx et al., 2005).

HRP-3/SHARP is a third HRP expressed by asexual blood stage of parasites. HRP-3 contains 28% Histidine, 28% alanine and 12% asparagine. There is a high level of sequence homology between HRP-3 and HRP-2, indicating that these two proteins have evolved from a common ancestral gene (Wellems and Howard, 1986). HRP-3 (Mr, 40000-55000) is distinguished by its lowest abundance compared with the other two HRP.

**Lactate Dehydrogenase**

*Plasmodium* Lactate dehydrogenase functions in the glycolytic cycle, an essential process that ensures the presence of PfLDH in each parasite, unlike HRP-2. Studies on sequence variability among PfLDH isoforms from different strains showed low diversity, which suggested that the antigen is conserved its sequence (Talman et al., 2007; Mariette et al., 2008). pLDH is a 316 amino acid protein coded by a single gene on chromosome 13 of *P. falciparum* and is expressed as a 1.6kb mRNA. The pLDH isoform can be distinguished from the human isoform on the basis of unique epitope within the pLDH protein and have also received considerable attention as a source of diagnostic tools. Blood stage malaria parasite synthesizes lactate dehydrogenase (LDH) as the terminal enzyme in their glycolytic pathway. The N-terminal amino acid sequence of pLDH has revealed that the DH is structurally different from that of the mammalian and bacterial LDH. The characteristic of the core technology for PfLDH have also been outlined in many references (Vander et al., 1981; Vander et al., 1990; Makler et al., 1998; Rajasekariah et al., 2003).

The amino acid sequence predicted from genomic and cDNA sequence indicates that essential catalytic residues are conserved, such as His195, Asp168, Arg109 and Arg 171 (Eventhoff et al., 1977). However, there are a number of interesting sequence difference between pLDH and human LDH, including an extra segment of five amino acids which are in a loop that helps to define the active site of LDH. Ile250 and Thr246, which from crystallographic analysis are critical active site residues (Grua et al., 1981; Dunn et al., 1991) that helps to define substrate and co-factor binding sites and are conserved in other LDH, are replaced by proline in pLDH. These sequences suggest that the kinetic properties of pLHD are quite distinct from those of human LDH. These studies used dipstick and ELISA to evaluate the characteristic of PfLDH.
They showed that PfLDH dipstick had a threshold effect of approximately 200 parasite/µl of the blood. Levels of PfLDH were more consistent with peripheral parasitemia than HRP-2. This effect was more obvious during the course of drug treatment. Additional studies showed that the parasite in patient blood sample had to be viable for them to be detectable for PfLDH (Piper et al., 1999).

Based on the unique characteristic of the HRP-2 and LDH antigen, detection of HRP-2 and pLDH serves as the basis for a serological assay for diagnosis of malaria using the antigen capture assay.

**Peptide Synthesis**

Two peptides from the HRP-2 proteins and three peptides from the pLDH were chosen for the study and all the peptides were synthesized using solid phase peptide synthesis. A dimmer of peptide with amino acid 15-26 was synthesized using F-moc chemistry on glycine Wang resin following standard procedure.

All the peptides were soluble in 20-30% acetic acid hence there was no problem in purifying the peptides by polar solvent using gel permeation chromatography and reverse phase HPLC technique. All the peptides were above 90% pure. The N terminal analysis and amino acid analysis authenticated the homogeneity of the peptides.

**Animal Model: Mice and Rabbit:**

Mice and Rabbits were chosen for the study because-

a. They are easy to produce, handle and maintain.

b. The breeding can be carried out on a large scale in a small interval of time.

c. They have genomic similarity to human and thus provide the closest model for correlating the study with humans.

The immune system and its regulation in mice, rabbit and humans are considered to be remarkably similar to the individual genetic restriction assessment, inbred mice of haplotypes (H2d) were taken, with different genetic makeup in an experimental group. Due to the lack of genetic variation, the consistent result was observed in an inbred population.

**PLGA microspheres:**

In the present study microsphere were used as the delivery system for generation of antibodies against all the five selected peptides of HRP-2 and pLDH antigen.

In this light, biodegradable microsphere based on poly-lactide-co-glycolide (PLGA) or poly lactide (PLA) are probably the most promising for all the antigens delivery
system studied. Microparticle based on biodegradable matrices can release proteins, hormones or drugs for the use of contraception. A biodegradable polymer is ideal for immunization purposes because it can release the antigen at a rate that mimics repeated administrations of the antigen and does not require an additional surgical step for retrieval of the depleted system. The sustained release technology can be applied to immunological adjuvants because release properties are one of the important functions of antigen. Microsphere have demonstrated excellent tissue compatibility (resorbable, synthetic polyesters), are nontoxic and have already been used for other biomedical applications including absorbable suture material and bone plates (Williams et al., 1988).

Microparticle can be used in targeted peptide delivery at the site of disease to improve the uptake of poorly soluble reagents, the targeting of peptides to a specific site, bioavailability. Several anti-cancer drugs including paclitaxel doxorubicin (Yoo HS, 2000), 5-fluorouracil (Bhadra D, 2003) and dexamethasone (Panyam J et al., 2004) have been successfully formulated using microparticles. Polylactic/glycolic acid (PLGA) and polylactic acid (PLA) based microparticles have been formulated to encapsulated dexamethasone, a glucocorticoid with an intracellular site of action. Dexamethasone is a chemotherapeutic agent that has anti-proliferative and anti-inflammatory effects. The drug binds to the cytoplasmic receptors and the subsequent drug receptor complex is transported to the nucleus resulting in the expression of certain genes that control cell proliferation (Panyam J et al., 2004). These drugs loaded microparticles formulations that release higher doses of drug for prolonged period of time completely inhibited proliferation of vascular smooth muscle cells.

The degradation rate of PLGA is controlled by factors including polymer molecular weight, polymer crystallinity and Lactate: glycolide ratio. Hence microparticles prepared with the range of different PLGA polymers may be utilized to develop the peptide delivery system. The polymer degrades by nonenzymatic hydrolysis to the metabolite lactic and glycolic acid and the rate of degradation can vary from days to a year. Lactic acid enters the TCA cycle and is metabolized and subsequently eliminated from the body as carbon dioxide and water.

The major drawback of PLGA system is that the microparticles need to be kept refrigerated of stored with desiccated or under vacuum to prevent rapid degradation and hence, PLGA system may not be suitable for use in areas where temperature controlled storage is not readily available.
Adjuvant

In the present study we have been using a CpGODN class B 1826 as an adjuvant. Immunostimulatoty DNA sequences containing unmethylated CpG dinucleotide in the context of particular base sequence (CpG motifs) exert strong stimulatory influence on the immune system. Such sequence which are either found naturally in bacterial NA or produced as synthetic oDNs directly activate human B cells and plamacytoid dendritic cells via Tir-9. CpG oligos act as polyclonal activated which directly activate B cells to proliferate and differentiate into IgG producing cells. CpG oligos also indirectly activate other cells such as monocytes and macrophases to produce a variety of pro-inflammatory cytokines and in particular those associated with these stimulatory influences CpG ODNs were capable of enhancing Cd4, Cd8 cytotoxic antibody response to a wide variety of antigens. As aresult of their strong adjuvanticity and low reactogenicity CpG ODNs are currently as one of the most promising adjuvants (Verthelji D et al., 2003).

Humoral response

After the generation of antipeptide antibodies using microsphere as a delivery system along with CpG ODN as adjuvant, peptide specific IgG level and peak titer were assessed by indirect ELISA. In the case of HRP-2 peptide I and peptide –II antiserum showed a peak titer ranging from 25,600- 102,400.

In the case of LDH peptide –I, high peptide specific IgG levels were maintained till 60 days post immunization. On end point titration of LDH peptide–I, peak titres ranges from 25,600- 51, 200 were observed. In case of LDH peptide-II antiserum IgG levels were maintained till days 60, peak-titer 25, 600-102,400 were observed. Similarly in case of antipeptide antibodies against LDH-III were also maintained till 60th days post immunization and peak titers ranges from 12,800- 51,200 and declined by 25,600 on days 90th.

Purification and Biotinylation and affinity of antibodies

The antibodies generated against the synthetic peptides of HRPII and LDH antigen were purified by affinity chromatography on a protein A column. IgG antibodies bind to the protein A chiefly by hydrophobic interactions and are disrupted by transient exposure to low pH.

After purification biotinylation of the antibodies was carried out using N-hydroxysuccinimide ester of biotin. Biotin reacts with α- amino groups of the peptide and protein to give stable bonds when used in from of N-hydroxysuccinimide spacer arm the caproic acid greatly improves the interaction between avidin and biotinylated
macromolecules by overcoming steric hindrance present at the binding site of avidin. The derivative is soluble in water and biotinylation proceeds at near neutral pH values. The extent of biotinylation and ratio of biotin to antibody was determined by avidin HABA assay. Since the affinity of avidin is higher for biotin than HABA, the absorption of the avidin-HABA complex at 500nm decreases proportionately with increased concentration of biotin as the HABA dye is displaced from avidin. The numbers of biotin molecules per IgG ratio were found to be 3-4.

Antibody affinity is important in determining the efficacy of a humoral response. It is a measurement of the strength of interaction between an antibody combining site and a complementary antigenic determinant. There is considerable evidence that high affinity antibodies are more effective than low- affinity antibodies in a variety of biological reactions (Steward et al., 1981). The $K_d$ value is a reciprocal measure of the affinity of the antibody i.e. higher the affinity lower the $K_d$ value.

The type of antibody response in terms of affinity is detrimental to the nature of the immune response generated. High affinity antibodies are important in the control of parasitemia so that even a small amount can be detected and neutralized.

**Standardization of assay and detection of HRP-2 and pLDH in patient’s sera**

In the present study, the performance of antibodies raised against two epitopes for PfHRP-2 and three different epitopes for PfLDH antigens was assessed for their use as Immunoreagents for development of ELISA-based diagnostic assay to assess the antigen load in a large number of Pf infected sera. The selected region of pLDH shows four to six amino acids different that is sufficient to generate antibodies that differentiate between the respective peptides and protein. All the selected regions were surface exposed to the native protein and hence generated antibodies against these peptides and thus it is able to differentiate between *P.falciparum* and *P.vivax*.

In the present study, we have selected peptides from two regions of HRP-2 and three unique and unexplored region of pLDH based on the structural data (by using different computer algorithm software including PYMOL for surface analysis of peptides), the peak antibody titres and affinity values for different antibodies, the antisera of PfHRP-2 peptide I and PfLDH peptide III (278-300 amino acid regions) were used as capture antibody and the antisera of PfHRP-2 peptide II and a cocktail mixture of PfLDH peptide I (33-51 amino acid region) and PfLDH peptide II (51-71 amino acid regions) were used as detecting antibody in the EIA. Also to enhance the immunogenicity, the microencapsulated peptides were co-entrapped with CpG Oligodeoxynucleotide as an adjuvant. It is well known that CpG adjuvant is more potent when used in conjunction with the delivery system largely as a consequence of
its improved delivery into the endosome of antigen presenting cell (APC), where it can more easily interact with TLR9 (Toll like receptor 9) (Molad Y et al., 2013; Suwarti S et al., 2013). Hence in this study, it was observed that when the length of peptide sequences of PfHRP-2 and PfLDH proteins was increased by tandem repeat and CpG was combined with the microsphere delivery, the dissociation constant of antisera was considerably reduced thereby indicating that antibodies have high affinity, which helped to detect low parasitemia levels up to ~75 parasites/μl blood in *P. falciparum* positive sera. The developed antibodies against *Plasmodium* lactate dehydrogenase have been used in the differential diagnosis of *P. falciparum* and *P. vivax* infection. The lower limits of detection for PfHRP-2 and PfLDH were comparable to those reported for other rapid diagnostic tests.

The recommended method and current gold standard used for the routine laboratory diagnosis of malaria is the microscopic examination of stained thin and thick blood films. By this gold standard method an expert technician can detect up to 50 parasites /μl (0.001% parasitemia) and can identify species with 98% specificity. Unfortunately, the microscopic procedure is time consuming and requires considerable training to obtain the necessary expertise. For many years, people have been making efforts to replace the traditional methods for parasite detection with better sensitivity and specificity than microscopy. Methods using fluorescence microscopy have helped to improve sensitivity but not the specificity. PCR has proven to be a sensitive method for diagnosis of all four species of human malaria parasite and can be expected to exceed the sensitivity of microscopic examination. The detection of <5 parasite/μl and identification to the species level make this excellent technique which to compare the sensitivity and specificity of other non-microscopic methods. Nested and multiplex PCR methods can give valuable information when difficult morphological problems arise during attempts to identify parasites to the species level. A number of PCR assays have been developed for the detection of malaria DNA from whole blood as either single or multiplex methods (Barker et al., 1992; Jelinek T et al., 1999; Kawamoto F et al., 1996; Seesod N et al., 1993; Sethabutr O et al., 1992; Snounou G et al., 1993; Wataya Y et al., 1993; Wataya Y et al., 1991). These assays have been used for the initial diagnosis, following the response to treatment, and as sensitive standards against which other non-molecular methods have been evaluated. However, PCR is an impractical standard against which to measure routine acute malaria diagnosis because of the time involved and the technical experience required.

Immunochromatographic dipsticks offer the possibility of more rapid methods for malaria diagnosis, however these tests are time consuming but easy to perform. There are two tests for diagnosis and detection of *P. falciparum* infections: The parasite F-
tests and the ICT malaria Pf tests. The parasite F-Immunochromatographic test for HRP-2 detection in *P. falciparum* in blood samples have shown an overall average sensitivity of 77 to 98% when 100 parasites/μl are present (0.002% parasitemia), with a specificity of 83 to 98% for *P. falciparum* compared with thick blood film microscopy (Beadle C et al., 1994; Brenier-Pinchart 2000; Dietz R et al., 1995; Kilian A et al., 1997; Kodisinghe HM et al., 1997; Premji Z et al., 1994; Snounou G et al., 1993). The lower sensitivity range obtained from different studies probably reflects the inability of the observer to detect parasites at densities as low as 200 parasites/μl by microscopy (many laboratories examine only 10 fields of a thick film as a routine) or the failure to read faint positive lines from the test strip. The lower level or absence of HRP-2 secretion of sexual forms may explain the negative results in some cases (Brenier-Pinchart, et al., 2000). Studies by Humar et al. (Humar, A. et al., 1997) and Pieroni et al. (Pieroni, P et al., 1998) investigated the sensitivity and specificity of the ParaSight F antigen capture test for *P. falciparum* compared with PCR. They demonstrated a sensitivity and specificity for the ParaSight F test of 88 and 97%, respectively. ICT Pf and PATH Falciparum Malaria IC They obtained a sensitivity and specificity of 96 and 99% with HRP-2 detection for *P. falciparum*, with discrepant results having 100 parasites /μl (0.002% parasitemia). But these tests have some limitations as there is an evidence that certain individuals may actually have a gene deletion for the production of HRP-2 and so will never give a positive result with these tests. Other limitations of tests for this antigen relate specifically to technical aspects of the HRP-2 test system. Several reports that the monoclonal IgG antibody used in the ParaSight F cross-reacts with serum rheumatoid factor, causing false-positive results, have been made (Iqbal J et al., 2000; Laferi H et al., 1997). The Amrad ICT Pf and PATH falciparum Malaria tests use a monoclonal IgM antibody, and reports of false-positive reactions occurring with rheumatoid factor are less frequent (Izumo A et al., 1987). Low-level parasitemias seen in areas of endemic infection because of constant exposure to the malarial parasites may result in positive results with doubtful clinical significance (Tjitra E et al., 1999).

A commercially available kit for LDH is optimal. A panel of monoclonal antibodies that can bind to active pLDH was developed from *P. falciparum* and *P. vivax* in this series are 94 and 88% respectively, with a specificity of 100 and 99%, respectively. Samples found positive by microscopy but negative by optimal (3%) had parasite parasites levels of >100 parasites/μl (0.002% parasitemia), the sensitivity obtained for OptiMAL was 97%. In a study of the OptiMAL assay for detection and identification of malaria infections in asymptomatic residents in Indonesia, Fryauff et al. (Benito, A et al., 1994) found an 88 to 92% sensitivity for detecting infection of 500-1000
parasites/μl. However, the system was markedly less sensitive than was expert microscopy for discriminating between malaria species.

In the present study, we have shown that \textit{P/LDH} and \textit{P/HRP-2} ELISA levels can be used to complement microscopy in our target population. Microscopy only detects circulating infected RBC and is not an accurate measure of parasite burden, particularly in \textit{P. falciparum} infections where mature stages are known to be sequestered in various tissues. It is now widely accepted that \textit{P/LDH} levels reflect current infection whereas \textit{P/HRP-2} levels indicates both past and current infection. Thus concurrent measurement of these two biomarkers by ELISA provides a better evaluation of parasite burden especially in areas, where malaria is endemic (Rajasekariah GH et al., 2003; Martin SK et al., 2009; Hopkins H et al., 2009). There are few RDT kits commercially available for the diagnosis of \textit{P. falciparum} specific HRP-2 and LDH antigen (Iqbal J et al., 2002). The overall sensitivity reported was 88.7% when parasite count <100 parasite/μl of blood and increased from 94.3 to 99.3% when the parasite count > 100 -10000 parasite/μl of blood with a specificity of 97.5% (Maltha J et al., 2010; Murray CK et al., 2009; Wongsrichanalai C et al., 2007). However in the present study we identified certain unique epitopes of \textit{P/HRP-2} and \textit{P/LDH} which are used for diagnosis of malaria by 96% and 100% sensitivity and 100% specificity when the parasite count was >75 parasite/μl blood. Furthermore our data provided a standardized protocol for the measurement of both \textit{P/LDH} and \textit{P/HRP-2} antigens in the same patient sample using separate capture or detecting antibodies specific to these antigens. Our data was supported by the work of Martin et al, who also developed a unified \textit{Plasmodium} LDH and HRP-2 protein for quantification of \textit{P. falciparum} (Martin SK et al., 2009). Our work highlights the unique and specific epitopes of \textit{P/HRP-2} and \textit{P/LDH} which are used for diagnosis of malaria in \textit{P. falciparum} positive patients by sandwich ELISA. This approach may be useful to many small-scale industries in developing country like India, which involves minimal cost for development of immunoreagents required for making an indigenous diagnostic kit based on ELISA for detection of \textit{P/HRP-2} and \textit{P/LDH} antigens.
SUMMARY AND CONCLUSION

The aim of the present study was to develop indigenously developed Immunoreagents for the rapid and sensitive detection of *P. falciparum* HRP-2 and LDH antigen using antigen capture assay.

To achieve the above goal, initially unique peptide sequence of the HRP-2 antigen (two region) and pLDH antigen (three region) were synthesized using F-moc chemistry on a manual peptide synthesizer. After the synthesis of the peptides, the peptides were cleaved from the resin and purified to homogeneity using a combination of gel chromatography and HPLC. After physiochemical characterization, all the peptides were found to be above 90% pure. After the purification of the peptides, antipeptide antibodies were raised in mice and rabbit using microsphere as the delivery system with CpG ODN adjuvant. Peptide specific IgG level and peak titres were measured by indirect ELISA.

**Humoral Response:** Peptide in microspheres elicited high peptide specific IgG antibody levels and peak titres and the immune response was maintained till 90 days post immunization in all the peptides and high peak titres were observed till 90 days post immunization in all the HRP-2 and LDH peptides. The incremental increase in antibody titres seen with microsphere delivery with adjuvant may release from sustained release of the antigen. Small amount of antigen continuously release from microsphere to the immune system to stimulate the correct repertoire of B-cells. (i.e. secreting antibodies of high affinity).

**Affinity studies:** High affinity antibodies were generated against the synthetic peptides of HRP-2 and LDH antigen using microsphere delivery system. The affinity of the antibodies ranged between 0.73nM-3.0nM. The evidence of microspheres generating high affinity sera provides a reason for the high titer, long lasting immune response seen with these formulations of peptides entrapped in microspheres. After the generation of high titer and high affinity antibodies, the antibodies were purified by affinity chromatography on a protein A column. Purified antibodies were biotinylated using N-hydroxysuccinimide ester of biotin.

**Detection studies:** Upon standardization of the various parameters, the HRP-2 and LDH antigen were detected in the culture supernatants and parasitized lysate of *in vitro* culture of *P. falciparum* using antigen capture assay. The assay allowed the detection of the HRP-2 and LDH antigen up to a low level of parasitemia of 0.001%, which is comparable to the detection limit being achieved by thick blood smear in light microscopy.
In conclusion, both the antigen capture assays were comparable to each other for
detection of *P. falciparum* infection at low levels of parasitemia (0.0015%). The
sensitivity limit (0.0015% parasitemia) of *Pf*HRP-2 and *Pf*LDH assays was
comparable to those achieved by microscopic examination of thick blood smears or
other RDTs viz., ICT test/ Malaria *P f/ P v* Test.

Hence, the study highlights the importance of the microsphere delivery system in the
generation of high titer against synthetic peptides of HRP-2 and LDH antigen. These
specific antibodies have diagnostic importance for the detection of *P.falciparum*
HRP-2 and LDH antigen in patients' blood. The present study signifies the importance
of indigenously developed Immunoreagents for the diagnosis of *P.falciparum* malaria
upto 75 parasites per microliter of blood. By using the antigen capture assay. A
positive correlation was observed between parasitemia and two antigens (as the
parasite count increases secretion of antigen also increases per microliter of patients' 
blood were seen in all the infected patients studied.

**Future Study:** These high affinity antibodies can be used in the future for
development of immunoreagents for immunochromatography tests to detect specific
*Pf*HRP-2 and *Pf*LDH in blood of *P. falciparum* positive patients. The benefits of *P. 
falciparum* HRP-2 and LDH antigen capture assays include its ease of use, its
sensitivity and specificity. It does not require any specialized equipment, requires no
skilled operator, and requires a small volume of whole blood will be useful in
epidemiological survey and ease of result interpretation. It is fast and cost-effective
and more importantly can be used in the field. Thus, antigen capture assays using
indigenously developed reagents were able to detect *P. falciparum* even at low
parasitemia in endemic regions.
Discussion, Summary & Conclusions
Group B: Chikungunya
GROUP B: CHIKUNGUNYA

The CHIKV strain that prevailed in Indian Ocean Island in 2005 and subsequently spread to India and Southeast Asia was phylogenitically derived from the Central/East African chikv group (Ng, L. C. et al., 2010; Shuffenecker et al., 2006). In terms of structural poly protein homology Tanzania strain S27 may be classified within Central/ East African Chikv group. The current Indian epidemic is caused by the Central/East African genotype S27. Chikungunya virus strains isolated in India are closely related to strains isolated in Reunion Islands (99.61% nucleotide sequence homology) and strains isolated in the Maharashtra province in 2000 (98.95% homology) (Khan AH et al., 2002; Yergolkar P et al., 2006). Howsoever, there is currently no commercial vaccine or specific treatment available for the disease, for better patient management, control of outbreaks and epidemiological studies rapid identification of virus is essential. Virus identification is needed for a void of drug resistance, and cross reactivity with other related alpha viruses. Currently, there are a few specific serological tests or molecular diagnostic tools available despite the facts that CHIKV resurgence is associated with epidemics of unprecedented magnitude.

Structure of the virion:

The alpha virus nucleocapsid contains the single stranded plus-sense RNA genome of approximately 11.7 kb complexed with multiple copies of a single species of capsid protein of about 30 kDa. The virion envelope consists of a lipid bilayer in which multiple copies of virus encoded E1 and E2 glycoprotein are embedded (Harisson SC, 1986; Strauss JH and Strauss EG, 1976). The lipid bilayer is composed of lipids derived from the plasma membrane of the host cell, and its composition closely approximates that of the host plasma membrane.

E1 and E2 glycoproteins:

Alpha virus glycoproteins are, during a natural infection, expressed on the surface of the host cell to allow budding of progeny virus. The two glycoproteins, E1 and E2 are 440 and 404 amino acid in length. The molecular mass of each of these two glycoproteins is approximately 50 kDa. The CHIKV envelope protein E1 and E2 are components of spikes, which composed of triplets of a heterodimer of E1 and E2 glycoproteins, and cover the viral surface in the form of membrane-anchored types. The viral spike proteins facilitate attachment to cell surfaces and viral entry into the cells. The E1 envelope protein is a class II fusion protein that mediates low pH-triggered membrane fusion during virus infection. The E2 envelope protein is a type I transmembrane glycoprotein and has been known to be responsible for receptor binding during the course of alphavirus cycle (Kielian M et al., 2006; Brehin AC et
E1 and E2 protein correlate with the serological response in human hosts (Simizu B et al., 1984) and E1 modulates penetration of the virus in the mosquito species. On partial E1 sequences from African and Asian isolates revealed the existence of three distinct Chikungunya virus phylogroups: first containing all isolates from West Africa, the second containing isolates from Asia, and third corresponding to East, Central, and South African isolates (Higgs S, 2006). They have a worldwide distribution and all alpha viruses are antigenetically related. The viruses are inactivated by acid pH, heat, lipid solvent, detergents, bleach, phenol, 70% alcohol and formaldehyde. Most of the viruses possess haemagglutination activities.

The production of CHIKV E1 and E2 insect cells using baculovirus vectors leads to glycosylation, (partial) furin processing and plasma membrane translocation and/or secretion of E1 and E2. E1 and E2 are expected to be transported to the cell membrane during CHIKV infection and be exposed at the cell surface. Thus, expression of E1 is correlated with the syncytium formation and E1 is functionally active as viral fusion protein in a defined pH range. The fusogenicity of CHIKV E1 is in the same order of magnitude of what has been found in the major envelope fusion protein F. The glycosylation patterns and resulting size changes found for CHIKV E1 and E2 appear to correspond with the postulated number of glycosylation sites in E1 and E2.

**Peptide synthesis**

A total of seven peptide from E1, seventeen peptide from E2 and one peptide from E3 envelope protein were chosen from the study and all the peptides were synthesized using solid phase peptide synthesis. Peptide with amino acid 10-20 was synthesized using F-moc chemistry on glycine wangi resin following standard procedure. All the peptides were soluble in 20-30% acetic acid hence there was no problem in purifying the peptides by polar solvent using gel permeation chromatography and reverse phase HPLC technique. All the peptides were above 90% pure. The N and C terminal analysis and amino acid analysis authenticated the homogeneity of the peptides.

**Synthetic Peptide based diagnostic reagents:**

The peptide based diagnostic reagents is based on the identification and chemical synthesis of B-cell and T-cell epitopes particularly those that are immunodominant and induce specific immune functions. The main advantage of synthetic peptide is that the product is easy to produce free from any contamination and stable at room temperature. The main disadvantage is low immunogenicity and genetic restriction in the host.
Herein, we have evaluated the seroreactivity of E1, E2 and E3 envelope peptides using anti-CHIKV positive and negative sera, as well as anti-dengue sera. The sensitivity of CHIKV E2 envelope peptides was found to be higher than that of CHIKV E1 and E3 envelope peptides. None of the CHIKV E2 peptides showed seroreactivity with thirty three dengue positive serum. All the ten E2 peptides showed absorbance close to the E2 protein with patient’s sera. This study clearly demonstrates the importance of peptide based diagnosis. All the peptides that showed seroreactivity have shown to be exposed on the surface of E2 protein.

In Chikungunya infection the envelope proteins have been reported to elicit a protective immune response (Luclle Warter et al., 2011; Anne-Claire Bréhina et al., 2008; Lee CY et al., 2011). As a result, most previous studies aimed at identifying B cell epitopes for CHIKV were focused on the envelope protein. Morey et. al., reported four potential peptides (RAGLLVRTSAPCT, GHFILARC, HGHPHEILYYEL and HGKELPSSTYVQSC) by screening 19 CHIKV positive sera against 17 synthetic peptides designed from the E2 glycoprotein of Chikungunya (Morey SH et al., 2010). Using La Reunion (IMT) or Singapore (SGP11) originated CHIK virus, Yiu-Wing Kam et al. reported multiple linear B-cell epitopes covering the entire CHIKV proteome (Kam YW et al., 2012).

Due to frequent explosive outbreaks of CHIKV, it is important to understand human IgM, IgG epitopic pattern in the development of a diagnostic reagent. In this study, we have dissected the antibody response to E1, E2 and E3 envelope peptides using anti-CHIKV sera, collected from different epidemic regions of India. The seroreactivity for E2 derived peptides was significantly higher as compared to E1 and E3 peptides. The difference in seroreactivity between CHIKV E1, E2 and E3 envelope protein could be that, the E1 and E3 might have been buried (cryptic in nature) thereby giving less exposure to host immune system. In cryptic sequence the peptide is able to recognize its own antibody but it fails to recognize the native antigen (Conway JF et al., 2003). Another reason for very low serum titer for E1 and E3 derived peptides could be that serum samples were collected during the early phase of the disease (7-15 days from the onset of symptoms). Previous reports showed that E2 protein is the only surface protein showing IgG reactivity in the early convalescent phase33 and others including E1 glycoprotein and capsid (Byungki Cho et al., 2008; Kowalzik S et al., 2008) are detected in varying levels at later stages of the disease. A specific mutation in E1 (Ala226Val) was initially absent in the viral strains but in the later strain >90% mutation was observed (Schuffenecker I et al., 2006). E1 is less conserved than E2; this could also be another reason why only E2 peptides are showing consistent seroreactivity with individual patient’s sera. Importantly, synthetic
peptides have advantages of low cost production, easier standardization and quality control as compared to Q-RT-PCR for Chikungunya in small laboratories. Of note, it mimics specific epitopes of infectious viral protein and hence has been exploited for diagnostic purpose. Earlier studies demonstrated the usefulness of peptides for the diagnosis of infectious diseases (Barbara Hosein et al., 1991; Deepak Tomar et al., 2006, Gokulan K et al., 1999; Shweta H Morey et al., 2010; zhengi et al., 2002). In another study role of fusogenic peptides in the serological diagnosis of dengue fever was reported (Pattanaik et al., 2006). Synthetic peptides offer the advantage of eliminating nonspecific reactions with enhanced specificity by averting the selection of cross reactive sequence from the designed peptides. Peptide selected from an immunodominant region of Chikungunya envelope protein on the basis of optimal seroreactivity with patients’ sera as seen in the present study can be used for diagnosis of disease in the small laboratories. It is comparatively easy to produce and easy to handle as it is stable at room temperature. With this intent, we used peptide based ELISA, rather than using whole envelope protein of Chikungunya virus to develop an in-house immunoassay for diagnosis.

Sometimes when the viraemia load was high in the patient sample, the acute phase of Chikungunya infection persists up to 15 days after the onset of the symptoms (Devi Goorah et al., 2009; Pialoux et al., 2007) as compared to other viral disease. Somehow, in few samples we missed the IgM seropositivity with selected peptides. The utilization of IgM/IgG antibodies to neutralize the excessive viral copies, in above samples, could have been one possible reason associated with this observation. In this pursuit, we may also state that the sero-conversion from IgM to IgG could be another plausible reason associated with missing IgG/IgM in above samples, as the sera were collected between acute and convalescent phase in these patients. Since the peptides are known to be HLA restricted, the reason for varied antibody generation among different individuals due to genetic polymorphism in HLA alleles could be another reason underlying to the above discrepancy. However, in the future these peptides can be exploited as multiple antigenic peptides (MAP) for efficient diagnosis of CHIKV. Above study show that antibodies are specific for E2 peptide, can also be exploited for identifying cell receptor recognition during viral entry.

Thus the above result clearly states that CHIKV E2 envelope peptides can be potential diagnostic reagents for serological assay. Furthermore, because of high levels of sensitivity and specificity these peptides can also useful for differentiation of dengue sera from Chikungunya positive sera.
SUMMARY AND CONCLUSION:

The aim of the present study was to develop E1, E2, E3 (envelope protein) peptide based diagnostic assay for the detection of Chikungunya specific IgM and IgG assay using in-house reagents.

To achieve the above goal, initially unique peptide sequence from E1, E2, E3 envelope protein of Chikungunya virus were selected using the computer algorithm program. All selected peptides from chikungunya envelope protein were synthesized using F-moc chemistry on a manual peptide synthesizer. After the synthesis of the peptides, the peptides were cleaved from the resin and purified to homogeneity using a combination of gel chromatography and HPLC. After physiochemical characterization, all the peptides were found to be above 90% pure.

Study subject: All the CHIKV infected patients were selected from different endemic region of India. A total of 123 CHIKV positive patients were enrolled in the study to evaluate the assay by indirect ELISA.

Our data suggest that many immunodominant peptides were identified throughout the length of E2 protein. Interestingly we observed that the epitopic expression for IgM, IgG is almost conserved with few exceptions. The peptides showing IgM seroreactivity also demonstrated IgG binding capacity with the sera. Out of 17 synthetic peptides of E2 protein, peptides E2P3, E2P11, E2P16 and E2P17 were found to generate IgM and IgG antibodies with high sensitivity. The peptides E2P8 and E2P12 showed negligible seroreactivity for IgM but a well-defined IgG was observed with the patient’s sera. This could due to consummation of IgM antibodies by the virus or class switching to IgG.

So far, the experimental results indicate, it is not essential, that an individual can have both IgM and IgG seroreactivity even for the same peptide. The level of antibody response generated by an individual patient was found to be different for different peptides. Also it was observed that the potential seroreactivity of an individual peptide is not same in all patients. This could be due to the genetic makeup of an individual.

Our study provides important insight into antibody profile against envelope antigens of Chikungunya S27 infection. In conclusion, CHIKV E2 peptides can be potential diagnostic reagents for serological assay. We found many seroreactive peptides covering the entire length of E2 antigen. In this study we observed four most immunodominant peptides (E2P3, E2P7, E2P11, E2P16 and E2P17) having significant high seroreactivity for IgM and IgG antibodies. All seroreactivity peptides
have shown to be exposed on the surface of the E2 protein using Bioinformatics study. Since these peptides showed high IgM and IgG reactivity, it appears that E2 protein has long lasting antibody response in CHIKV infected individuals. We can conclude that these peptides can be assembled as multiple antigen peptide (MAP) or attaching to multiplexed microbeads in the near future for diagnosis in early and late phase of the disease.