A STUDY ON CORRELATION OF CLINICAL FEATURES WITH SERODIAGNOSIS AND MOLECULAR CHARACTRISATION OF CHIKUNGUNYA

Dissertation submitted to

THE TAMILNADU DR.M.G.R.MEDICAL UNIVERSITY

in partial fulfillment of the regulations

for the award of the degree of

M.D. (MICROBIOLOGY)

BRANCH – IV

MADRAS MEDICAL COLLEGE

CHENNAI

APRIL 2011

CERTIFICATE

This is to certify that this dissertation titled **"A STUDY ON CORRELATION OF CLINICAL FEATURES WITH SERODIAGNOSIS AND MOLECULAR CHARACTRISATION OF CHIKUNGUNYA"** is a bonafide record of work done by **DR.E.PADMAVATHI,** during the period of her Post graduate study from June 2008 to April 2011 under guidance and supervision in the Institute of Microbiology, Madras Medical College and Government General Hospital, Chennai-600003, in partial fulfillment of the requirement for **M.D. MICROBIOLOGY** degree Examination of The Tamilnadu Dr. M.G.R. Medical University to be held in April 2011.

Dr. J.MOHANA SUNDARAM.,M.D Ph.D,DNB
Dean
Madras Medical College &
Government General Hospital,
Chennai -600 003

DR.S.GEETHALAKSHMI.,M.D.,Ph.D
Director i/c
Institute of Microbiology,
Madras Medical College &
Government General Hospital
Chennai -600 003

DECLARATION

I declare that the dissertation entitled **"A STUDY ON CORRELATION OF CLINICAL FEATURES WITH SERODIAGNOSIS AND MOLECULAR CHARACTRISATION OF CHIKUNGUNYA"** *submitted by me for the degree of M.D. is the record work carried out by me during the period of* ***May 2009 to May 2010*** *under the guidance of Professor* ***Dr.G.JAYALAKSHMI M.D.,DTCD.,*** *Professor of Microbiology, Institute of Microbiology, Madras Medical College, Chennai. This dissertation is submitted to the Tamilnadu Dr.M.G.R. Medical University, Chennai, in partial fulfillment of the University regulations for the award of degree of M.D., Microbiology (Branch IV) examination to be held in April 2011.*

Place: Chennai ***Signature of the Candidate***
Date : ***(Dr.E.PADMAVATHI)***

Signature of the Guide
Prof.Dr.G.JAYALAKSHMI,MD.,DTCD.,
Institute of Microbiology,
Madras Medical College,
Chennai.

ACKNOWLEDGEMENT

I humbly submit this work to the **Almighty** who has given the health and ability to pass through all the difficulties in the compilation and proclamation of this blue print.

I wish to express my sincere thanks to our Dean, **Dr.J.MOHANASUNDARAM M.D.,Ph.D.,DNB** for permitting me to use the resources of this institution for my study.

I feel indebted to Prof. **Dr. S.GEETHALAKSHMI M.D.,Ph.D.,** Director i/c & Professor, Institute of Microbiology for her constant encouragement, innovative ideas and timely suggestions during my work.

I owe special thanks to our former Director, Prof. **Dr.G.SUMATHI M.D.,Ph.D.,** for her constant support, invaluable suggestions, erudite guidance in my study and for being a source of inspiration in my endeavours.

I also owe special thanks to Prof. **Dr.G.JAYALAKSHMI M.D.,DTCD.,**Institute of Microbiology for her constant support, invaluable suggestions, erudite guidance in my study and for being a source of inspiration in my endeavours.

I would like to thank my Professors **DR. J.SASIKALA M.D.,Dr. THASNEEM BANU.S M.D.,** and **Dr.N.DEVASENA M.D.,** for their valuable assistance in my study.

I extend my whole hearted gratitude to our Assistant professor **Dr.R.DEEPA M.D.,** for her valuable guidance in my study.

I also express my sincere thanks to our Assistant professors **Dr. LATA SRIRAM,M.Sc.,Ph.D., Dr.J.EUPHRASIA LATHA M.D., Dr.P.BALAPRIYA,M.D., D.A.,Dr.T.SABEETHA M.D.,Dr.N.RATHNA PRIYA M.D., Dr. K.G.VENKATESH M.D.,Dr.C.S.SRIPRIYA M.D.,Dr.N.LAKSHMIPRIYA M.D.,**for their support in my study.

I would like to thank **Dr.P.GUNASEKARAN, M.D.,Dr.K.KAVERI,M.D.,**Deputy Directors, King Institute of Preventive Medicine, for giving me permission to conduct a part of my study at their department. I would also like to acknowledge **Mr.K.SARAVANAMURALI** who helped me in my PCR study.

I would like to thank all my colleagues and all staff of Institute of Microbiology,Madras Medical College and Chennai-3 for their help and encouragement.

I would like to thank the Institutional Ethical Committee for approving my study. I acknowledge my thanks to Mr .Yuvaraj, Lecturer in Statistics, for his help during my study in statistical analysis.

Finally I am indebted to my husband, my son and my family members who have been solid pillars of everlasting support and encouragement and for their heartfelt blessings.

INDEX

INTRODUCTION

Chikungunya fever is an important arthropod-borne virus (arbo virus) disease.[47,48,49] Until recently it attracted only minor interest in the medical community and did not evoke the fear associated with other arboviruses, such as dengue and west nile viruses. The recent resurgence of chikugunya fever has drawn global attention due to its explosive onset, rapid spread, high morbidity and myriad clinical manifestations.[17,59,61,76,79]

Documented first time from an outbreak in 1952[41,67]. Explosive outbreaks of epimedics of the disease have occurred after periods of long quiescence in different parts of the world. After an extensive outbreak during the beginning of the current millenium in the French territory of Reunion Islands in the Indian ocean, Chikungunya fever has been reported from almost 40 countries from different regions of the world[88]. After an interval of 32 years, India witnessed a massive epidemic in 2005, which is still ongoing in different parts of the country[48]. The disease has affected millions of people and left many with crippling disabilities[88].

Chikungunya virus (CHIK V) produces a dengue-like illness in humans, characterized by fever, rash, and severe arthralgia persisting for a few weeks to several months. The most significant characteristic of Chikungunya is the prolonged arthralgic syndrome that primarily affects the

peripheral small joints associated with excruciating pain.[61] The disease is generally non-fatal and the acute phase resolves within 3–4 days leaving the arthralgic syndrome persisting for some more time. The clinical course of the acute phase of infection has been well characterised during previous epidemics in African countries, the Indian subcontinent and Southeast Asia.[88] It involves an incubation period lasting between two to six days, followed by the abrupt onset of fever associated with intense diffuse muscle and joint pain. Headache, photophobia, nausea, vomiting, diarrhea and a maculopapular or morbiliform skin rash may accompany these symptoms. Treatment is mainly symptomatic, with remission observed in most patients some days after the infection.

A human-mosquito-human cycle is responsible for the maintenance of the virus in the South East Asia region in contrast to the sylvatic transmission cycle occurring in the African continent.[88] The re-emergence of chikungunya fever has been attributed to a multitude of factors including mutation of the virus, absence of herd immunity, lack of efficient vector control activities and globalization and emergence of another vector, Aedes albopictus (A. albopictus) in addition to Aedes aegypti (A.aegypti) as an efficient transmitter of chikungunya virus.[88]

Currently, the diagnosis of Chikungunya infection is accomplished through either virus isolation or detection of virus specific antibodies by

enzyme linked immunosorbent assay (ELISA) or genomic detection by RT–PCR.[74,78]

Although isolation is considered as gold standard, however, it is practically difficult, time consuming and requires live viable virus. The RT–PCR-based molecular assays are now being increasingly utilized for rapid, early diagnosis particularly during acute phase of the illness.

The re-emergence and persistence of CHIKV suggests the need for continuous monitoring and identification of the newly evolving variants and their genetic divergence with a view to plan for appropriate strategies for vaccine development. Further studies on the vector prevalence, the environmental conditions that favour the persistence of CHIKV, serosurveys in humans and mosquitoes in the affected areas are required to better understand the dynamics of Chikungunya in this region and prevent further spread of the virus to other parts of the country.[88]

This study was undertaken to study the incidence, clinical presentation and molecular characterisation of chikungunya virus in patients presenting with fever and arthralgia in Government General Hospital, Chennai.

AIMS OF THE STUDY

1. To study the incidence of chikungunya among patients with fever in Government General Hospital, Chennai.
2. To determine the seropositivity of chikungunya cases.
3. To determine the molecular characterisation of chikungunya virus.

REVIEW OF LITERATURE

Historical Background

There are historical accounts of epidemics of fever, arthralgias/arthritis and rash, resembling what is now called "Chikungunya fever" dating back to 1824 from India and elsewhere.[87] Chikungunya fever was first described in 1952,[41,67] following an outbreak on the Makonde Plateau, along the border between Tanganyika and Mozambique [The mainland part of modern day Tanzania] in the Nawala district. The chikunugunya fever was first described by Marion Robinson and W.H.R.Lumsden in 1955[41].

Etymology and History of the Name "Chikungunya"

For a long time, it was erroneously reported that the word "Chikungunya" was derived from the "Swahili" language both in lay press and the media and reputed medical journals[7]. The word "Chikungunya" is derived from the Makonde language, spoken by an ethnic group in south-east Tanzania and northern Mozambique from the root verb "kungunyala", meaning "to dry up or become contorted," and signifies the cause of a contortion or folding[4,41]. Literally, the word "Chikungunya" translates to "that which bends up" in reference to the stooped posture developed due to the rheumatological manifestations of the disease. In Congo, it has been

called “buka-buka”, meaning “broken-broken” once again reflecting the incapacitating arthralgias that are common acute manifestations of Chikungunya fever[51]. In concurrent research, Robinson glossed the Makonde term more specifically as "that which bends up."[41]

Natural history and transmission

Chikungunya fever epidemics display secular, cyclical, and seasonal trends. These epidemics are characterized by explosive outbreaks interspersed by periods of disappearance ranging from several years to a few decades. The exact reason for this behavior still remains a mystery. Several mechanisms including a complex interaction between various factors such as the susceptibility of humans and the mosquito vectors to the virus, conditions facilitating mosquito breeding resulting in a high vector density, ability of the vector to efficiently transmit the virus are all thought to play a role.[47,59,76] International travel has facilitated the introduction of the virus from endemic areas to other areas resulting in outbreaks of the illness, and imported cases of Chikungunya fever have been documented in France, Italy, Australia, and United States of America also[14,31,60,76].

The natural cycle of the virus is human-mosquito-human; evidence is available regarding the existence of epizootic cycles that may maintain the virus during the interepidemic period.[17,47,59,61,76,70,79] During epidemics, human beings serve as the Chikungunya virus reservoirs; during

interepidemic periods, several vertebrates such as monkeys, rodents, birds, have been implied as the reservoir.

Little is known regarding whether and how the Chikungunya virus is maintained in the wild in Asia. Unlike dengue virus, there is no evidence for transovarial transmission of Chikungunya virus in mosquitoes. Variations in the geographical strains of Aedes mosquitoes regarding their susceptibility to infection and ability to transmit the virus may be crucial factors in determining endemicity of Chikungunya virus in a given region.[17,47,59,61,70,76,79] Vertical maternal-fetal transmission has been documented in pregnant women affected by Chikungunya fever.[66]

The virus

Chikungunya virus is a Group IV positive sense single stranded RNA virus belonging to family Togaviridae with genus Alphavirus and species Chikungunya virus (CHIKV). It has a genome consisting of a linear, positive-sense, approximately 11.8 kb single stranded ribonucleic acid (RNA) molecule, a 60-70 nm diameter capsid and a phospholipid envelope[59,61,76]. The complete genome was found to be 11,805 nucleotides in length. Coding sequences consisting of two large open reading frames of 7422 nucleotide and 3744 nucleotide encoding the non-structural polyprotein (2474 amino acids) and the structural polyprotein (1248 amino acids), respectively. The non-structural polyprotein is the precursor of proteins

nsP1 (535 amino acids), nsP2 (798 amino acids), nsP3 (530 amino acids) and nsP4 (611 amino acids) and the structural polyprotein is the precursor of protein C (261 amino acids), E3 (64 amino acids), E2 (423 amino acids), 6K (61 amino acids) and E1 (439 amino acids)[59,61,76].

Studies on Chikungunya virus isolates collected from various geographical areas indicate that three lineages with distinct genotypic and antigenic characteristics exist. These include the West-African; the East, Central, Southern African (ECSA) phylogroups that have contributed to epidemics in Africa; and the Asian phylogroup. Phylogenic analysis based on partial sequences of NS4 and E1 genes showed that isolates from India during the present epidemic and the isolates from the ongoing Indian Ocean outbreak represent a distinct clade within the ECSA phylogroup[37,47,57,90], while all earlier isolates from India (1963 to 1973) were Asian phylogroup.[32,50,56,58,74] This finding corroborates that CHIK virus originated in Africa and subsequently was introduced into Asia. These different genotypes exhibit differences in their transmission cycles: in Asia, the virus appears to be maintained in an urban cycle with *Aedes aegypti* mosquito vectors, while CHIK virus transmission in Africa involves a sylvatic cycle, primarily with Aedes furcifer and Aedes africanus mosquitoes. Complete genomic sequence of CHIKV has been determined[20].

INACTIVATION BY PHYSICAL AND CHEMICAL AGENTS

Chikungunya virus is inactivated by 70% ethanol, 1% sodium hypochlorite, 2% glutaraldehyde, lipid solvents, moist or dry heat > 58° C, as well as drying.[87]

Mutations in the Chikungunya virus genome and infectivity

A mutation at residue 226 of the membrane fusion glycoprotein E1 (E1-A226V- substitution of alanine at position 226 with valine) was detected in more than 90% of Chikungunya virus isolates from Reunion Islands after September, 2005. By reducing the cholesterol dependence of the virus to infect mosquito hosts, this mutation is postulated to have facilitated the replication and transmission of the virus by this mosquito.[72,83] Because mosquitoes often do not have enough cholesterol for viruses to efficiently infect the host cells, the E1:A226V mutation might have helped in replication and transmission of the virus. This rapid pathogen adaptation to ecologic perturbation appears to be a unique example of the "evolutionary convergence" occurring in nature.[20]

PATHOGENESIS

In humans, CHIKV produces disease about 48 hours after mosquito bite. Patients have high viraemia during the first 2 days of illness. Viraemia declines around 3 or 4 days, usually disappearing by day 5.[53,74]

Haemagglutination inhibition (HI) and neutralizing antibodies can usually be detected after day 5 with fading viraemia.[53] Neuro-invasive cases and haemorrhagic manifestation related to CHIKV infection have been conclusively documented in scientific literature. "Silent" CHIKV infections do occur, but how commonly this happens is not yet known. CHIKV infection (whether clinical or silent) is thought to confer lifelong immunity.[87]

Clinical laboratory findings are not remarkable. Few patients may present with leucopoenia with relative lymphocytosis; however, most patients will have normal blood count. The platelet count may be moderately less. Erythrocyte sedimentation rate is significantly elevated and C-reactive protein is positive in acute-cases.[28,49]

Molecular pathogenesis

Little information is available on the molecular pathogenesis of Chikungunya fever. Given the similarities between the two, molecular pathogenesis of Chikungunya fever may be similar to the observations made on the molecular and cellular aspects of Ross River virus (RRV) that also causes epidemics of fever, polyarthritis (including rheumatic symptoms), and mucocutaneous manifestations similar to that seen with Chikungunya fever.[28] A defective cell-mediated immune response (CMI) where CD8+ T-

lymphocytes are absent or inactive has been thought to be the cause of chronic disease and viral persistence. Oversecretion of toxic chemokines or apoptosis are postulated as causes of cell/tissue destruction and associated clinical symptoms.[61] An antibody-dependent enhancement (ADE) mechanism similar to that suggested for dengue viruses[69] has also been implicated in the pathogenesis. Cell tropism of Chikungunya virus in the murine brain; susceptibility of human adherent cells (epithelial and endothelial cells, primary fibroblasts, and macrophages) to Chikungunya virus whose replication induces a cytopathic effect and apoptosis;[76,78] are all leads that need to be studied in greater detail. Presence of viral antigen in muscle progenitor cells (also called muscle satellite cells) in a muscle biopsy from a patient who had a clinical relapse some weeks after disease onset[64,76] suggests the possibility of persistence of Chikungunya virus in some patients long after initial viremia. Further research is required in this area to clarify these issues.

The vector

In Asia and the Indian Ocean region, Chikungunya fever is transmitted by the bite of mosquitoes of the genus Aedes (which also transmit the dengue virus). Aedes aegypti is considered to be the principal vector and Aedes albopictus (Asian Tiger mosquito) has also recently emerged as an important vector. A. aegypti predominantly breeds in stored

fresh water, such as desert coolers, flower vases, water-tanks, etc., and in peri-domestic areas (discarded household junk items like vehicular tyres, coconut shells, pots, cans, bins, etc.,) in urban and semiurban environments.[71,88] Adult mosquitoes rest in cool and shady areas and bite humans during the daytime. Insecticide treated bed nets are, therefore, of limited use against Aedes mosquitoes. The bite of only the female mosquito is considered to be infective because a blood meal is required for the formation of the egg.

The same vectors can sometimes transmit several arboviruses, and mixed epidemics of Chikungunya fever and dengue fever;[15,59] Chikungunya fever, dengue fever, and Plasmodium falciparum malaria[86] have sometimes been documented.

ANIMAL PATHOGENECITY AND CELL/ TISSUE TROPISMS

Pathogenicity studies of CHIKV infection could not be studied due to the lack of a proper animal model. There is a recent report that explains the development of a mouse model and that CHIK virus replicates first in the liver, and targets muscle, joints and skin, closely resembling the cell/tissue tropism observed in biopsy samples of CHIK infected humans. It also states the dissemination of virus to choroids plexuses and the leptomeninges in the CNS in severe infections.[18] There was also evidence for the presence of virus in the leg muscles even after the disappearance of viraemia, which

lasted for 6-7 days. Histopathologic studies revealed focal necrosis and inflammation in the skeletal muscle followed by fibrosis and distrophic calcification. Distrophic calcification was also observed in the joint cartilages.[19]

EPIDEMIOLOGY

Global scenario

Following the report from Tanganyika in 1952,[41,67] Chikungunya epidemics have been reported from several parts of the world including Africa, Asia, and elsewhere. In South-East Asia, epidemics have been documented in India, Pakistan, Sri Lanka, Myanmar, Thailand, Indonesia, the Philippines, Cambodia, Vietnam, Hong Kong, and Malaysia.[5,47,59,88] Since 2003, there have been outbreaks in the islands of the Pacific Ocean, including Madagascar, Comoros, Mayotte the Seychelles, and Mauritius, and the Reunion Island (French overseas district in the Indian Ocean).[5]

India

Since the first Indian report from Kolkata (Calcutta then),[74] several outbreaks of Chikungunya fever have been documented from different parts of India including Vellore,[30] Chennai (then called Madras) in Tamil Nadu, and Puducherry (then called Pondicherry),[82] Visakhapatnam, Rajahmundry, and Kakinada in Andhra Pradesh,[32,47,59,88,90] Nagpur,[68] and Barsi[56] in

Maharastra. Occasional cases were recorded in Maharastra State between 1983 and 2000.[50] Keeping with the character of the disease, Chikungunya fever has re- emerged in India after nearly 32 years in October 2005[47,59,88] and the outbreak is ongoing.

In the investigation carried out by the National Institute of Virology[90] from several districts in Andhra Pradesh, Karnataka, and Maharastra (n = 1938), the occurrence of Chikungunya epidemic was confirmed. During the current outbreak as per the current release by the Ministry of Health and Family Welfare, Government of India, 13,91,165 cases suspected to be Chikungunya fever have been recorded from several parts of India during the year 2006.[47,88] The corresponding figures for the subsequent years have been 59,535 cases (2007) and 71,222 (till November 2008),[47,88] and 13,117 (till June 2009).[21] Some workers have approximated that these figures are underestimates and the actual figures could be up to five times these numbers.[45]

Clinical Manifestations

Acute stage

Chikungunya fever affects all age groups, and both genders are equally affected. The incubation period ranges from 3 to 12 days (usually 3-7 days). In susceptible populations, the attack rates can be as high as 40-85%[17, 47,59,61,76,79]

Onset

Prodromal symptoms are rare. In the acute stage, the onset is usually abrupt and sudden with high-grade fever (usually 102-105 °F), severe arthralgias, myalgias, and skin rash.[17,47,59,61,76,79] Headache, throat discomfort, abdominal pain, and constipation may also be evident. Conjunctival congestion, persistent conjunctivitis, cervical, or sometimes generalized lymphadenopathy may be present.

Mucocutanoeus Manifestations

Several mucocutaneous manifestations, such as morbilliform eruption, scaling, macular erythema, intertrigo, hypermelanosis, xerosis, excoriated papules, urticaria, and petechial spots have been described in patients with Chikungunya fever.[3,29]

Arthropathy

The Chikungunya viral polyarthropathy frequently involves the small joints of the hand, wrist, and ankles and the larger joints such as knee and shoulder; more than ten joint groups may be involved.[17,59,61,75,76,79] Axial involvement is common. The joints are swollen and the involvement can be asymmetric sometimes; disabling acute tenosynovitis is frequently present.[17,59,61,79] Sometimes, atypical features such as Baker's cyst may be present. Sometimes sternoclavicular and temporomandibular joints may be

involved; hips are relatively spared.[8,75,76] Swollen tender joints and crippling arthritis are usually evident in almost all the patients at presentation. The pain may be severe enough to immobilize the patient and interfere with sleeping in the night. Joint pain may worsen with movement and back ache may also be present. Radiological findings are usually normal, and biological markers of inflammation may be normal or moderately elevated.[24,34]

Effect on pregnancy

Chikungunya fever appears to have a direct impact on pregnancy with a higher risk of abortion in the first trimester and mother-to-child transmission in the last trimester.[17,59,61,76,79] In a study from the Reunion Islands outbreak, three out of nine miscarriages before 22 weeks of gestation were attributed to the Chikungunya virus infection documented by positive reverse transcription polymerase chain reaction (RT-PCR) in amniotic fluid.[38]

Others

Several neurological, ocular, and hemorrhagic manifestations of fulminant hepatitis have been documented during the recent epidemic.[22] Other uncommon manifestations include myocarditis after acute febrile

illness,[54,55] mixed cryoglobulinemia in the first months after disease onset, and long persistence of symptoms.[76]

Course of the Disease

The fever is usually of short duration and usually resolves in 3 to 4 days. In some patients, a biphasic pattern of fever has been described with a febrile episode of 4 to 6 days, followed by a fever-free period of a few days followed by recurrence of fever (usually 101-102 °F) that may last a few days.[76]

Chronic stage

In a majority of the patients, the joint pains resolve in 1 to 3 weeks. However, the arthritis can persist in about 33% of patients for 4 months, 15% for 20 months, and in 12% for 3-5 years.[12,24,34,59] The chronic stage is characterized by unpredictable relapses that include sensation of fever, asthenia, and exacerbation of arthralgias and stiffness. Affected patients may manifest inflammatory polyarthritis, severe subacute tenosynovitis/bursitis (consequently nerve tunnel syndromes) in hands, wrists, and exacerbation of pain on movement in previously injured joints.[75,76] Older individuals and those with underlying rheumatic and traumatic joint disorders seem to be more vulnerable to develop the chronic stage.[17,59,61,76,79] Rarely, rheumatic manifestations resulting in joint destruction before resolution after 15 years

have been reported.[13] Some studies have documented occurrence of rheumatoid arthritis following Chikungunya fever, suggesting that the viral infection may have a role in the initiation or unmasking of rheumatoid arthritis.[10]

Chikungunya Fever in Neonates and Children

Neonates

Mothers afflicted with Chikungunya fever in the perinatal period (−4 days to +1 days) can transmit Chikungunya fever to neonates by vertical transmission.[73] Intrapartum transmission also contributes; caesarean section does not appear to prevent the transmission.[26,73,76] Neonatal Chikungunya fever is associated with fever, poor feeding, pain, distal edema, various skin manifestations, seizures, meningoencephalitis, and echocardiographic abnormalities in the newborn.[73,76]

Children

Chikungunya fever in children resembles that observed in adults with important differences.[73] Common clinical manifestations include abrupt onset of high-grade fever, skin rashes, minor hemorrhagic manifestations, arthralgia/arthritis, lymphadenopathy, conjunctival congestion, swelling of eyelids, and pharyngitis. Rare clinical features include neurological manifestations including seizures, altered level of consciousness, blindness

due to retrobulbar neuritis, and acute flaccid paralysis. Rheumatological manifestations are somewhat less frequent in children. Pediatric subjects may also experience febrile seizures, vomiting, abdominal pain, and constipation.[40,73]

Cardinal Features Observed in the Current Epidemic in India

Majority of the patients had presented with fever, arthralgias, arthritis, and mucocutaneous manifestations. In addition to these series[2,33,36,40,47,80], several rare manifestations such as neurological, ocular and haemorrhagic manifestations have also been documented in other publications from India.

Hemorrhagic manifestations

Unlike dengue fever, hemorhhagic manifestations are uncommon in Chikungunya fever. When present, they are mild and more frequently encountered in Asian compared with African patients.[17,59,61,76,79] When present, these manifestations include epistaxis, bleeding from the gums, positive Hess test, subconjunctival bleed, and petechial/purpuric rash.

Others

Sudden sensorineural hearing loss[6] and hypokalemic periodic paralysis[63] are other rare manifestations that have been reported. Severe systemic disease with multiple organ system involvement has also been observed during the recent epidemic.[81]

Economic Impact

In India, the national burden of Chikungunya was estimated to be 25,588 disability adjusted life years (DALYs) lost during 2006 epidemic, with an overall burden of 45.26 DALYs per million (range 0.01 to 265.62 per million in different states). Persistent arthralgia was found to account for 69% of the total DALYs. The productivity loss in terms of income foregone was estimated to be a minimum of Rs. 391 million. The actual loss is thought to be substantially higher than these estimates.[65]

Differential Diagnosis

Chikungunya fever has to be distinguished from the dengue fever which has the potential for much worse outcomes including death. The two diseases can often occur together in the same patient. Observations from previous outbreaks in Thailand and India, have characterized the principal features distinguishing chikungunya from dengue fever. In the former, shock or severe haemorrhage is not observed. The onset is more acute and the duration of fever is much shorter in chikungunya fever. In chikungunya fever, maculopapular rash is more frequent than in dengue fever. In chikungunya fever leucopenia is more frequent than dengue fever. In dengue fever thrombocytopenia is more frequent than chikungunya fever. In the early stage when rash is absent, malaria has to be ruled out. With rash, measles or German measles need to be ruled out. Differential diagnosis with

other arthropod borne viruses of the Alphavirus genus (Ross River, Barmah Forest, o'nyong nyong, Sindbis, and Mayaro viruses) is difficult, but these are comparatively rare.[87]

Laboratory Diagnosis of Chikungunya Fever:

As the clinical manifestations of chikungunya fever resemble those of dengue and other fevers caused by arthropod borne viruses of the *Alphavirus* genus, laboratory confirmation is critical to establish the diagnosis.

Types of Laboratory tests available and specimens required:

Three main laboratory tests are used for diagnosing chikungunya fever:

1. virus isolation, 2. serological test and 3. molecular technique of Polymerase Chain Reaction (PCR).

Specimen is usually blood or serum but in neurological cases with meningo-encephalitic feature, CSF (cerebro-spinal fluid) may also be sent.

Virus isolation

Virus isolation is the most definitive test. Between 2-5 ml of whole blood is collected during the first week of illness in a heparinized tube and transported on ice to the laboratory. The isolation of CHIKV is comparatively more simple and effective owing to the highly cytopathic and fast-growing nature of the virus, which will grow to very high titers.[74] CHIKV replicates in various cell lines, including insect cells, for example,

C6/36 and other non human cell lines such as , Vero, chick embryo fibroblast-like cells, BHK21, L929 and human cell lines, for example, HeLa, MRC5 and Hep-2 cells in which it will often induce a significant cytopathic effect. Human epithelial and endothelial cells, primary fibroblasts and, to a lesser extent, monocyte-derived macrophages, are susceptible to infection and permit viral production. By contrast, CHIKV does not replicate in lymphoid and monocytoid cell lines, primary lymphocytes and monocytes, or monocyte-derived dendritic cells.[28] However, the isolation process is time-consuming and the degree of success is dependent on a number of complicating factors, for example, time of collection, transportation, maintenance of cold chain, storage and processing of samples.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.

RT-PCR.

A specimen for PCR is heparinized whole blood. 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 E-2 and part of Envelope E1 . PCR results can be available in 1-2 days. RT-PCR is also a confirmatory test for isolated virus.[14]

Serological diagnosis

For serological diagnosis, serum obtained from 10-15 ml of whole blood is required. An acute phase serum must be collected immediately after the onset of illness and the convalescent phase serum 10-14 days later. The blood specimen is transported at 4 degrees Celsius and not frozen for immediate transfer to the laboratory. Only if the testing cannot be done immediately, the serum specimen should be separated and then stored and shipped frozen. Serologic diagnosis can be made by demonstration of four-fold rise in antibody titre in acute and convalescent sera or by 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. The later viruses are relatively rare in South East Asia and if further confirmation is required by ruling these viruses out, it can be done by neutralization tests and Hemagglutination Inhibition Assay (HIA). Haemagglutination inhibition antibodies appear with the cessation of viraemia. All patients will be positive by day 5-7 of illness. Neutralization antibodies parallel HI antibody.[48,61] The CHIK antigen for HI Test is available from NIV, Pune.

Interpretation of results:

Sero-diagnosis rests on demonstrating a four-fold increase in CHIK IgG titre 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. A positive virus culture supplemented with neutralization is taken as the definitive proof for the presence of chikungunya virus. Positive PCR result for E1 and C genome either singly or together from the specimen (serum, cerebro-spinal fluid, etc) also constitutes a positive evidence of chikungunya virus infection.[52]

MANAGEMENT

Treatment of Chikungunya fever is nonsteroidal symptomatic and supportive. Adequate fluid intake must be ensured. Paracetamol or anti-inflammatory drugs (NSAIDS) may be used for symptom relief. Aspirin should be avoided due to its effect on platelets. In a recently conducted double-blind, placebo-controlled, randomized trial[19] assessing the efficacy of chloroquine in patients with acute Chikungunya fever, the authors[19] suggested that there was no justification for the use of chloroquine to treat acute Chikungunya infection.

Published evidence does not support the use of corticosteroids, antibiotics, or antiviral drugs in the management of Chikungunya fever, and

indiscriminate use of these agents can be hazardous. Electrolyte imbalance, pre-renal acute renal failure and bleeding manifestations should be watched carefully and managed accordingly.[47] The optimal treatment strategy for viral arthropathy of Chikungunya fever is not known. Further randomized controlled studies with a large sample size are required to clarify the role of chloroquine/ hydroxychloroquine in the treatment of Chikungunya arthropathy.

Mortality

Observations from the earlier epidemics including the experience from India till mid-1970s indicate that Chikungunya fever is a self-limiting disease; mortality was not documented. The official figures in the current release[21] and earlier releases by the Ministry of Health and Family Welfare, Government of India,[21,47,49] also do not document mortality due to Chikungunya. However, there have been reports of mortality due to Chikungunya fever during the present epidemic in some publications.[1,44,45,85] Mavalankar et al.[44] compared the mortality rates in 2006 with those in 2002-2005 for Ahmedabad and found that 2,944 excess deaths occurred during the Chikungunya epidemic (August-November 2006) when compared with the average number of deaths in the same months during the preceding 4 years. These authors[44] suggested that the excess deaths were attributable to this epidemic. Indiscriminate use of

corticosteroids, NSAIDS (especially aspirin), and antibiotics which contribute to thrombocytopenia, gastrointestinal bleeding, nausea, vomiting, and gastritis. This may lead to dehydration, pre-renal acute renal failure, dyselectrolytemia, and sometimes hypoglycemia. These can indirectly contribute to the mortality due to Chikungunya fever.[47]

Prevention

Patients with Chikungunya fever should be advised to avoid being bitten by mosquitoes as the disease can be transmitted to others. Personal protection measures such as applying insect repellents to the exposed skin can keep out A.aegypti. Insect repellents containing 30% N,N- Diethyl-m - Tolumide (DEET) have shown to provide an average of five hours of complete protection against A.aegypti after a single application on a exposed skin. However,in field conditions, perspiration, rain and raising temperature may make DEET less effective.[19]

VACCINES

Presently no commercial vaccine is available for Chikungunya fever in India, although some candidate vaccines are being tested in human beings. The widespread geographic distribution and recurrent epidemics causing severe morbidity have necessitated the need for an efficient vaccine. Levitt *et al* (1986) reviewed the developments in vaccine development taken place

through sixties and early 1970s.[39] Among the many preparations, the cell culture based formalin inactivated vaccine developed by Harrison *et al* (1971) was the most promising as it elicited high levels of neutralizing antibody in human volunteers in a phase 1 trial. The study also demonstrated excellent immunogenic response without showing any side effects or untoward reactions in the vaccinees making it an ideal candidate for future. Edelman *et al* (2000) pursued the work and developed an attenuated vaccine by serial passaging in MRC-5 cell line. The vaccine was highly immunogenic and well within the tolerable limits.[23,39] In the phase II safety and immunogenicity study the investigators observed sero-conversion in 98% of the vaccinees by day 28 and the presence of N-antibodies in 85% volunteers for 12 months. Attempts are being made to revive the project by the US Army Medical Research Institute for Infectious Diseases on a request from the French government after the 2005-06 epidemic.[61]

Vector surveillance and control

Vector surveillance and control is a key element in containing Chikungunya fever epidemics. This can be done by active involvement of community and public health authorities with regard to hygiene and mosquito control measures is essential to stand a chance in the war against the mosquitoes.[76]

Future Directions

Chikungunya fever is emerging as a global disease. Several issues related to Chikungunya fever, such as, the reason(s) for the mysterious behavior of dramatic outbreaks interspersed by periods of prolonged absence; virus survival in nature and factors triggering outbreaks; reasons for ECSA strain replacing the Asian strain during the current epidemic, among others, need to be further studied. The quest for an effective vaccine is still on.[87]

MATERIALS AND METHODS

PLACE OF STUDY

The study was conducted at the Institute of Microbiology, Madras Medical College and Government General Hospital, Chennai in association with the fever outpatient clinic. Samples collected were processed at the Institute of Microbiology, Chennai and molecular diagnostic study was done at the Department of Virology, King Institute of Preventive Medicine, Chennai.

STUDY PERIOD

The cross sectional study was conducted from October 2009 to September 2010.

SAMPLE

Blood samples from 150 patients with clinical features suggestive of Chikungunya fever were included in this study. The samples were collected aseptically and serum was separated and stored at -70^{0}C.

INCLUSION CRITERIA

The following patients were included in this study.

- Patients who had sudden onset of fever, rash, joint pain, chills, headache, nausea, vomiting, low back pain, conjunctival congestion and abdominal pain.
- All age groups .
- Both males and females.

EXCLUSION CRITERIA

Patients with known history of chronic osteoarthritis, autoimmune diseases like rheumatoid arthritis, Systemic Lupus Erythematoses and any history of previous surgery of the joints were excluded.

CASE DEFINITION

Though case diagnosis can only be made by laboratory means,

- Chikungunya should be suspected when epidemic occurs with the characteristic triad of fever, rash and joint manifestations.
- The chikungunya case definition here is adapted from that proposed by the European Centre for Disease Control (ECDC).

Clinical criteria:

- Acute onset of fever >38.5°C.
- Severe arthralgia/arthritis not explained by other medical conditions.

Epidemiological criteria:

- Residing or having visited epidemic areas,
- Having reported transmission within 15 days prior to the onset of symptoms

Laboratory criteria:

At least one of the following tests in the acute phase:

- Virus isolation.
- Presence of viral RNA by RT-PCR.
- Presence of virus specific IgM antibodies in single serum sample collected in acute or convalescent stage.
- Four-fold increase in IgG titres in samples collected at least three weeks apart.

On this basis, cases are to be categorized as

Possible case: A patient meeting the clinical criteria

Probable case: A patient meeting both the clinical and epidemiological criteria

Confirmed case: A patient meeting the laboratory criteria, irrespective of the clinical presentation.

SOURCE OF SAMPLE

The samples were collected from fever clinic and from in- patients with features suggestive of chikungunya fever at Madras Medical College and General Hospital, Chennai - 3.

ETHICAL CONSIDERATIONS

Written consent to participate in the study was obtained from the subjects or their guardians after the full explanation of the study was provided to them. The study was reviewed and approved by Institutional Ethical Committee, Madras Medical College and General Hospital, Chennai-3. All data were handled confidentially and anonymously.

STATISTICAL ANALYSIS

The proportional data of this cross-sectional study was tested using Pearson′s Chi-Square (X^2) analysis test, Two sample binomial proportion test, Stastical analyses were carried out using Statistical Package for Social Sciences (SPSS) and Epi-Info softwares.

METHODS

The samples were subjected to IgM capture ELISA for chikungunya , Macroscopic Slide Agglutination Test for Leptospirosis, Standard Diagnostics(SD) Rapid immunochromatographic Duo Cassette method for

Dengue at Institute of Microbiology, Madras Medical College and General Hospital, Chennai. Reverse Transcriptase PCR (RT-PCR) for Chikungunya was done for 40 samples at Department of Virology at King Institute of Preventive Medicine, Guindy, Chennai.

COLLECTION, STORAGE AND TRANSPORTATION OF SAMPLES:

Proper collection, processing, storage and transportation of the specimens are an essential aspect of the laboratory diagnosis.

Collection of samples for isolation & molecular diagnosis

Sample: Serum, or plasma or whole blood (in heparinized tube).

Time of collection: Within first five days of illness

To collect serum:

- Aseptically collect 4-5 ml of venous blood in a tube or a vial.
- Allow blood to clot at room temperature, centrifuge at 2000 rpm to separate serum. Collect the serum in a clean dry vial.
- Use adhesive tape marked with pencil, indelible ink, or a typewritten self adhesive label to identify the container. The name of the patient, identification number and date of collection must be indicated on the label.

- All clinical samples should accompany the clinical information as per the proforma.

Sample: Blood in plain vial / serum

Time of collection:

1st sample: 5 days after the onset of symptoms for IgM detection as these Antibodies appear at this time.

2nd sample: At least 7 to 14 days after the first sample or, in the event of fatality, at the time of death.

Other types of specimen for laboratory investigation:

Specimens: CSF in meningo-encephalitis cases.

Synovial fluid in arthritis with effusion.

Autopsy tissues - liver, spleen, lymph nodes and thymus.

Mosquitoes collected in nature.

Transportation of samples

- Transport specimens to the laboratory at 2 –8 ^{0} C (ice box) as soon as possible.

- Do not freeze whole blood, as haemolysis may interfere with serology test results.

- If more than 24-hour delay is expected before specimens can be submitted to the laboratory, the serum should be separated and stored at refrigerated temperature.
- Samples for virus isolation and molecular diagnosis should always be stored and transported frozen.

CHIKUNGUNYA ANTIBODY ELISA

REQUIREMENTS

1. Anti-Human IgM coated microwells(Assay plates)
2. Chikungunya antigen.
3. Biotinylated Anti-Chikungunya Monoclonal Antibody: (CHIK-B)
4. Positive Controls and Negative controls
5. Avidin-HRP(Horse- Radish Peroxidase)
6. Wash Buffer Concentrate(10X)
7. Sample Dilution Buffer
8. Tetramethyl benzidine (TMB) 3,3′, 5,5′- the substrate, tetramethyl benzidine, hydrogen peroxide in a citric-acid citrate buffer (pH 3.5-3.8)
9. 1N $H_2 SO_4$ –Stop solution.

Chikungunya-IgM Capture ELISA

Principle of the test

IgM antibodies in the patient′s blood are captured by Anti-human IgM that are coated on to the solid surface (wells). In the next step, CHIK antigen is added which binds to capture IgM, if the IgM and antigen are homologus. Unbound antigen is removed during the washing step. In the subsequent step Biotinylated Anti- Chikungunya Monoclonal Antibody (CHIK-B) is added followed by Avidin-HRP. Subsequently, substrate /chromogen is added and watched for the development of colour. The reaction is stopped by 1N H_2SO_4. The intensity of the colur /optical density is monitored at 450nm. OD readings are directly proportional to the amount of Chikungunya virus specific IgM antibodies in the sample.

Procedure

Serum predilution

1. The PC, NC and CO and patient samples were diluted using suitable test tubes or microtitre plate.

2. 990 µl of serum diluents wass added to 10µl of serum and mixed well to get 1:100 dilution.

Wash Buffer Concentrate

120ml of wash buffer concentrate was provided with each kit. Before use, wash buffer concentrate was diluted to get 1:10 dilution by adding 9 parts of distilled water to 1 part of concentrate. For assay of eight samples 100 ml of diluted wash buffer was needed.

ELISA PROCEDURE

1. The sample was diluted 1:100 with sample dilution buffer.
2. The coated microtitre wells were washed thrice with wash buffer.
3. 50µl of diluted samples were transferred to the appropriate wells.
4. 50 µl of Positive Control and Negative Control were transferred to the respective wells.
5. The plate was kept in a humidified box (A bread box with a soaked cotton or tissue paper) and incubated the plate at 37^0 C for one hour.
6. At the end of incubation, the plate was washed for five times with wash buffer. The plate was tapped after the last wash on a tissue paper.
7. 50 µl of CHIK antigen was added to each well and incubated the plate at 37^0 C for one hour and the plate was washed for five times with wash buffer.

8. 50 µl of CHIK-B was added to each well and incubated the plate at 37^0 C for one hour and the plate was washed for five times with wash buffer.
9. 50 µl of Avidin-HRP was added to each well and incubated the plate at 37^0C for half an hour and the plate was washed for five times.
10. 100 µl of substrate (TMB/H_2O_2) was added to each well. The plate was incubated in dark at room temperature for 10 minutes. Observed for the development of colour. The reaction was stopped with 100 µl of1N H_2SO_4.
11. The absorbance was measured at 450 nm within 10 minutes on a spectrophotometer.

INTERPRETATION OF THE RESULTS

The test was considered to be valid if

Positive OD values $\geq$ 0.5

Negative OD value $\leq$ 0.18

The sample was considered as positive, if OD value of the sample tested exceeds OD value of Negative control by a factor 2.1, (Sample OD $\geq$ Negative OD x2.1)

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

PRINCIPLE

Polymerase chain reaction (PCR) allows the exponential copying of part of a DNA molecule using a DNA polymerase enzyme that is tolerant to elevated temperatures.

1. mRNA is copied to cDNA by reverse transcriptase using an oligo dT primer (random oligomers may also be used. A PCR mix is then set up which includes a heat-stable polymerase (such as Taq polymerase), specific primers for the gene of interest, deoxynucleotides and a suitable buffer.
2. cDNA is denatured at more than 90 degrees (~94 degrees) so that the two strands separate. The sample is cooled to 50 to 60 degrees and specific primers are annealed that are complementary to a site on each strand. The primers sites may be up to 600 bases apart but are often about 100 bases apart, especially when real-time PCR is used.
3. The temperature is raised to 72 degrees and the heat-stable Taq DNA polymerase extends the DNA from the primers. Four cDNA strands (from the original two) is obtained. These are denatured again at approximately 94 degrees.

4. Again, the primers are annealed at a suitable temperature (somewhere between 50 and 60 degrees).
5. Taq DNA polymerase binds and extends from the primer to the end of the cDNA strand. There are now eight cDNA strands.
6. Again, the strands are denatured by raising the temperature to 94 degrees and then the primers are annealed at 60 degrees.
7. The temperature is raised and the polymerase copies the eight strands to sixteen strands.
8. The strands are denatured and primers are annealed.
9. The fourth cycle results in 32 strands.
10. Another round doubles the number of single stands to 64. Of the 32 double stranded cDNA molecules at this stage, 75% are the same size, that is the size of the distance between the two primers. The number of cDNA molecules of this size doubles at each round of synthesis (exponentially) while the strands of larger size only increase arithmetically and are soon a small proportion of the total number of molecules.

After 30 to 40 rounds of synthesis of cDNA, the reaction products are usually analyzed by agarose gel electrophoresis. The gel is stained with ethidium bromide.

PROCEDURE

Selected samples, subjected to RT PCR as well as isolation in C6/36 cell line and the results evaluated. Standard RT-PCR using primers targeting E2 gene of ECSA phylogroup was performed.

Primer Table:

Primer details:	**Genomic Position**
Forward Primers: TATCCTGACCACCCAACGCTCC	9403-9424
Reverse primers: ACATGCACATCCCACCTGCC	9693- 912

RNA was extracted using QIA amp Viral RNA mini kit according to manufacturer's instruction (Qiagen, UK). A one step RT- PCR test was done using Superscript III one step RT-PCR platinum - Taq kit (Invitrogen, UK). A 25 µl volume reaction was used containing 12.5 µl 2X reaction mix, 10 µl water, 0.2 µM (primer Table) primers, 0.5 µl RT/Taq and 1 µl extracted RNA. The thermo cycling conditions were 50°c for 30 min, 94°c for 2 min, 40 cycles of 94°C 15 seconds, 55°c for 30 seconds and 68°C for 2 min and 20 seconds with a final extension at 68°C for 5 minutes and final cooling step 4°C for 30 seconds.

The Amplication product was 305 bp within gene that codes for the viral envelope protein E2.

MACROSCOPIC SLIDE AGGLUTINATION TEST (MSAT)

PRINCIPLE:

MSAT is a slide agglutination test which uses formalinised and heat killed antigen from leptospires(L.) belonging to different serovars commonly prevalent in around Chennai. The different serovars used were L.ictero haemorrhagiae,

L.australis , L.autumnalis, L.griphotyphosa, L.hebdomadis, L.pomona, L.patoc, L.javanica and L.sejroe.

PROCEDURE

One drop of (5µl) antigenic suspension was mixed with equal amount of serum both neat and diluted; on a depression slide and rotated on a slide rotator at 180 rpm for 4 minutes. It was examined macroscopically, for the presence of agglutination. Positive and negative controls were included.

INTERPRETATION OF RESULTS

1	Clumps of agglutination with complete clearing of leptospiral antigen suspension	4+
2	Obvious agglutination but partial clearing of suspension	3+
3	50% agglutination	2+
4	25% agglutination	1+
5	No agglutination and uniformity of serum antigen mixture	Negative

A 4+ agglutination titre was indicative of recent infection and considered significant.

1. RAPID DENGUE DUO IMMUNOCHROMATOGRAPHIC CASSETTE METHOD

The Standard cassette contains a square well for addition of buffer solution, a circular well for serum sample and a lateral flow membrane with colloidal gold complexes containing recombinant dengue 1-4 antigens and a control.

Principle of the test

If dengue specific IgM & IgG antibodies are present in the patients sample, they bind to Anti-human IgM or IgG antibodies immobilized in two

lines across the cassette membrane. Colloidal gold complexes containing recombinant dengue 1-4 antigens are captured by the bound patient′s IgM or IgG to give visible pink lines. A control is included to indicate that the assay has been performed correctly.

Serological sensitivity of the test - 96.3%

Serological specificity of the test - 95%

Procedure

1. 10µl of whole blood, serum or plasma was added to the circular well in the cassette using a micropipette.
2. The sample was allowed to absorb entirely into the specimen pad in the circular well.
3. The buffer (Phosphate buffer saline) bottle was held vertically and 1cm above the square well, adjacent to the circular well in the cassette and 2 drops of buffer was added.
4. The result was read exactly 15 min after adding the buffer to the cassette.
5. Any trace of a pink line in the test area indicated a positive result.
6. Any results read after 15 min considered invalid and repeated.

Interpretation of results

Interpretation should be based on the combined results of the IgG and IgM test lines.

C - Control line
M - IgM test line
G - IgG test line

Primary infection

- Pink bands in the IgM & control regions
- The test was positive for IgM antibodies and is suggestive of primary dengue infection.

Secondary infection

1. Pink bands in IgM, IgG and control regions.

   The test was positive for IgM & IgG antibodies and was suggestive of secondary dengue infection.

2. Pink band in IgG and control regions.

   The test was positive for IgG antibodies and was suggestive of secondary dengue infection.

Negative

• A pink band in the control region only.
• No detectable IgG or IgM antibodies to dengue.

Invalid

• No pink band in control region.

RESULTS

Total number of samples tested: 150

- Chikungunya – IgM capture ELISA, SD rapid immunochromatographic cassette method for Dengue, MSAT for Leptospira were done for the samples at Institute of Microbiology, Madras Medical College & Government General Hospital, Chennai.
- Reverse Transcriptase PCR (RT-PCR) was done for 40 samples at Institute of Virology, King Institute of Preventive Medicine, Chennai.
- Clinical data was collected for all the patients.

TABLE - 1 INCIDENCE RATE OF CHIKUNGUNYA

Total number of fever cases	Suspected number of cases	Chikungunya positive cases	Incidence
12477	150	62	0.49%

Incidence rate = 62 /12477×100= 0.49%

(0.38 % to 0.60 %) with 95% confidence interval

Burden of the disease = 1 out of 102 patients

TABLE - 2 SEROPOSITIVITY OF CHIKUNGUNYA

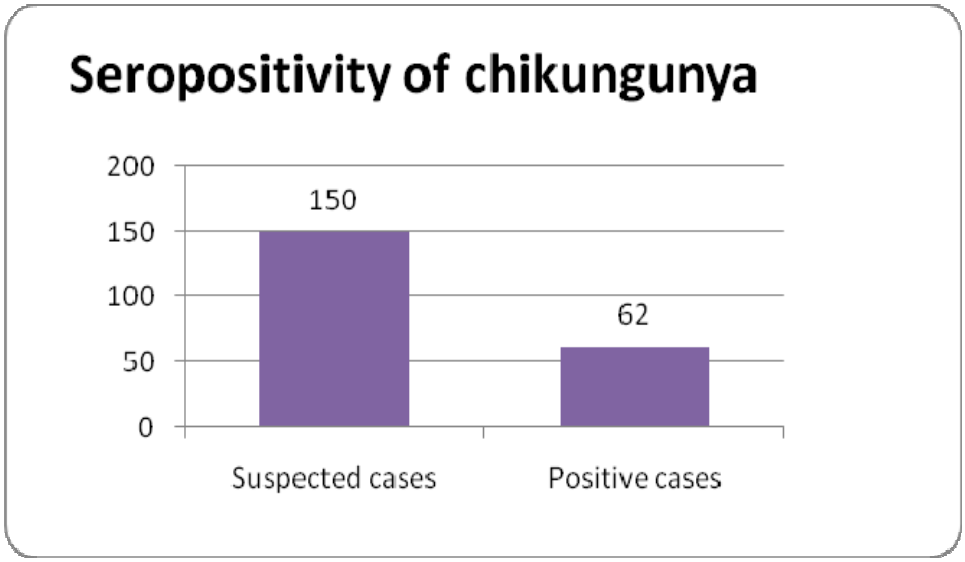
Total number of suspected cases	Total number of positive cases	Percentage
150	62	41.33%

Seropositivity = 41.33% (Range =35-48%) with 95% confidence interval

TABLE - 3 AGE DISTRIBUTION OF CHIKUNGUNYA CASES (n=62)

Age group	Total cases	Positive cases	Percentage
0-20 years	30	7	23.3
21-40 years	70	34	48.5
41-60 years	47	20	42.5
61-80years	3	1	33.3

Age group 21-40 years was commonly involved in both sexes.


Seropositivity of chikungunya
200
150
100
50
0
150
62
Suspected cases
Positive cases

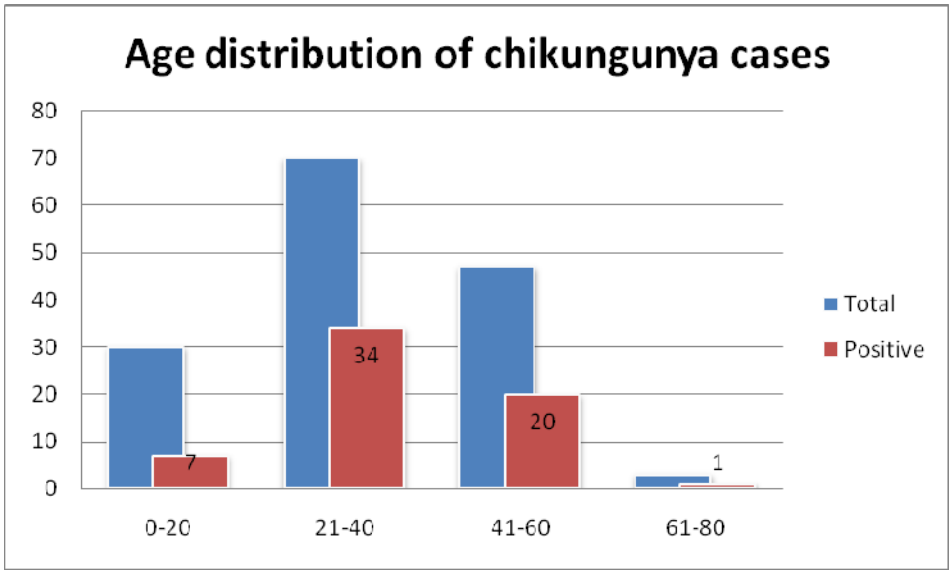

Age distribution of chikungunya cases
80
70
60
50
40
30
20
10
0
7
34
20
1
0-20
21-40
41-60
61-80
Total
Positive

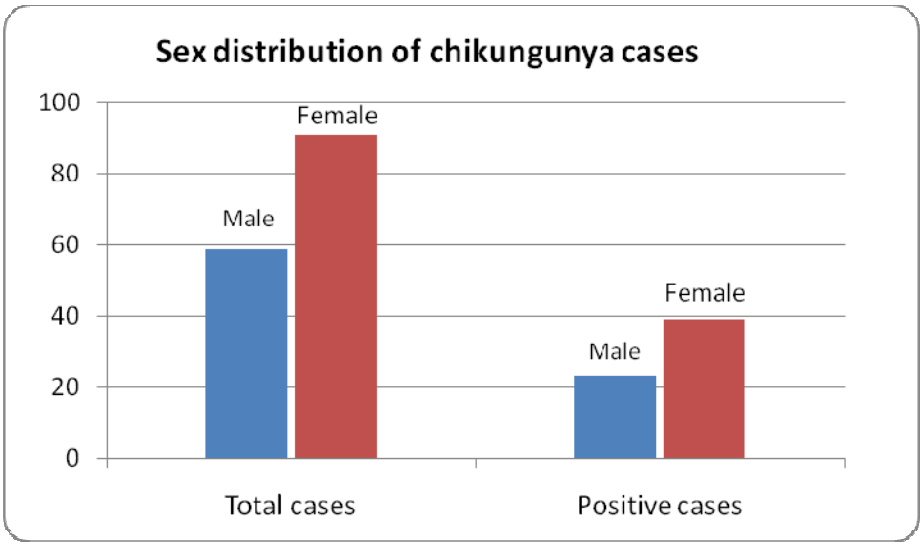
TABLE - 4 SEX DISTRIBUTION OF CHIKUNGUNYA CASES

Sex	Total cases	Positive cases	Percentage
Males	59	23	37.1
Females	91	39	62.9
Total	150	62	100.0

TABLE - 5 PRESENTING CLINICAL FEATURES (n=62)

Sl.no	Clinical features	Number of patients	Percentage of patients
1	Fever	60	96.7
2	Arthralgia	62	100
3	Joint swelling	47	75.8
4	Rash	37	59.7
5	Itching	33	53.2
6	Conjunctival congestion	21	33.9
7	Cough	5	8
8	Vomiting	3	4.8

Arthralgia was a presenting feature in all the cases. Other than Arthralgia, fever and joint swelling were the other predominant symptoms followed by rash.


Sex distribution of chikungunya cases
100
80
60
40
20
0
Male
Female
Male
Female
Total cases
Positive cases

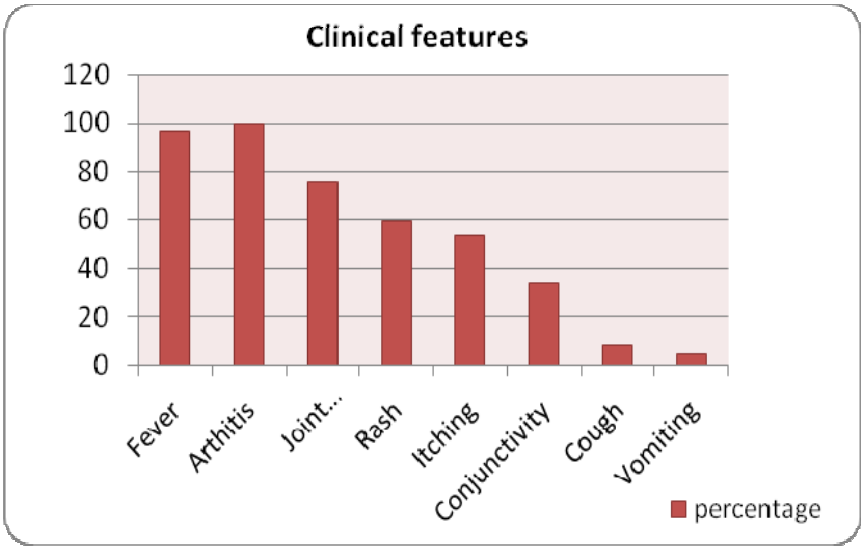

Clinical features
120
100
80
60
40
20
0
Fever
Arthitis
Joint...
Rash
Itching
Conjunctivity
Cough
Vomiting
percentage

TABLE - 6 PLATELET COUNTS IN CHIKUNGUNYA CASES (n=62)

Platelet count (lakhs/cu.mm)	Total number of cases	Percentage
50,000-1,00,000	5	8
1,00,000-1,50,000	27	43.5
>1,50,000	30	48.3

92.0 % of the patients had a platelet count of more than one lakh/ cu.mm.

TABLE - 7 DISTRICTWISE DISTRIBUTION OF CHIKUNGUNYA CASES (n=62)

Sl.no	Districts	Number of patients	Percentage of patients
1	Chennai	43	69.3
2	Tiruvallur	5	8
3	Kancheepuram	4	6.4
4	Vellore	10	16.1

69.3% of the chikungunya cases were from Chennai.

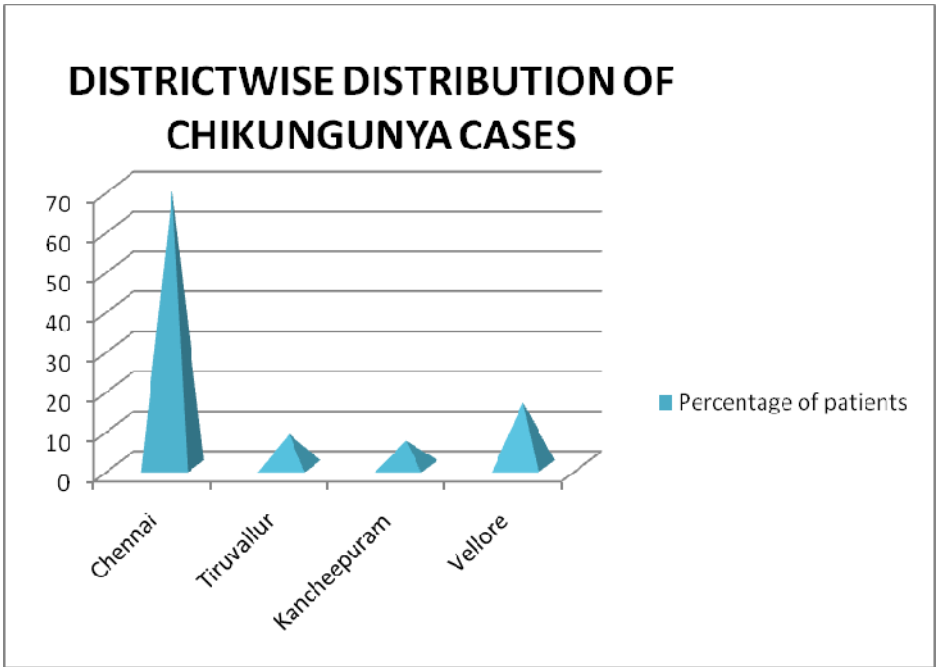

DISTRICTWISE DISTRIBUTION OF CHIKUNGUNYA CASES
70
60
50
40
30
20
10
0
Chennai
Tiruvallur
Kancheepuram
Vellore
Percentage of patients

TABLE - 8 ANTIBODY RESULTS IN EARLY AND LATE FEBRILE PERIOD (n=62)

Sl.no	Duration of fever	IgM ELISA positive cases	Percentage
1	1 -5 days	8	12.9
2	6-10 days	39	62.9
3	More than 10 days	15	24.2

IgM positivity was more in the early febrile period between 6-10days.

Chi square test =25.66 $p < 0.0001$(Significant)

TABLE - 9 SEASONAL DISTRIBUTION OF CHIKUNGUNYA CASES (n=62)

Month / year	Total number of suspected cases	Total number of positive cases	Percentage of patients
October 2009	22	9	14.5
November 2009	25	13	20.9
December 2009	28	16	25.8
January 2010	23	12	19.3
February 2010	13	5	8.0
March 2010	5	1	1.6
April 2010	7	1	1.6
May 2010	5	1	1.6
June 2010	4	--	--
July 2010	6	2	3.2
August 2010	4	--	--
September 2010	8	2	3.2

The incidence of chikungunya is increased during November to January , that is during monsoon and post monsoon period.

TABLE - 10 DISTRIBUTION OF CHIKUNGUNYA CASES WITH COINFECTION (n=62)

	DENGUE IgM positive	Lepto MSAT positive	Total
Chikungunya IgM positive	5	6	11

Dengue co-infection and Leptospiral co-infections were identified.

TABLE - 11 DENGUE SERO POSITIVITY

Total no of samples	DENGUE IGM positive	Percentage
150	5	8

Dengue co-infection with chikungunya was 8%.

TABLE - 12 LEPTO SERO POSITIVITY

Total no of samples	LEPTO MSAT positives	Percentage
150	6	9

Leptospiral co-infection with chikungunya was 9%.

TABLE - 13 RESULTS OF RT-PCR SAMPLES

Total no of samples	RT-PCR negatives	RT-PCR positives	Percentage
40	21	19	47.5

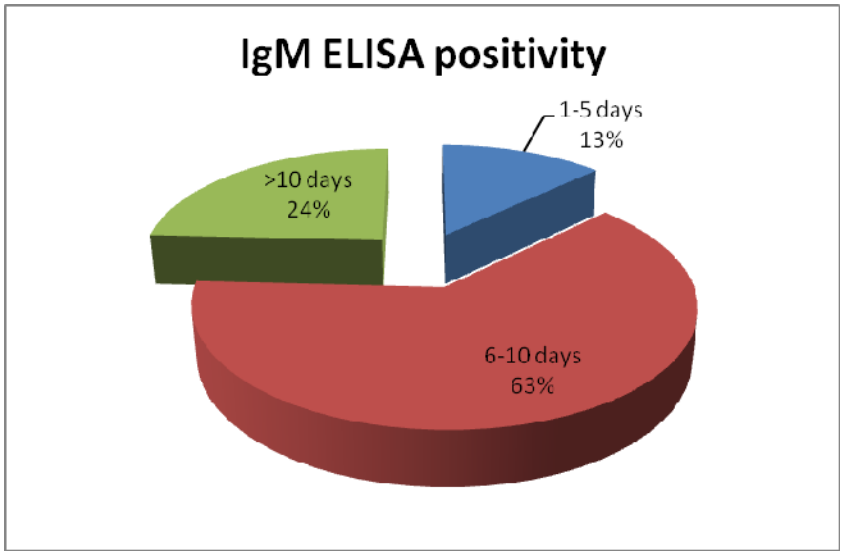
RT-PCR was found to be positive in nineteen samples out of forty samples (47.5%).

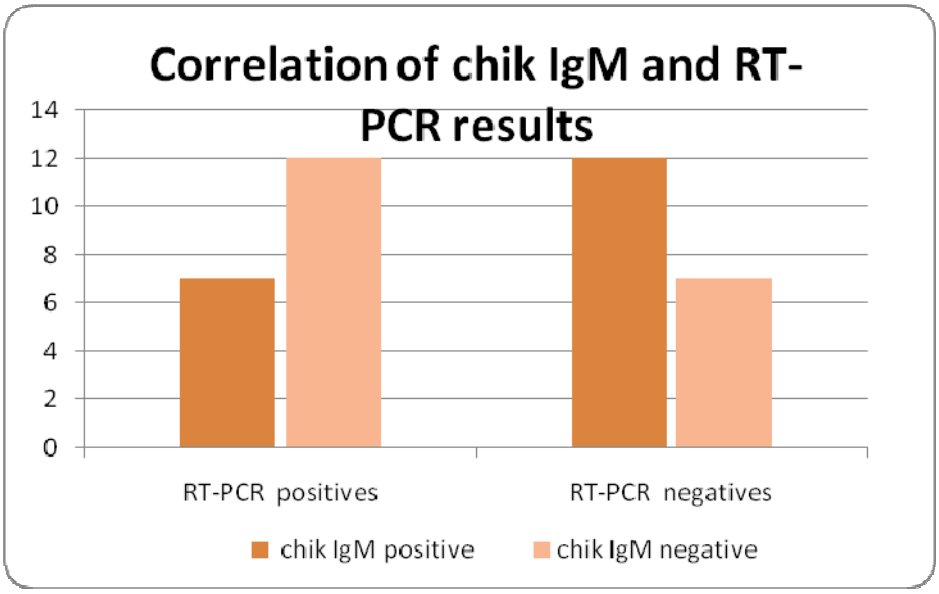
TABLE – 14 CORRELATION OF CHIK IgM AND RT-PCR RESULTS (n=40)

	RT-PCR positives (n=19)	Percentage	RT-PCR negatives (n=21)	Percentage
Chikungunya chik IgM positive (n=16)	7	17.5	9	22.5
Chikungunya chik IgM negative (n=24)	12	30	12	30

PCR test positive for 12 samples (30%) which were negative by IgM capture ELISA.

PCR test positive for 7 samples (17.5%) which were positive by IgM capture ELISA.

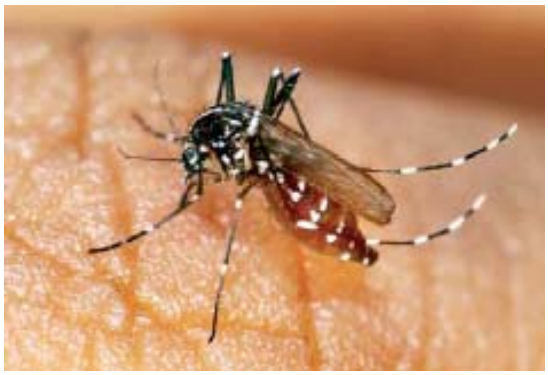

IgM ELISA positivity
1-5 days
13%
>10 days
24%
6-10 days
63%


Correlation of chik IgM and RT-PCR results
14
12
10
8
6
4
2
0
RT-PCR positives
RT-PCR negatives
chik IgM positive
chik IgM negative

ELECTRON MICROSCOPY OF CHIKUNGUNYA VIRUS

AEDES AEGYPTI

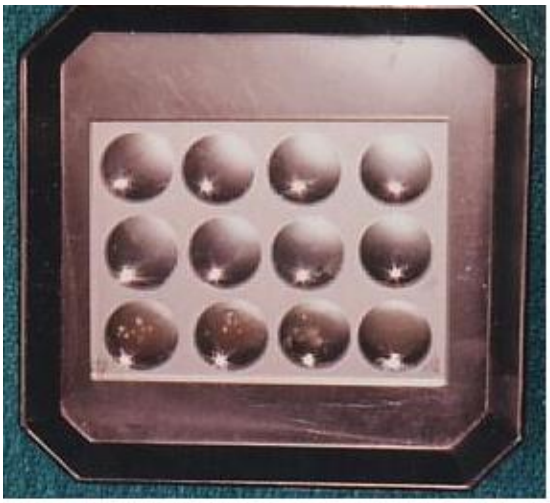
AEDES ALBOPICTUS



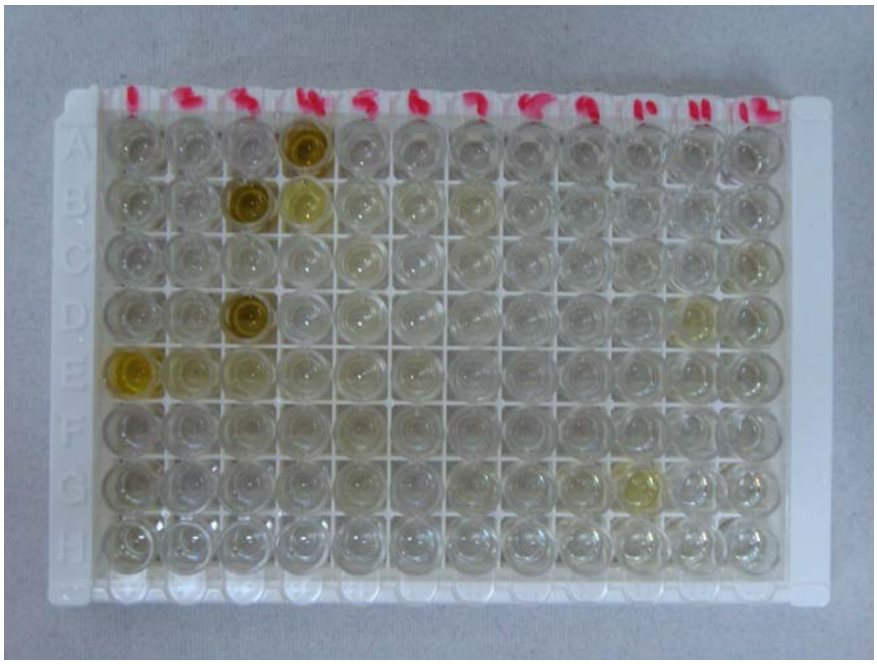
DENGUE RAPID CARD TEST

POSITIVE

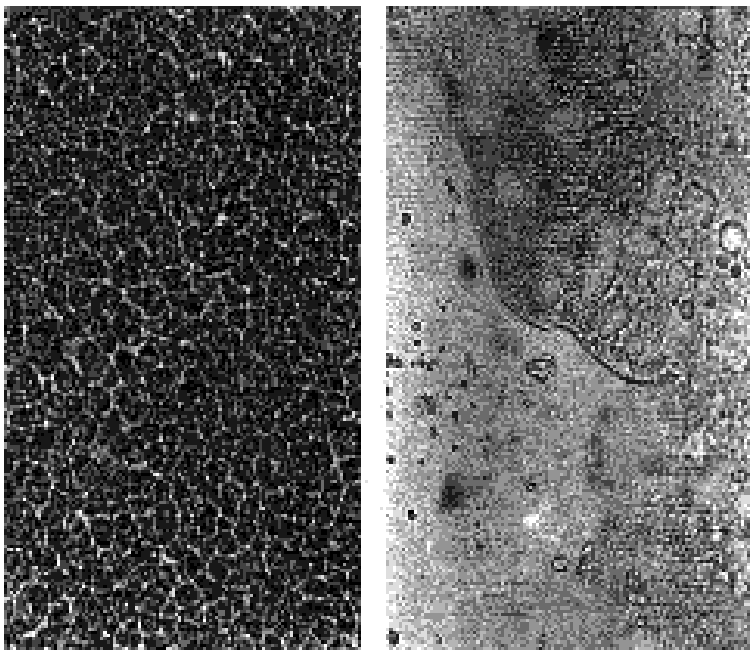
MSAT



CHIKUNGUNYA IgM ELISA



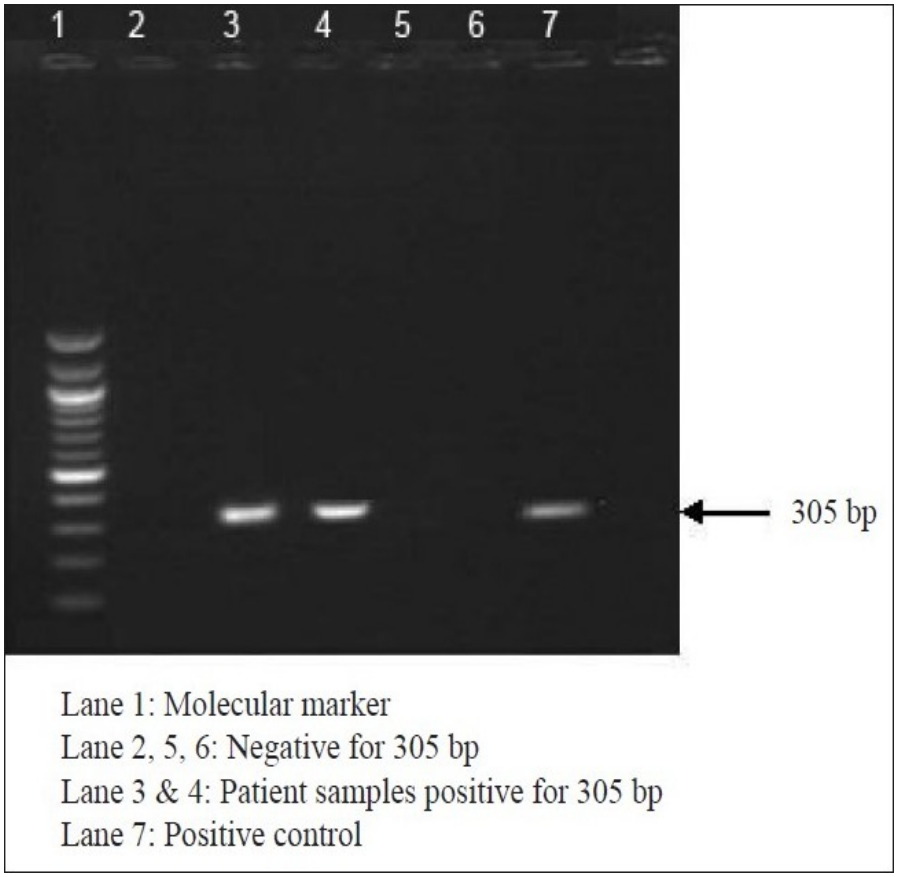
CHIKUNGUNYA CULTURE ON C6/36 CELL LINE



A – Normal Cell line

B – Cytopathic effects

RT-PCR



Lane 1: Molecular marker
Lane 2, 5, 6: Negative for 305 bp
Lane 3 & 4: Patient samples positive for 305 bp
Lane 7: Positive control

DISCUSSION

Chikungunya has been increasingly recognised as an emerging infectious disease affecting large number of people and produced one of the biggest outbreaks in 2006.

The high prevalence of Chikungunya cases at Chennai in the recent years, makes it necessary to evaluate the incidence of Chikungunya and to find out the seropositivity of Chikungunya cases.

Rapid diagnosis of Chikungunya is crucial for proper patient care and effective vector control measures. As IgM antibodies appear early during the disease course, its detection is a valuable tool for rapid diagnosis.

This study was done with 150 serum samples from patients with clinical symptoms suggestive of Chikungunya.

The incidence of Chikungunya fever in this study over a period of one year (from October 2009 to September 2010) was 0.49%.[Table:1] among fever cases attending the fever clinic and those admitted in our hospital.

The seropositivity of chikungunya cases [Table:2] in the present study among clinically suspected fever cases was 41.33%. The study conducted by Suryawanshi et al in 2006 in Maharashtra showed a seropositivity of chikungunya cases of 52.4%.[80]

The higher distribution of chikungunya cases in the present study was seen in the 29-40 year age group (48.5%) (Table:3) followed by 40-60 years age group (42.5%) This was similar to the study conducted by Suryawanshi et al in 2006 in Maharashtra which showed 63% of cases in less than 30 years age group.[82]

In the present study, Chikungunya was found more among female patients (42.8%) as compared to male patients (38.9%) (Table-4). This is similar to the study done by Thiruvenkadam et al in 2006 in Chennai which showed more number of Chikungunya cases in female patients (66.6%).[82]

The predominant symptoms with which patients presented in the present study (Table -5) were arthralgia (100%), fever (96.7%), joint swelling (75.8%), rash (59.7%), itching (53.2%), conjunctival congestion (33.9%), cough (8%), vomiting (4.8%). In the study conducted by Thiruvenkadam et al in 2006, the predominant symptoms were fever (91.2%), headache (94.8%), myalgia (95.2%), arthralgia (92.8%), neck stiffness (41%), edema (24.2%), rashes (2.6%). There was no associated case fatality in both the studies.[82] In a study conducted by Suryawanshi et al in 2006, the predominant symptoms were fever (100%), joint swelling (100%), headache (48%) and rash (27%).[80]

In the present study, maximum cases were reported in the month of November and December [Table-8]. In the study conducted by Suryawanshi

et al in 2006, maximum cases were reported in the month of August.[82] The incidence of chikungunya is higher following rainfall. True to this, a clear cut incidence of chikunguya cases was seen between September to December when TamilNadu receives rainfall from North East monsoon and post monsoon period.

In the present study maximum number of cases (69.3%) of chikungunya were from Chennai District, followed by Vellore (16.1%), Tiruvallur(8%) and Kanchipuram(6.4%) (Table-7). As this is tertiary care centre situated in Chennai, patients were more from in around Chennai.

In the present study, Dengue coinfection with the Chikungunya cases was 8% (Table -11).This is similar to the study conducted by Yergolkar et al in 2006. Another study which reported 3% Dengue coinfection with Chikungunya was conducted by Carey DE et al in 1964.[14] This is due to the fact that the same vector Aedes transmits both Chikungunya and Dengue viruses.

In this present study, Leptospiral co-infection in Chikungunya cases was 4.6% (Table-12).There was no study conducted for Leptospiral co-infection to compare the results.

In the present study 92% of the patients presented with platelet count of more than one lakh (Table-6). This is similar to the findings in the study conducted by Thiruvenkatam et al in 2006.[82]

In this present study IgM capture ELISA for chikugunya positivity was more in the early febrile period(Table-9). IgM antibodies were detected in 41.33% of the samples. In a study conducted by Suryawanshi et al in 2006, showed 52.4% of IgM positivity.[82]

RT-PCR was done using primer targeting E_1 and E_2 genes to detect ECSA phylogroup for 40 samples in the early febrile period . 16 samples which were negative by IgM capture ELISA and 24 samples positive by IgM capture ELISA were subjected to RT-PCR. Nineteen samples were positive by RT-PCR (47.5%) (Table-13). Among IgM positive cases 56% of cases were RT-PCR negative (Table-14). This could be due to the decrease in the viral load because of seroconversion.[58] Out of RT-PCR positive cases, 63.1% were IgM negative showing that RT-PCR could diagnose early cases of Chikungunya even before seroconversion. From this it is inferred that RT-PCR is more sensitive than IgM capture ELISA for samples collected in the early febrile period.[53]

The RT-PCR technique was useful in detecting the viral infection in the acute phase and also indicates the usefulness of molecular techniques in an outbreak scenario. A report by Lewthweite et al in 2009 highlighted the

importance of PCR in early detection of CHIKV Central Nervous System infections.[40] PCR is also useful in tracing the evolution of the virus by phylogenetic analysis . PCR is a more sensitive method for detection of the virus in the first week of disease onset. Further studies on CHIKV that correlate molecular diagnostics with disease onset and progression may provide a better understanding.

SUMMARY

- 150 clinically suspected cases of chikungunya were included in the study.
- IgM capture ELISA, Reverse Transcriptase PCR for Chikungunya, Rapid dengue Duo cassette method, MSAT for Leptospirosis tests were done for the suspected cases.
- Arthralgia was most common presenting symptom(100%) followed by fever (96.7%), joint swelling (75.8%), Rash (59.7%), Itching (53.2%), conjuctival congestion (33.9%), cough (8%) and vomiting (4.8%).
- Incidence of chikungunya among fever patients in the study was 0.49%.
- Preponderance of chikungunya was found in females (62.9%), males(37.1%).
- Out of 150 samples tested, 62 (41.33%) patients were found to be positive for IgM antibodies in the early and late febrile period.
- Increased incidence of chikungunya was found during September to January months, during monsoon and post monsoon period.
- Increased number of cases of chikungunya were from Chennai followed by Vellore, Kanchipuram and Tiruvallur.
- RT-PCR was positive for nineteen patients with symptoms of chikungunya.
- Dengue co-infection with chikungunya cases was 8%.
- Leptospiral co-infection with chikungunya was 9%.

CONCLUSION

- The incidence of chikungunya was 0.49% among patients attending the fever clinic and patients admitted in Government General Hospital, Chennai.
- The seropositivity of chikungunya cases was 41.33%.
- Dengue co-infection with chikungunya was 8%.
- Leptospiral co-infection with chikungunya was 9%.
- RT-PCR positivity among forty patients was 47.5%.
- Serum samples need to be collected at a very early period (1-3 days) within onset of fever to determine the serotype by RT-PCR.
- Serological diagnosis should be done in all clinically suspected Chikungunya cases to help in early initiation of treatment, thereby, minimizing the morbidity.

PROFORMA

- Name: IP/OP no.
- Age: Ward:
- Sex:
- Occupation:
- Address:
- Economic status:
- Fever
- Joint pains
- Chills
- Headache
- Vomiting
- Joint swelling
- Low back pain
- Rash
- Photophobia
- Retro orbital pain
- Abdominal pain
- Examination
- Site of joint pain
- Icterus

Investigations

- ❖ CHIK IgM capture ELISA
- ❖ RT – PCR
- ❖ MSAT
- ❖ Dengue rapid duo cassette method

BIBLIOGRAPHY

1. Bandyopadhyay B, Bandyopadhyay D, Bhattacharya R, De R, Saha B, Mukherjee H, et al. Death due to chikungunya. Trop Doct. 2009;39:187–8.

2. Bandyopadhyay B, Pramanik N, De R, Mukherjee D, Mukherjee H, Neogi DK, et al. Chikungunya in West Bengal, India. Trop Doct. 2009;39:59–60.

3. Bandyopadhyay D, Ghosh SK. Mucocutaneous features of Chikungunya fever: a study from an outbreak in West Bengal, India. Int J Dermatol. 2008;47:1148–52.

4. Benjamin M. Chikungunya is not a Swahili word, it is from the Makonde language! Available from: http://kamusiproject.org/en/node/144.

5. Bhatia R, Narain JP. Re-emerging chikungunya fever: some lessons from Asia. Trop Med Int Health. 2009;14:940–6. Epub 2009 Jun 28.

6. Bhavana K, Tyagi I, Kapila RK. Chikungunya virus induced sudden sensorineural hearing loss. Int J Pediatr Otorhinolaryngol. 2008;72:257-9.

7. Bodenmann P, Genton B. Chikungunya: an epidemic in real time. Lancet. 2006;368:258.

8. Borgherini G, Poubeau P, Jossaume A, Gouix A, Cotte L, Michault A, et al. Persistent arthralgia associated with chikungunya virus: A study of 88 adult patients on reunion island. Clin Infect Dis. 2008;47:469–75.

9. Borgherini G, Poubeau P, Staikowsky F, Lory M, Le Moullec N, Becquart JP, et al. Outbreak of chikungunya on Reunion Island: Early clinical and laboratory features in 157 adult patients. Clin Infect Dis. 2007;44:1401–7.

10. Bouquillard E, Combe B. Rheumatoid arthritis after Chikungunya fever: A prospective follow-up study of 21 cases. Ann Rheum Dis. 2009;68:1505–6.

11. Brighton SW. Chloroquine phosphate treatment of chronic Chikungunya arthritis. An open pilot study. S Afr Med J. 1984;66:217-8.

12. Brighton SW, Prozesky OW, de la Harpe AL. Chikungunya virus infection. A retrospective study of 107 cases. S Afr Med J. 1983;63:313–5.

13. Brighton SW, Simson IW. A destructive arthropathy following Chikungunya virus arthritis-a possible association. Clin Rheumatol. 1984;3:253–8.

14. Centers for Disease Control and Prevention (CDC) Chikungunya fever diagnosed among international travelers - United States, 2005-2006. MMWR Morb Mortal Wkly Rep. 2006;55:1040–2.

15. Chahar HS, Bharaj P, Dar L, Guleria R, Kabra SK, Broor S. Co-infections with chikungunya virus and dengue virus in Delhi, India. Emerg Infect Dis. 2009;15:1077–80.

16. Chandak NH, Kashyap RS, Kabra D, Karandikar P, Saha SS, Morey SH, et al. Neurological complications of Chikungunya virus infection. Neurol India. 2009;57:177–80.

17. Chevillon C, Briant L, Renaud F, Devaux C. The Chikungunya threat: an ecological and evolutionary perspective. Trends Microbiol. 2008;16:80–8.

18. Couderc T, Chrétien F, Schilte C, Disson O, Brigitte M, Guivel-Benhassine F, Touret Y, Barau G, *et al* 2008 A mouse model for Chikungunya: Young age and ineffi cient type-I interferon signaling are risk factors for severe disease.

19. De Lamballerie X, Boisson V, Reynier JC, Enault S, Charrel RN, Flahault A, et al. On chikungunya acute infection and chloroquine treatment. Vector Borne Zoonotic Dis. 2008;8:837–9.

20. De Lamballerie X, Leroy E, Charrel RN, Ttsetsarkin K, Higgs S, Gould EA. Chikungunya virus adapts to tiger mosquito via evolutionary convergence: a sign of things to come? Virol J. 2008;5:33.

21. Directorate of National Vector Borne Disease Control Pogramme. Status report on Dengue and Chikungunya as on 30.06.09. Available from: http://nvbdcp.gov.in/Doc/den-chikun-report-june09.pdf

22. Economopoulou A, Dominguez M, Helynck B, Sissoko D, Wichmann O, Quenel P, et al. Atypical Chikungunya virus infections: Clinical manifestations, mortality and risk factors for severe disease during the 2005-2006 outbreak on Réunion. Epidemiol Infect. 2009;137:534–41.

23. Edelman R, Tacket CO, Wasserman SS, Bodison SA, Perry JG, Mangiafico JA. Phase II safety and immunogenicity study of live chikungunya virus vaccine TSI-GSD-218. Am J Trop Med Hyg. 2000;62:681–5.

24. Fourie ED, Morrison JG. Rheumatoid arthritic syndrome after chikungunya fever. S Afr Med J. 1979;56:130–2.

25. Fradin MS,Day JF. Comparative efficacy of insect repellents against mosquito bite. N Engl J Med, 2002; 347:13-18.

26. Gérardin P, Barau G, Michault A, Bintner M, Randrianaivo H, Choker G, et al. Multidisciplinary prospective study of mother-to-child chikungunya virus infections on the island of La Réunion. PLoS Med. 2008;5:E60.

27. Grivard P, Le Roux K, Laurent P, Fianu A, Perrau J, Gigan J, et al. Molecular and serological diagnosis of Chikungunya virus infection. Pathol Biol (Paris) 2007;55:490–4.

28. Harley D, Sleigh A, Ritchie S. Ross River virus transmission, infection, and disease: a cross-disciplinary review. Clin Microbiol Rev. 2001;14:909–32.

29. Inamadar AC, Palit A, Sampagavi VV, Raghunath S, Deshmukh NS. Cutaneous manifestations of chikungunya fever: Observations made during a recent outbreak in south India. Int J Dermatol. 2008;47:154–9.

30. Jadhav M, Namboodripad M, Carman RH, Carey DE, Myers RM. Chikungunya disease in infants and children in Vellore: a report of clinical and haematological features of virologically proved cases. Indian J Med Res. 1965;53:764–76.

31. Johnson DF, Druce JD, Chapman S, Swaminathan A, Wolf J, Richards JS, et al. Chikungunya virus infection in travellers to Australia. Med J Aus. 2008;188:41–3.

32. Jupp PG, McIntosh BM. Chikungunya virus disease. In: Monath TP, editor. The arboviruses: epidemiology and ecology. Vol II. Boca Raton: CRC Press; 1988. pp. 137–57.

33. Kannan M, Rajendran R, Sunish IP, Balasubramaniam R, Arunachalam N, Paramsivan R, et al. A study on chikungunya outbreak during 2007 in Kerala, south India. Indian J Med Res. 2009;129:311–5.

34. Kennedy AC, Fleming J, Solomon L. Chikungunya viral arthropathy: A clinical description. J Rheumatol. 1980;7:231–6.

35. Khan AH, Morita K Del Carncn Parquit M, Hasebe F, Matchenge EG, Igarashi A. Complete neucleotide sequence of Chikungunya virus and evidence for aninternal polyadenylation site. J. Gen. Virology 2002; 83: 3075-84.

36. Lakshmi V, Neeraja M, Subbalaxmi MV, Parida MM, Dash PK, Santhosh SR, et al. Clinical features and molecular diagnosis of Chikungunya fever from South India. Clin Infect Dis. 2008;46:1436–42.

37. Lakshmipathy DT, Dhanasekaran D. Molecular epidemiology of Chikungunya virus in Vellore district, Tamilnadu, India in 2006. East Afr J Public Health. 2008;5:122–5.

38. Lenglet Y, Barau G, Robillard PY, Randrianaivo H, Michault A, Bouveret A, et al. Chikungunya infection in pregnancy: Evidence for intrauterine infection in pregnant women and vertical transmission in the parturient. Survey of the Reunion Island outbreak. J Gynecol Obstet Biol Reprod (Paris) 2006;35:578–83.

39. Levitt NH, Ramsburg HH, Hasty SE, Repik PM, Cole FE, Jr, Lupton HW. Development of an attenuated strain of chikungunya virus for use in vaccine production. Vaccine. 1986;4:157–62.

40. Lewthwaite P, Vasanthapuram R, Osborne JC, Begum A, Plank JL, Shankar MV, et al. Chikungunya virus and central nervous system infections in children, India. Emerg Infect Dis. 2009;15:329–31.

41. Lumsden WH. An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53; II. General description and epidemiology. Trans R Soc Trop Med Hyg. 1955;49:33–57.]

42. Mahendradas P, Ranganna SK, Shetty R, Balu R, Narayana KM, Babu RB, et al. Ocular manifestations associated with chikungunya. Ophthalmology. 2008;115:287–91.

43. Mahesh G, Giridhar A, Shedbele A, Kumar R, Saikumar SJ. A case of bilateral presumed chikungunya neuroretinitis. Indian J Ophthalmol. 2009;57:148–50.

44. Mavalankar D, Shastri P, Bandyopadhyay T, Parmar J, Ramani KV. Increased mortality rate associated with chikungunya epidemic, Ahmedabad, India. Emerg Infect Dis. 2008;14:412–5

45. Mavalankar D, Shastri P, Raman P. Chikungunya epidemic in India: A major public-health disaster. Lancet Infect Dis. 2007;7:306–7.

46. Mittal A, Mittal S, Bharathi JM, Ramakrishnan R, Sathe PS. Uveitis during outbreak of Chikungunya fever. Ophthalmology. 2007;114:1798.

47. Mohan A. Chikungunya fever: clinical manifestations & management. Indian J Med Res. 2006;124:471–4.

48. Mohan A. Chikungunya fever strikes in Andhra Pradesh. Natl Med J India. 2006;19:240.

49. Mohan A, Sharma SK. Chikungunya fever. In: Singal RK, editor. Medicine update. Mumbai: Association of Physicians of India; 2007. pp. 634–8.

50. Mourya DT, Thakare JR, Gokhale MD, Powers AM, Hundekar SL, Jayakumar PC, et al. Isolation of chikungunya virus from Aedes aegypti mosquitoes collected in the town of Yawat, Pune District, Maharashtra State, India. Acta Virol. 2001;45:305–9.

51. Muyembe-Tamfum JJ, Peyrefitte CN, Yogolelo R, Mathina Basisya E, Koyange D, Pukuta E, et al. Epidemic of Chikungunya virus in 1999 and 2000 in the Democratic Republic of the Congo. Med Trop (Mars) 2003;63:637–8.

52. National Institute of Communicable Disease, New Delhi. Chikungunya fever. CD Alert. 2006;10:6–8.

53. Nimmannitya S,Halstead SB, Cohen SN, Margiotta MR. Dengue and chikungunya virus infection in man in Thailand, 1962-1964. I. Observations on hospitalized patients with hemorrhagic fever. Am J Trop Med Hyg. 1969;18:954–71.

54. Obeyesekere I, Hermon Y. Myocarditis and cardiomyopathy after arbovirus infections (dengue and chikungunya fever) Br Heart J. 1972;34:821–7.

55. Obeyesekere I, Hermon Y. Arbovirus heart disease: myocarditis and cardiomyopathy following dengue and chikungunya fever—a follow-up study. Am Heart J. 1973;85:186–94.

56. Padbidri VS, Gnaneswar TT. Epidemiological investigations of chikungunya epidemic at Barsi, Maharashtra state, India. J Hyg Epidemiol Microbiol Immunol. 1979;23:445–51.

57. Paramasivan R, Philip Samuel P, Thenmozhi V, Rajendran R, Victor Jerald Leo S, Dhananjeyan KJ, et al. Chikungunya virus isolated in Lakshadweep islands in the Indian Ocean: Evidence of the Central/East African genotype. Jpn J Infect Dis. 2009;62:67–9.

58. Pastorino B, Bessaud M, Grandadam M, Murri S, Tolou HJ, Peyrefitte CN. Development of a TaqMan RT-PCR assay without RNA extraction step for the detection and quantification of African Chikungunya viruses. J Virol Methods. 2005;124:65–71.

59. Pialoux G, Gaüzère BA, Jauréguiberry S, Strobel M. Chikungunya, an epidemic arbovirosis. Lancet Infect Dis. 2007;7:319–27.

60. Pistone T, Ezzedine K, Schuffenecker I, Receveur MC, Malvy D. An imported case of Chikungunya fever from Madagascar: use of the sentinel traveller for detecting emerging arboviral infections in tropical and European countries. Travel Med Infect Dis. 2009;7:52–4.

61. Powers AM, Logue CH. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. J Gen Virol. 2007;88:2363–77.

62. Rampal, Sharda M, Meena H. Neurological complications in Chikungunya fever. J Assoc Physicians India. 2007;55:765–9.

63. Rampal, Sharda M, Meena H. Hypokalemic paralysis following Chikungunya fever. J Assoc Physicians India. 2007;55:598.

64. Reiskind MH, Pesko K, Westbrook CJ, Mores CN. Susceptibility of Florida mosquitoes to infection with chikungunya virus. Am J Trop Med Hyg. 2008;78:422–5.

65. Rishnamoorthy K, Harichandrakumar KT, Krishna Kumari A, Das LK. Burden of chikungunya in India: estimates of disability adjusted life years (DALY) lost in 2006 epidemic. J Vector Borne Dis. 2009;46:26–35.

66. Robillard PY, Boumahni B, Gérardin P, Michault A, Fourmaintraux A, Schuffenecker I, et al. Vertical maternal fetal transmission of the chikungunya virus. Ten cases 84 among pregnant women. Presse Med. 2006;35:785–8.

67. Robinson MC. An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53; I. Clinical features. Trans R Soc Trop med Hyg. 1955;49:28–32.

68. Rodrigues FM, Patankar MR, Banerjee K, Bhatt PN, Goverdhan MK, Pavri KM, et al. Etiology of the 1965 epidemic of febrile illness in Nagpur city, Maharashtra State, India. Bull World Health Organ. 1972;46:173–9.

69. Rulli NE, Suhrbier A, Hueston L, Heise MT, Tupanceska D, Zaid A, et al. Ross River virus: molecular and cellular aspects of disease pathogenesis. Pharmacol Ther. 2005;107:329–42.

70. Sam IC, AbuBakar S. Chikungunya virus infection. Med J Malaysia. 2006;61:264–9.

71. Samuel PP, Krishnamoorthi R, Hamzakoya KK, Aggarwal CS. Entomo-epidemiological investigations on chikungunya outbreak in the Lakshadweep islands, Indian Ocean. Indian J Med Res. 2009;129:442–5.

72. Schuffenecker I, Iteman I, Michault A, Murri S, Frangeul L, Vaney MC, et al. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. PLoS Med. 2006;3:e263.

73. Sebastian MR, Lodha R, Kabra SK. Chikungunya infection in children. Indian J Pediatr. 2009;76:185–9.

74. Shah KV, Gibbs CJ, Jr, Banerjee G. Virological investigation of the epidemic of haemorrhagic fever in Calcutta: isolation of three strains of Chikungunya virus. Indian J Med Res. 1964;52:676–83.

75. Simon F, Parola P, Grandadam M, Fourcade S, Oliver M, Brouqui P, et al. Chikungunya infection: an emerging rheumatism among travelers returned from Indian Ocean islands. Report of 47 cases. Medicine (Baltimore) 2007;86:123–37.

76. Simon F, Savini H, Parola P. Chikungunya: a paradigm of emergence and globalization of vector-borne diseases. Med Clin North Am. 2008;92:1323–43.

77. Singh SS, Manimunda SP, Sugunan AP, Sahina, Vijayachari P. Four cases of acute flaccid paralysis associated with chikungunya virus infection. Epidemiol Infect. 2008;136:1277–80

78. Sourisseau M, Schilte C, Casartelli N, Trouillet C, Guivel-Benhassine F, Rudnicka D, et al. Characterization of reemerging chikungunya virus. PLoS Pathog. 2007;3:E89.

79. Sudeep AB, Parashar D. Chikungunya: an overview. J Biosci. 2008;33:443–9.

80. Suryawanshi SD, Dube AH, Khadse RK, Jalgaonkar SV, Sathe PS, Zawar SD, et al. Clinical profile of chikungunya fever in patients in a tertiary care centre in Maharashtra, India. Indian J Med Res. 2009;129:438–41.

81. Tandale BV, Sathe PS, Arankalle VA, Wadia RS, Kulkarni R, Shah SV, et al. Systemic involvements and fatalities during Chikungunya epidemic in India, 2006. J Clin Virol. 2009;46:145–9.

82. Thiruvengadam KV, Kalyanasundaram V, Rajgopal J. Clinical and pathological studies on chikungunya fever in Madras city. Indian J Med Res. 1965;53:729–44.

83. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog. 2007;3:e201.

84. Valamparampil JJ, Chirakkarot S, Letha S, Jayakumar C, Gopinathan KM. Clinical profile of Chikungunya in infants. Indian J Pediatr. 2009;76:151–5

85. Wadia RS. A neurotropic virus (Chikungunya) and a neuropathic aminoacid (homocysteine) Ann Indian Acad Neurol. 2007;10:198–213.

86. World Health Organization. Outbreak news. Chikungunya and dengue, south-west Indian Ocean. Wkly Epidemiol Rec. 2006;81:106–8.

87. World Health Organization Regional Office for South-East Asia. Chikungunya fever, a re-emerging disease in Asia. Available from: http://www.searo.who.int/en/Section10/Section2246.htm.

88. World Health Organization, Regional Office for South-East Asia. Guidelines for prevention and control of Chikungunya fever. New Delhi: World Health Organization, Regional Office for South-East Asia; 2009.

89. World Health Organization, Regional Office for South-East Asia. Proposed case definition of Chikungunya fever. Available from: http://www.searo.who.int/LinkFiles/Chikungunya_Def_Chikungunya_Fever.pdf

90. Yergolkar PN, Tandale BV, Arankalle VA, Sathe PS, Sudeep AB, Gandhe SS, et al. Chikungunya outbreaks caused by African genotype, India. Emerg Infect Dis. 2006;12:1580–3.