Diagnosis of Chikungunya fever in an Indian population with an indirect ELISA protocol based on an antigen detection assay: A prospective cohort study

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Running Title: CHIKV diagnosis using indirect antigen detection ELISA
Abstract

Background:

A Chikungunya virus (CHIKV) outbreak continues in India. Monitoring the clinical features of CHIKV infection is an important component of assessing the disease process. Diagnosis is usually made by immunoglobulin IgM/ IgG Enzyme Linked Immunosorbent Assay. However, these assays show extremely low sensitivity in the majority of CHIKV patients in the acute stage of infection (during 1-4 days after infection). In our laboratory, a sensitive ELISA protocol for antigen detection has been developed for CHIKV infection in the acute stage.

Objectives:

We assessed the usefulness of antigen detection using an ELISA-based system for CHIKV infection.

Study Design:

We performed a prospective, double blinded study of 205 Indian patients with suspected CHIKV in the Nagpur district. All patients underwent full clinical assessment, and their serum samples were analyzed for the presence of antigens and of IgM and IgG using an ELISA protocol.

Results:

In patients with CHIKV, the sensitivity of antigen detection was 85%, which was significantly higher (p < 0.001) than that of IgM (17%) or IgG (45%) detection. The sensitivity of detecting IgM (20%) or IgG (25%) was significantly lower than the antigen assay (95%) in patients with acute infections (i.e. from day 1 to 5 after infection).
Conclusion: Antigen detection not only gives a positive confirmatory result in the early phase of the disease, but it is also useful in the prodromal/subclinical stage and may be useful for field applications to rapidly detect CHIKV infection.

Introduction

Chikungunya virus (CHIKV), an alphavirus of the Togaviridae family, is a relatively uncommon infection caused and spread by *Aedes aegypti* mosquito bites (19). It was first identified in West Africa in 1952. CHIKV is geographically distributed in Africa, India and Southeast Asia (6, 13). In Africa, the virus is maintained through a sylvatic transmission cycle between wild primates and mosquitoes such as *Aedes luteocephalus*, *A. furcifer*, and *A. taylori* (4, 9, 15). CHIKV symptoms include sudden onset of fever, chills, headache, nausea, vomiting, joint pain with or without swelling, lower back pain, and rash.

Recently CHIKV has caused one of the largest Chikungunya fever outbreaks reported in the past 40 years (3, 11, 18) in many parts of the world. India has previously experienced CHIKV epidemics in Kolkata, Vellore, Barsi, and Nagpur (1, 10, 12, 14). Since April 2006, a CHIKV fever outbreak has been ongoing in three states in India (Karnataka, Maharashtra, and Andhra Pradesh), and it may have spread to neighboring states. The initial cases were reported from Hyderabad and Secunderabad as well as from the Anantpur district of Andhra Pradesh as early as November and December 2005, and the epidemic has continued unabated. Some deaths have been reported, but these have been attributed mainly to inappropriate management. The major causes of morbidity include severe dehydration, electrolyte imbalance, and loss of glycemic control.
In May 2006, there was a large outbreak in the Nagpur district of Maharashtra state in India. In Nagpur city alone, according to a communication from the District Health Officer, an average of 50-100 cases of CHIKV were seen in government dispensaries each day (personal observation). The number of patients treated by private medical practitioners may be much greater. No specific drug treatment for CHIKV is available; thus, the treatment of CHIKV fever is only palliative, involving bed rest, fluids, and analgesics. In a few cases, symptoms are severe enough to warrant hospitalization.

Monitoring the clinical features of CHIKV infection is an important component of assessing the disease process in humans so as to assess that which organ system including the nervous system is affected. Laboratory tests are necessary to confirm the diagnosis of CHIKV. The immunoglobulin M (IgM) capture ELISA method, which provides evidence of CHIKV infection, is widely used to lend support to clinical findings in the assessment of patients with suspected CHIKV (8). However, the sensitivity of IgM capture ELISA is low in the majority of patients in the acute stage (days 1-5); therefore, a negative IgM capture ELISA does not rule out the diagnosis. The specificity of IgM capture ELISA is also limited because of cross-reactivity with other alphavirus related infections.

IgG cannot be detected in CHIKV patients in the acute stage.

A rapid antigen detection test using ELISA for CHIKV infection may be a more accurate diagnostic method for patients in the acute stage of infection. In the present study, we compared the usefulness of antigen detection with that of antibody detection (IgM and IgG) by ELISA. Our goal was to determine whether antigen detection by ELISA improves the diagnostic assessment of patients with CHIKV infection in routine medical practice. We performed antigen and IgM and IgG detection at the time of initial
assessment and compared the test results with the final clinical, immunological and molecular (real-time RT-PCR) diagnosis. This study was done in Nagpur, India, the location of the CHIKV epidemic.

The Institutional Ethics Committee of the CIIMS, Nagpur, India approved the study.

**Materials and methods**

**Selection of patients**

Patients were enrolled from July 2006 through September 2006 at the Central India Institute of Medical Sciences (CIIMS), Nagpur, India and from the Bhilgaon village (Nagpur District). All patients between the ages of 5 and 85 years with symptoms considered to be the clinical features of CHIKV were included in this study; these symptoms included fever, headache, myalgia, joint pains with or without swelling, and the presence or absence of rash on the body. An acute case of CHIKV was defined as any case with clinical features consistent with CHIKV and had CHIKV infection confirmed by either reverse transcription polymerase chain reaction (RT-PCR) and/or real time PCR or virus isolation. Serum from 205 participants, comprising 55 from the Bhilgaon village (rural locality) and the remaining 150 from the CIIMS in Nagpur (urban locality), were studied. A slight majority of the subjects (122, 59%) were male. The median age was 45 years (range, 5-85 years). All patients gave oral consent. Written consent was deemed unnecessary since all diagnostic tests are routinely used in clinical practice. Clinically, patients were divided into confirmed, suspected and non-CHIKV categories as outlined in the panel.
Preparation of anti-CHIKV

Pooled sera from CHIKV were collected, and IgG was purified by protein G affinity column chromatography (IgG purification kit, Bangalore Genei, India) according to the manufacturer's instructions and used at a dilution of 1:10,000. The specificity of the antibody was evaluated using different alphavirus antigens [Chikungunya Virus (CHIKV) and Ross River Virus (RRV)] by ELISA.

Antigens

CHIKV and RRV antigens used in the present study were a kind gift from Dr. P.V.L. Rao, Department of Virology, Defense Research and Development Establishment (DRDE; Gwalior, India).

An Indian strain of Chikungunya virus CHIKV ISW HYD06 (Gene Bank accession number 876190) was used to prepare antigen. The virus belonging to novel East Central South African (ECSA) genotype was isolated from a RT-PCR and serologically confirmed human patient, from Hyderabad, India during 2006 epidemic. CHIKV ISW HYD06 was initially isolated in C6/36 (cell line derived from larvae of Aedes albopictus) and then serially passaged in Vero cell line to increase adaptability as well as virus titer. The virus infected culture supernatant was clarified by centrifugation at 3000 rpm for 10 min. The clarified infected culture supernatant was then concentrated by polyethylene glycol precipitation. The purification of PEG pelleted viral preparation was carried out by discontinuous sucrose gradient centrifugation in an ultracentrifuge employing 50% and 20% sucrose cushions according to method of Gould and Clegg, 1985. The sucrose gradient purified chikungunya viral antigen used in this study was obtained from DRDE, Gwalior.
Standardization of antigens

Antigen titration was carried out by ELISA using the human anti-CHIKV IgG in order to prepare the virus antigens (Fig. 1). Briefly, wells of the ELISA plate (Nunc) were coated with 100 µl of viral antigen (in 1:2 dilution) in phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (PBS-T) for 90 min and then blocked with 0.5% BSA in PBS-T for 60 min. After 60 min of incubation at room temperature, the wells were washed with PBS-T and then reacted with anti-CHIKV IgG (pooled from sera from CHIKV patients), and the plate was incubated at 37 ºC for 60 min. The wells were washed, the secondary antibody (goat anti human IgG-HRPO, 1:10,000) was added, and the plate was incubated for 60 min at 37 ºC. After another washing with PBS-T, 100 µl of TMB/H₂O₂ substrate solution was added to the wells, which were then incubated at room temperature for approximately 10 min. The reaction was then stopped with 100 µl of 2.5 N H₂SO₄. The absorbance of each well was read at 450 nm.

CHIKV Antigen detection

100 µl of serum samples (1:100) from CHIKV patients were added to the microtiter wells and then blocked with 0.5% BSA in PBS-T for 60 min. After the samples were washed with PBS-T, anti-CHIKV IgG was added (1:10,000) and the plates were incubated at 37 ºC for 60 min. After incubation wells were washed and Goat anti human IgG-HRP (Horseradish peroxidase) secondary antibody (1:10,000 dilution) was added. Then samples were incubated for 60 min at 37 ºC. After another wash with PBS, 100 µl of TMB/H₂O₂ substrate solution were added to the wells, which were incubated at room temperature for approximately 10 min. The reaction was then stopped with 100 µl of 2.5 N H₂SO₄. The absorbance of each well was read at 450 nm.
CHIKV IgM/IgG detection

The immunoglobulin (IgM) and IgG antibodies to CHIKV were detected by ELISA. An indirect ELISA protocol allowed the detection of IgG and IgM antibodies directed against CHIKV antigens. The viral antigens (in 1:2 dilution) in PBS-T were coated onto the microtitre wells. After 90 min of incubation at room temperature, the wells were washed with PBS-T, and after blocking with 0.5% BSA in PBS-T for 60 min, serum samples (1:100) of CHIKV patients were added to the wells and incubated for 60 min. After the incubation of the diluted patient serum, 100µl of goat anti-human IgG or IgM conjugated with horseradish peroxidase was added. After another washing with PBS, 100 µl of TMB/H₂O₂ substrate solution was added to the wells, which were incubated at room temperature for approximately 10 min. The reaction was then stopped with 100 µl of 2.5 N H₂SO₄. The absorbance of each well was read at 450 nm.

Real-time RT-PCR:-

Real-time PCR of samples was performed at DRDE, Gwalior.

Primers

The oligonucleotide primers used for real-time amplification of CHIKV were designed from the E1 gene of the S-27 African CHIKV prototype strain (GenBank accession number AF369024). The potential target regions were selected by identifying highly conserved regions among strains belonging to all the different CHIKV genotypes using ClustalW programme available in the Lasergene 5 package (DNAStr, USA) (Fig. 1). In addition, the comparative analysis with other related alphaviruses viz. O’Nyong Nyong virus (ONNV), Semliki Forest virus (SFV), Sindbis virus (SINV), Mayaro virus (MAYV)
and Ross River virus (RRV) was also attempted and maximum care has been taken to reduce the cross-reactivity due to sequence similarities.

**RNA extraction**

The genomic viral RNA was extracted from 140 µl of infected culture supernatant with a known PFU of virus and 140 µl of patient serum samples using QIAamp viral RNA mini kit (QIAGEN, Germany), according to the manufacturer’s protocols. The viral RNA was eluted from the QIAspin columns in a volume of 70 µl of elution buffer and was stored at −70 ºC until used.

**SYBR Green I-based real-time RT-PCR**

SYBR Green I-based one-step real-time quantitative RT-PCR amplification was performed in the MX 3000P quantitative PCR system (Stratagene, USA). Following optimization with standard RNA using ‘Brilliant SYBR Green Single-Step QRT-PCR Master Mix’ (Stratagene), test samples were assayed in a 25 µl reaction mixtures containing 12.5 µl of 2× reaction mix, 0.4 µl of reference dye (ROX), 1 µl (10 pmol) of each forward and reverse primers, 1 µl of RNA, 0.1 µl of reverse transcriptase and 9.0 µl of nuclease free water. No template, no primer and buffer controls were also included. The thermal profile consist of 30 min of reverse transcription at 50 ºC one cycle and 10 min of polymerase activation at 95ºC, followed by 40 cycles of PCR at 95ºC for 30 s, 55ºC for 60 s and 72ºC for 30 s. Following amplification, a melting curve analysis was performed to verify the authenticity of the amplified product by its specific melting temperature (Tm) with the melting curve analysis software of the Mx3000 according to the instructions of the manufacturer.
Conventional RT-PCR

In order to compare the sensitivity of real-time RT-PCR assay, the conventional RT-PCR was performed with the same primer sets targeting the 205 bp of the E1 gene of the viral genome. The amplification was carried out in 50 µl total reaction volume by using Access Quick RT-PCR kit (Promega, USA) as per the manufacturer’s protocol.

Statistical analysis

The results are expressed as mean ± SD together with the range. Comparisons of the proportions of positives were made between the different tests and subgroups using the Kruskal-Wallis test (non-parametric ANOVA) with the Dunnett post test. A P value less than 0.05 was considered significant. Cut-off values for absorbance of CHIKV antigen, IgM, and IgG were calculated using the mean ± SD of the absorbance of CHIKV antigen/IgM/IgG in the healthy control group. The sensitivity (true positive rate) for the test was calculated as: [the number of samples in the CHIKV group with absorbance ≥ (mean ± SD) of absorbance in the healthy group, divided by the total number of samples in the CHIKV group] x 100. The specificity (true negative rate) for the test was calculated as: [the number of samples in the CHIKV group with absorbance < (mean ± SD) of the absorbance in the healthy group, divided by the total number of samples in the healthy group] x 100.

Results

Figure 1 shows the breakdown of the number of patients in each category of diagnosis. Of a total of 205 patients, 29 patients were not included in the analysis, since not all the immunological tests could be performed. Thus, 176 patients were considered for further
analysis. Of these, 109 patients were in the “Confirmed” CHIKV category, 48 were in the “suspected” category, and 19 were in the “non-CHIKV” category.

The majority of patients (81%) in all diagnostic categories were between the ages of 20 and 60 years. Only five patients (2%) were below the age of 10 years (Table 1). Fever, arthralgia without swelling, myalgia, and skin rash were the most common symptoms in nearly all of the diagnostic categories (Table 2).

**Figure 2** shows the increase in absorbance at 450 nm with increasing concentration of CHIKV antigen (5-100 ng/ml) during the standardization of the ELISA method with anti-CHIKV IgG. **There was no cross-reactivity observed with RRV antigen.**

Antigen detection in CHIKV patients was positive in the majority of patients (85%), while this was not the case for detection of IgM (17%) or IgG (45%) (Table 3). It is worth noting that in the ‘confirmed’ group, the CHIKV antigen detection rate was 90% compared to 73% in the ‘suspected’ group. In the non-CHIKV group, antigen detection was positive in only 7% of cases (Table 3).

Table 4 shows the occurrence of CHIKV antigen in sera from the CHIKV and non-CHIKV groups as determined by the indirect ELISA method along with mean absorbance (with range). Cutoff value of 0.92 was determined for ELISA test using ROC analysis.

The serum positivities for CHIKV antigen in cases of confirmed and possible CHIKV patients were 90% (98/109) and 73% (35/48) respectively, while the positivity for patients in the Non-CHIKV group was 11% (02/19). **In healthy control group CHIKV antigen was detected in only one case.** Overall, the indirect ELISA method yielded 85% sensitivity (95% confidence interval [CI] 67 to 93%) and 89% specificity (95% CI, 57 to 98%) for the diagnosis of CHIKV. The mean absorbance value of CHIKV antigen in the
CHIKV patients was 1.28 ± 0.39 (range 0.88–2.23), significantly higher than in the non-
CHIKV group (0.72 ± 0.19; range 0.10–1.02; \( P < 0.001 \)). There was a significant
difference in the mean CHIKV antigen activity between the confirmed CHIKV patients
(1.69 ± 0.46; range 0.79 – 2.23) and the possible CHIKV patients (1.19 ± 0.29; range
0.42 – 1.86; \( P < 0.0001 \)).

The results of IgM assays for all groups, expressed as OD are shown in Table 5. The
mean absorbance value of IgM in CHIKV group was 0.898 ± 0.18 [(range 0.30–1.28), \( p < 0.0001 \)] significantly higher than the non-CHIKV group 0.48 ± 0.16 [(range 0.08–0.68),
\( p < 0.0001 \)]. Overall, the ELISA method showed 17% sensitivity and 95% specificity for
the diagnosis of CHIKV. Cut off value of >0.523 was determined for ELISA test using
ROC analysis. Table-6 shows the positivity for IgG assay in cases of CHIKV patients and
non-CHIKV patients, showing sensitivity of 45% and specificity of 53%. The mean IgG
level 1.71 ± 0.32 [(range 0.82–2.38), \( p < 0.0001 \)] in CHIKV group were significantly
higher than in non CHIKV 0.61 ± 0.14 [(range 0.15–1.37), \( p < 0.0001 \)]. Cut off value of
>0.95 was determined for ELISA test using ROC analysis.

Out of 157 CHIKV patients (confirmed and suspected), 46 patient samples were
subjected to RT-PCR assay and compared with IgM, IgG and antigen detection
assay. Figure 3 shows a comparison between the antigen, IgM assays and RT-PCR
in CHIKV patients. Out of 46 CHIKV patients, antigen detection was positive in 36
patients (78%) and negative in 10 patients (22%). Of the positive group, 5 (14%) were
IgM-positive and the rest 31 (86%) were IgM-negative. However RT-PCR was positive
in 61% (19/31) of IgM negative patients and 60% (3/5) of IgM positive patients. Of the
10 patients in the antigen-negative group, IgM was positive in 1 (10%) with RT-PCR also
positive for the same and IgM negative in 9 (90%) cases. On the other hand, RT-PCR was positive in 56% (5/9) of IgM negative cases. Thus, the IgM assay was helpful in only a small percentage of patients with antigen-negative results. Figure 4 shows a comparison between the antigen, IgG and RT-PCR assays in CHIKV patients. In patients positive for antigen (36), IgG detection was negative in 47% (17) and positive in the remainder 19 (53%). In the same group RT-PCR was found positive in 63% (12/19) of IgG positive cases and 59% (10/17) of IgG negative cases. However, in the antigen-negative group (10), IgG detection was positive in 4 (40%) cases of which RT-PCR positivity was noted in 3 cases (75%), while in RT-PCR was positive in 50% (3/6) of the IgG negative cases. Thus this observation seems dependent on the duration of illness before blood collection for immunological tests.

Discussion

CHIKV has caused several large outbreaks in India. Early diagnosis of CHIKV remains difficult because the clinical picture of CHIKV infection is similar to that of other viral infections, which results in frequent diagnostic uncertainty (2). Detection of most alphaviruses currently depends on isolating virus from the blood of viremic patients, infected tissues, or blood-feeding arthropods, which is time-consuming and complicated by the lack of required resources. It is therefore necessary to develop a simple and rapid test that can discriminate CHIKV infection from other viral infections.

Detection of CHIKV immunoglobulin M (IgM) and G (IgG) is a widely used diagnostic method for CHIKV infection (5, 7, 16, 17). Some patients with secondary infection have been reported to produce IgM more slowly, and approximately 5% of patients have
undetectable levels of IgM. Similarly, ELISA for IgG estimation is not very useful for the
diagnosis of acute virus infection. Tests for viral nucleic acid have recently been introduced,
but these methods require sophisticated technology and well-trained personnel. In addition,
viral RNA is not usually detected in the serum after defervescence (20).

Detection of the viral antigen can be an alternative to isolating the virus or detecting viral
nucleic acids, IgG, or IgM. To our knowledge, there have been no studies evaluating a viral
antigen detection assay in CHIKV patients. In our laboratory, we have developed an antigen
detection assay that can be used with the serum of clinically suspected CHIKV patients for
early diagnosis. Since this test is aimed at the detection of a viral antigen, we expect the
result to be positive not only in the early phase of the disease but also in the prodromal/
subclinical stage. The antigen detection assay measures the viral protein in the blood, which
is detectable earlier than IgG and IgM during acute infection. This ability to detect infection
early is due to the initial burst of virus replication and is associated with high levels of
viraemia, during which time the individual is highly infectious. Pooled sera from CHIKV
patients were collected, IgG (antibody) was purified by protein G affinity column
chromatography, and purified antibodies were standardized using different concentrations of
CHIKV antigens. Using purified antibodies, an indirect ELISA protocol was developed for
the detection of antigen in acute CHIKV infection.

We assessed the clinical usefulness of antigen, IgM, and IgG detection assays based on the
ELISA method for CHIKV infection in suspected CHIKV patients. The study population had
a high prevalence of CHIKV infection. The sensitivity of the antigen detection assay was
significantly higher than that of the IgM and IgG detection assays in CHIKV patients. Our
results indicate that antigen detection has a higher sensitivity and specificity than IgM and
IgG detection. The sensitivity of IgM detection in CHIKV patients was only 17% and therefore less useful in diagnosis. Similarly, the diagnostic sensitivity of IgG detection was not very impressive (45%), whereas the sensitivity of antigen detection was 85%. The increased sensitivity of the antigen detection assay results from the fact that among CHIKV patients for which all three tests results are available, 126 were IgM-negative but antigen-positive and 68 were IgG-negative but antigen-positive, whereas 60 were IgG-positive but antigen-negative. This difference in the results may reflect differences in the infection and the time of sample collection during the infection. The observation that the rate of positive antigen detection was highest (95%) in the acute phase (first 5 days) and then gradually diminished is an important finding, because it indicates the possibility of definitive diagnosis of this viral disease at a very early stage. It may also be possible to detect the disease in the prodromal/subclinical stage when viraemia is present. Detection of viral antigen throughout the infection suggests the presence of a high number viral particles (viral load) in the blood. No serum specimens from children younger than 5 years old suspected of having CHIKV were received. According to local physicians, they have not seen many cases of children with CHIKV symptoms. The reasons for this phenomenon are unclear. CHIKV is thought to occur more commonly among elderly patients with underlying medical conditions. However in our study, we included subjects from all age groups, including the very young and very old, and our results do not show such observation or occurrence. Moreover, our tests showed similar performance regardless of the patient’s age. Nevertheless, CHIKV infection has a somewhat different clinical picture in younger patients.
Arthralgia and arthritis occur but are less prominent and shorter-lasting, and rashes may occur in small proportions of patients. In our study, we selected study subjects from both rural and urban areas. It was expected that CHIKV infection would be more common in remote populations (rural areas); however, the results from our study showed a similar prevalence of CHIKV infection between the two areas. However, the biggest problem with this study is that we were unable to quantify to what extent residents of rural areas had contact with the urban population. Similarly, age was not significantly associated with CHIKV infection. However, 50% of the study population with suspected CHIKV infection fell within the age range of 30-50 years, suggesting that Chikungunya is predominantly high in this age group. When the seroprevalence data for CHIKV infection were stratified by sex, a significant difference in prevalence was observed between women and men, suggesting that the prevalence of CHIKV infection is higher in women.

The results indicate that the indirect ELISA method used in this study is sensitive, specific, rapid, and cost-effective, and it can be adopted by any laboratory with an ELISA reader and an incubator. It may thus be useful in laboratories with limited resources, especially in underdeveloped and developing countries.

Overall, our study suggests that the diagnostic sensitivity of CHIKV antigen detection using an ELISA-based system is higher than that of IgM and IgG detection and helpful in the detection of antigen throughout the infection, even in the earlier stages of infection. Therefore, we conclude that CHIKV antigen detection can be very effective for diagnosis not only in the early stage of the disease but possibly also in the prodromal/subclinical stage.
Acknowledgements:

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References:


Legends
Panel: Diagnostic classification of patients presenting with chikungunya

Figures
Figure 1 Recruitment and diagnostic classification of participants.

Figure 2 The absorbance at 450 nm is shown with increasing concentrations of CHIKV antigen (5, 10, 20, 50, 100 ng/ml) during the standardization of the indirect ELISA method.

Figure 3: Flowchart showing a comparison between the antigen and IgM assays in patients with CHIKV.
Figure 4: Flowchart showing a comparison between the antigen and IgG assays in patients with CHIKV.

Tables

Table 1: Distribution of age in the confirmed, suspected and non-CHIKV groups of patients.

Table 2: Assessment of clinical symptoms in the highly probable, possible, and non-CHIKV patient groups of patients.

Table 3: Proportion of patients with antigen, IgM, and IgG assays arranged according to duration of CHIKV infection.

Table 4: Demonstration of CHIKV antigen in sera from chikungunya and non-chikungunya subjects by ELISA along with mean absorbance (with range).

Table 5: Demonstration of IgM in sera from chikungunya and non-chikungunya subjects by ELISA along with mean absorbance (with range).

Table 6: Demonstration of IgG antigen in sera from chikungunya and non-chikungunya subjects by ELISA along with mean absorbance (with range).
Panel:

<table>
<thead>
<tr>
<th>Confirmed chikungunya</th>
<th>Suspected chikungunya</th>
<th>Not chikungunya</th>
</tr>
</thead>
<tbody>
<tr>
<td>• RT-PCR and/or real time PCR positive or virus isolation</td>
<td>• Low grade fever</td>
<td>• Fever</td>
</tr>
<tr>
<td>• High grade fever</td>
<td>• Joint pain with &amp; without swelling</td>
<td>• Cough /cold</td>
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<td>• Headache</td>
<td>• Extreme fatigue</td>
<td>• Upper respiratory infection</td>
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<tr>
<td>• Joint pain with &amp; without swelling</td>
<td>• Rash with pruritis</td>
<td>• Generalized body ache</td>
</tr>
<tr>
<td>• Myalgia</td>
<td>• Headache</td>
<td></td>
</tr>
<tr>
<td>• Maculopapular rash</td>
<td>• Nausea /Vomiting</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1:

![Diagram showing the flow of patients with suspected, confirmed, and not confirmed CHIKV](attachment:diagram.png)

- 205 patients with suspected CHIKV
- 176 patients with antigen, IgG and IgM results not available
- 109 patients with confirmed CHIKV
- 48 patients with possible CHIKV
- 19 patients with not confirmed CHIKV
Table 1:

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Confirmed CHIKV (n=119)</th>
<th>Possible CHIKV (n=67)</th>
<th>Non CHIKV (n=19)</th>
<th>Total (n=205)</th>
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<tr>
<td>&lt;10</td>
<td>01 (0.8%)</td>
<td>01 (1.5%)</td>
<td>03 (16%)</td>
<td>05 (2%)</td>
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<td>10-20</td>
<td>07 (6%)</td>
<td>04 (3%)</td>
<td>03 (16%)</td>
<td>14 (7%)</td>
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<td>20-30</td>
<td>14 (12%)</td>
<td>10 (15%)</td>
<td>04 (21%)</td>
<td>28 (14%)</td>
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<td>30-40</td>
<td>28 (24%)</td>
<td>15 (22%)</td>
<td>02 11%)</td>
<td>45 (22%)</td>
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<tr>
<td>40-50</td>
<td>37 (31%)</td>
<td>22 (33%)</td>
<td>03 (16%)</td>
<td>62 (30%)</td>
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<td>50-60</td>
<td>20 (17%)</td>
<td>07 (10%)</td>
<td>04 (21%)</td>
<td>31 (15%)</td>
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<tr>
<td>&gt;60</td>
<td>12 (10%)</td>
<td>08 (12%)</td>
<td>-</td>
<td>20 (10%)</td>
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TABLE 2:

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<th>Symptoms</th>
<th>Confirmed CHIKV (n=119)</th>
<th>Possible CHIKV (n=67)</th>
<th>Non CHIKV (n=19)</th>
<th>Total (n=205)</th>
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</thead>
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<tr>
<td>Fever</td>
<td>114 (96%)</td>
<td>62 (96%)</td>
<td>15 (79%)</td>
<td>191 (93%)</td>
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<tr>
<td>Arthragia without swelling</td>
<td>112 (94%)</td>
<td>54 (81%)</td>
<td>8 (42%)</td>
<td>174 (85%)</td>
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<tr>
<td>Arthragia with swelling</td>
<td>94 (79%)</td>
<td>55 (81%)</td>
<td>8 (42%)</td>
<td>157 (77%)</td>
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<td>Myalgia</td>
<td>84 (71%)</td>
<td>38 (57%)</td>
<td>12 (63%)</td>
<td>134 (65%)</td>
</tr>
<tr>
<td>Rash</td>
<td>116 (97%)</td>
<td>29 (43%)</td>
<td>-</td>
<td>145 (71%)</td>
</tr>
<tr>
<td>Low back pain</td>
<td>25 (21%)</td>
<td>40 (60%)</td>
<td>07 (37%)</td>
<td>72 (35%)</td>
</tr>
<tr>
<td>Other</td>
<td>08 (7%)</td>
<td>6 (9%)</td>
<td>9 (47%)</td>
<td>23 (11%)</td>
</tr>
</tbody>
</table>

TABLE 3:

<table>
<thead>
<tr>
<th>Duration of Disease (number of CHIKV cases)</th>
<th>Antigen assay</th>
<th>IgM assay</th>
<th>IgG assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 5 days (n=61)</td>
<td>58 (95%)</td>
<td>12 (20%)</td>
<td>15 (25%)</td>
</tr>
<tr>
<td>5-10 days (n=38)</td>
<td>34 (89%)</td>
<td>9 (32%)</td>
<td>12 (32%)</td>
</tr>
<tr>
<td>10-20 days (n=38)</td>
<td>27 (71%)</td>
<td>3 (8%)</td>
<td>30 (79%)</td>
</tr>
<tr>
<td>&gt; 20 days (n=20)</td>
<td>14 (70%)</td>
<td>3 (15%)</td>
<td>8 (40%)</td>
</tr>
</tbody>
</table>
### TABLE 4:

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Positivity for CHIKV antigen (cutoff 0.92)</th>
<th>Negativity for CHIKV antigen</th>
<th>Absorbance (Mean ± 2SD)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chikungunya (n=157)</td>
<td>133 (85%)</td>
<td>24 (15%)</td>
<td>1.28 ± 0.39</td>
<td>(0.88-2.23)</td>
</tr>
<tr>
<td>• CHIKV (n=109)</td>
<td>98 (90%)</td>
<td>11 (10%)</td>
<td>1.69 ± 0.46</td>
<td>(0.79-2.23)</td>
</tr>
<tr>
<td>• Possible CHIKV (n=48)</td>
<td>35 (73%)</td>
<td>13 (27%)</td>
<td>1.19 ± 0.29</td>
<td>(0.42-1.86)</td>
</tr>
<tr>
<td>Non CHIKV (n=19)</td>
<td>02 (11%)</td>
<td>17 (89%)</td>
<td>0.72 ± 0.19</td>
<td>(0.10-1.02)</td>
</tr>
<tr>
<td>• Dengue subjects (n=05)</td>
<td>01 (20%)</td>
<td>4 (80%)</td>
<td>0.46 ± 0.12</td>
<td>(0.03-0.98)</td>
</tr>
<tr>
<td>• Rheumatoid arthritis subjects (n=04)</td>
<td>-</td>
<td>-</td>
<td>0.29 ± 0.10</td>
<td>(0.03-0.8)</td>
</tr>
<tr>
<td>Healthy Control (n=10)</td>
<td>1 (10%)</td>
<td>9 (90%)</td>
<td>0.19 ± 0.13</td>
<td>(0.01-1.1)</td>
</tr>
<tr>
<td>Subjects</td>
<td>Positivity for IgM assay (cutoff- 0.523)</td>
<td>Negativity for IgM</td>
<td>Absorbance (Mean± 2SD)</td>
<td>Range</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------------------------------</td>
<td>--------------------</td>
<td>------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>Chikungunya</strong> (n=157)</td>
<td>27 (85%)</td>
<td>130 (15%)</td>
<td>0.838 ± 0.27</td>
<td>(0.38-1.28)</td>
</tr>
<tr>
<td>• CHIKV (n=109)</td>
<td>19 (17%)</td>
<td>92 (83%)</td>
<td>0.898 ± 0.18</td>
<td>(0.30-1.28)</td>
</tr>
<tr>
<td>• Possible CHIKV (n=48)</td>
<td>08 (17%)</td>
<td>40 (83%)</td>
<td>0.692 ± 0.21</td>
<td>(0.08-0.98)</td>
</tr>
<tr>
<td><strong>Non CHIKV</strong> (n=19)</td>
<td>1 (05%)</td>
<td>18 (95%)</td>
<td>0.48 ± 0.16</td>
<td>(0.08-0.68)</td>
</tr>
<tr>
<td>• Dengue subjects (n=05)</td>
<td>1 (20%)</td>
<td>04 (80%)</td>
<td>0.39 ± 0.9</td>
<td>(0.23-0.68)</td>
</tr>
<tr>
<td>• Rheumatoid arthritis</td>
<td></td>
<td></td>
<td>0.18 ± 0.6</td>
<td>(0.03-0.43)</td>
</tr>
<tr>
<td>subjects (n=04)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Healthy Control</strong> (n=10)</td>
<td>1 (10%)</td>
<td>09 (90%)</td>
<td>0.21 ± 013</td>
<td>(0.12-0.51)</td>
</tr>
</tbody>
</table>
### TABLE 6:

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Positivity for IgG assay (cutoff- 0.95)</th>
<th>Negativity for IgG</th>
<th>Absorbance (Mean ± 2SD)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chikungunya</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=157)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• CHIKV (n=109)</td>
<td>71 (45%)</td>
<td>86 (55%)</td>
<td>1.46 ± 0.28</td>
<td>(0.91-2.38)</td>
</tr>
<tr>
<td>• Possible CHIKV (n=48)</td>
<td>49 (45%)</td>
<td>60 (55%)</td>
<td>1.71 ± 0.32</td>
<td>(0.82-2.38)</td>
</tr>
<tr>
<td><strong>Non CHIKV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Dengue subjects (n=05)</td>
<td>09 (47%)</td>
<td>10 (53%)</td>
<td>0.61 ± 0.14</td>
<td>(0.15 - 1.37)</td>
</tr>
<tr>
<td>• Rheumatoid arthritis subjects (n=04)</td>
<td>01 (20%)</td>
<td>04 (80%)</td>
<td>0.33 ± 0.12</td>
<td>(0.18 - 1.21)</td>
</tr>
<tr>
<td><strong>Healthy Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=10)</td>
<td>01 (10%)</td>
<td>09 (90%)</td>
<td>0.11 ± 0.09</td>
<td>(0.12 - 0.51)</td>
</tr>
</tbody>
</table>