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ORIGINAL RESEARCH ARTICLE

Isolation and Detection of Chikungunya Virus from patients Serum samples using RT- PCR and Real Time RT-PCR

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ABSTRACT

Chikungunya is a viral disease transmitted by *Aedes mosquitoes*. The disease typically consists of an acute illness with fever, skin rash, and arthralgia. The word chikungunya used for both the virus and the disease means "to walk bent over" in some East African languages. Chikungunya is a specifically tropical disease but it is geographically restricted and outbreaks are relatively uncommon. The objective of this study was to develop a rapid detection method to determine of CHIK viral virus in the serum samples collected from chikungunya infected patients in an around Chennai, India. Serum specimens were analyzed for CHIK viral RNA by RT- PCR and Real Time RT-PCR using specific primers. Thirty out of 12 samples were positive for CHIK virus. This result suggests that molecular diagnosis is always having hands higher than the serological diagnosis and viral culture methods. The advantages of RT-PCR and Real Time PCR method adds to early and specific diagnosis of the viral infection before the development of IgM, which is the basis for all the serological assays. The detection procedure used in this study was simple, sensitive and rapid.

Keywords: RT- PCR, Chikungunya, CHIK virus, Real Time PCR

INTRODUCTION

Chikungunya fever is an acute arthropod borne viral illness reported from many parts of Africa, South East Asia, Western Pacific and India. The causative agent Chikungunya virus (CHIKV) is a single strand positive RNA enveloped virus member of the genus *Alpha virus* of Togaviridae family and is transmitted from primates to humans generally by *Aedes aegypti* [1,2]. The name derived from the Makonde word meaning "that which bends up" in reference to the stooped posture developed as result of the arthritic symptoms of the disease [3,4]. The sudden onset of the disease including crippling arthralgia and frequent arthritis that accompanying fever, chills, headache, nausea, vomiting, low back pain and rash are clinically distinctive. The disease is almost self limiting and rarely fatal [5].

Chikungunya virus (CHICV) was first isolated from the serum of a febrile human in Tanganyika(Tanzania) in 1953.Between the 1960 and 1980s,the virus was isolated repeatedly from numerous countries in central and southern Africa as well as in Senegal and Nigeria in western Africa [6,7,8] . During the same period, the virus also identified in many parts of Asia [5]. In India CHIKV was first reported in 1963 in Kolkata, and subsequently, the virus was isolated from numerous well-documented outbreaks from 1963 to 1973. Although no major outbreak was reported in India since the 1973 outbreak in Barsi, Maharastra, however, the virus was isolated from sporadic cases in humans and mosquitoes during random surveillance and dengue virus outbreaks [9,10]. The recent outbreak of Chikungunya virus infection of an unprecedented magnitude in many parts of southern India is a point of major concern. This is the largest and most severe epidemic, affecting more than 1,000,000 persons in the Andhra Pradesh, Maharastra, and Karnataka states of India, and is spreading to several new areas, causing huge public health and administrative concerns for management of the situation [11].

Diagnosis of CHIKV infection is mostly based on serological and PCR techniques[12]. A virus culture followed by detection of viral antigens is a

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sensitive method but must be performed under BSL3 biosafety conditions and is time consuming. This method is therefore used only for virus identification at the beginning of an epidemic. Serological methods are reliable but are not appropriate in early stage infection. To date, only conventional reverse transcription (RT)-PCR and Real Time RT-PCR methods have been suggested for the study of CHIKV replication in supernatants and clinical samples or for epidemiological survey [2,3]. The aim of this study was to develop a rapid, sensitive method to detect and quantify CHIKV in acute-phase patient serum samples.

MATERIALS AND METHODS

Sample collection (CHIKV Patient's sera)

A total of 30 acute-phase serum samples collected from patients with a clinical diagnosis of Chikungunya fever who were admitted to Government Hospital in an around Chennai,Tamilnadu, were used for evaluation in this study. The samples were collected during the acute phase periods after the onset of symptoms. All the samples were transported to the laboratory under conditions and stored at -80°C until further investigation.

Primer sequences

Primer sequences were designed using primer premier for the structural poly protein region of Chikungunya virus

Name	Primer sequence	Position	Tm value	Length
CK-F	ACCACATGCCAGCAGACGCA	776-795	60.73	20 bp
CK-R	TGCTGCGGTCGGGAATGGAA	982-1001	60.17	20 bp

Primer sequences for real Time PCR: (Telles et al., 2009)

CHIKVR: 5'-GCC TGG ACA CCT TTC GAC-3'

CHIKVR 5'- AAT TCT AAT ACG ACT CAC TAT AGG GGC TCT TAC CGG GTT TGT TGC -3'

CHIKV probe: 5'-CGA GCG ACT CTC AGG CAC CAT CTG GCT CGC TCG-3' FAM labeled)

RNA Extraction

The genomic Viral RNA was extracted 200 µl of patient serum samples by using the Invitrogen PureLink Viral RNA/DNA isolation kit. The RNA was eluted from the spin columns in a final volume of 50 µl of elution buffer and was stored -80˚c till further application.

PCR Amplification

8 microliters of the purified RNA suspension were combined with 20 pmole each of oligonucleotide primers CHIKVR (5'GCC TGG ACA CCT TTC GAC-3'); and CHIKVR (5'AAT TCT AAT ACG ACT CAC TAT AGG GGC TCT TAC CGG GTT TGT TGC3'); 4 µl of M-MLV 5x reaction buffer, 1 µl of 100 mM dntps, M-MLV RT 200 units (2 µl) units of reverse-transcriptase enzyme, and 20 µl using Nuclease free water. The primers were designed to amplify a 100 base pair fragment of the gene region. Primer coordinates are based on a published CHIK RNA structural gene sequence (5'-CGA GCG ACT CTC AGG CAC CAT CTG GCT CGC TCG-3' (FAM labeled). The mixture was incubated at 48ºC for 45 minutes for reverse transcriptase reaction and subsequently subjected to 40 cycles of PCR, consisting of denaturation at 94ºC for 38 seconds, primer annealing at 60ºC for 1 minute, and primer extension at 68ºC for 2 minutes. A second nested PCR reaction was done using the product from the first reaction. For this reaction, 5 picomoles each of the internal primers CHIKVR (5'GCC TGG ACA CCT TTC GAC-3'); and CHIKVR (5'AAT TCT AAT ACG ACT CAC TAT AGG GGC TCT TAC CGG GTT TGT TGC3') were combined with 5 µl of 10 xPCR BufferII, 200 units of reverse-transcriptase enzyme. The mixture was then incubated at 95ºC for 10 minutes and subjected to 40 cycles of amplification, with each cycle consisting of 95ºC for 30 seconds, 55ºC for 30 seconds, and 72ºC for 30 seconds. Following the last cycle, the mixtures were held at 72ºC for 7 minutes. Amplified PCR products were resolved by electrophoresis on 2% agarose gel and ethidium bromidestain. Positive samples resulted in a 250 base-pair fragment.

Real Time RT-PCR

Extracted RNA from the serum samples were further to Real-time PCR using Taq Man probe RT-PCR kit in a Real Time PCR Machine. The reaction was done as per manufacturer's instruction. The reaction was cycled with real-time detection through the following thermal regime; 95°C for 15 min followed by 45 cycles of 95 °C for 15 s and 60°C for 1 minute.

RESULTS AND DISCUSSION:

Among the 30 samples collected from in an around Chennai, 12 were tested positive, observed 226 base pair product in Agarose Gel Electrophoresis (Figure 1). The positive samples were further confirmed using Real time PCR. But only 4 were tested positive for ELISA at the time

of collection. Further the positive samples were confirmed using Real time PCR (Figure 2, 3 & 4).

The results show that when 8 samples failed to show positive in the ELISA technique but has formed a well established band after RT PCR gel electrophoresis which proves the ability of the RT PCR for diagnosing the initial viraemic phase (day 0 to day 7) [13,14] but classic serological methods are simpler (Haemagglutination Inhibition, complement binding, Immuno-fluorescence, and ELISA) [15] and IgM is detectable after an average of 2 days by ELISA Immuno-fluorescent assay (1–12 days) and persists for several weeks to 3 months [16]. IgG is detected in convalescent samples and persists for years. The sensitivity and specificity of these tests are poorly established, and so is the possibility of false-positive reactions resulting from cross-reactivity with dengue or other chikungunya.

The result indicated that RT-PCR was sensitive enough to detect Chikungunya positive samples, which went undetected by ELISA. Same kind of results were observed by Joseph et al [17] in Kerala patients who observed 5 Positive samples by RT PCR method and only 3 positives by ELISA method among 20 samples they have processed

Individual serological testing is not particularly useful, except when faced with atypical or severe forms, or in travelers returning from an epidemic zone. Parola and colleagues [18] analyzed sera from four infected travelers returning from Indian Ocean islands by using a quantitative real-time RT-PCR-based method. They detected a viral load of 10 copies per ml in one case. Such high levels of viraemia are uncommon in arthropod-borne diseases such as dengue fever and West Nile disease [19].

Here we confirmed the RT-PCR positive samples by Quantitative real time PCR which also showed the positive peek during observation. Also the study reveals the re emergence/ persistence of Chikungunya infection in Chennai city.

From the study it has been concluded that molecular diagnosis (nucleic acid methods) are always having a hands higher than the serological diagnosis and viral culture methods. The advantages of RT-PCR method adds to early and specific diagnosis of the viral infection before the development of IgM, which is the basis for all the serological assays. The study further concludes about the persistence of the infection in Chennai although with lesser severity, it is emerging as an endemic disease in the state of Tamilnadu and India. As the treatment for the diseases are very scarcely available and not proven to be of 100% effectiveness, the available ways are to manage and prevent. The easiest way for prevention is to prevent us from mosquito bites as the arbovirus group pathogen is transmitted through mosquito bites.

Fig 1: Agarose Gel Electrophoresis. M-100 bp DNA Marker, (-) – Negative control,(+) – Positive control 1-30 –Test samples, Product size – 226 base pair

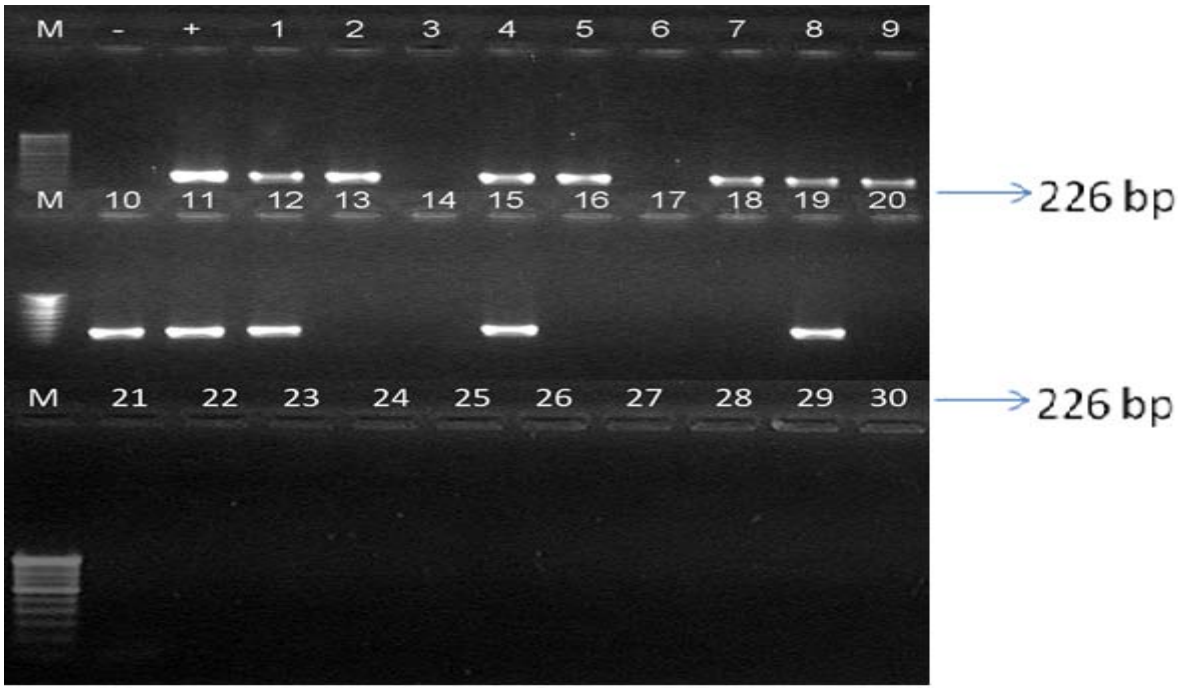


Fig 2: Amplification Plot

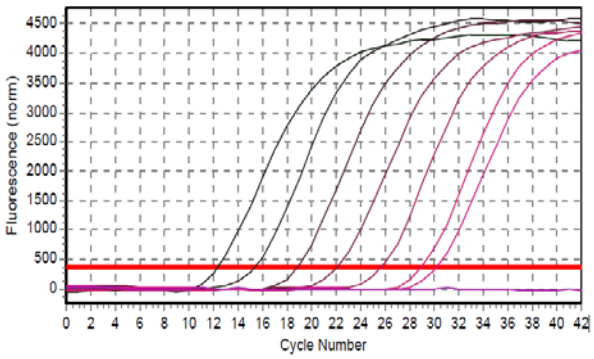


Fig 3: Standard Curve

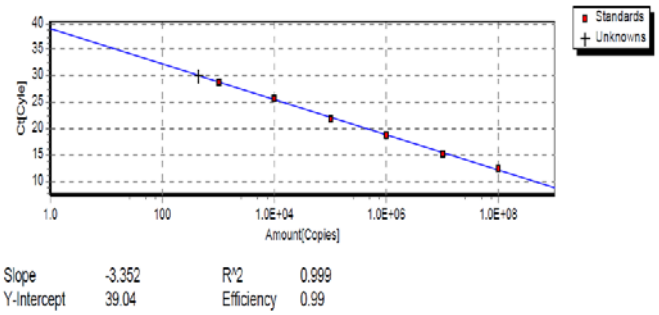
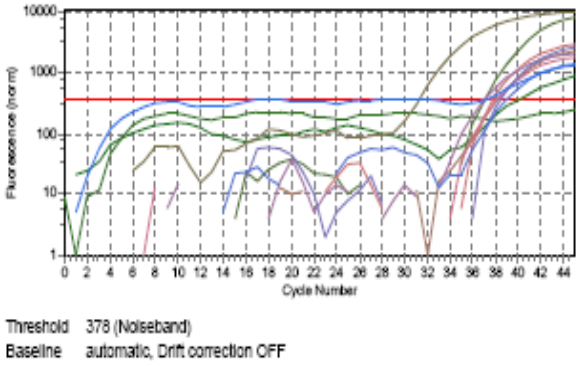


Fig 4: Real Time PCR for serum samples



CONCLUSION

CHIKV, a member of the Alphavirus genus, is of considerable public health importance in India. Transmission between species and individuals occurs via mosquito vectors, making the Alphaviruses a member of Arboviruses, or arthropod borne viruses. After quiescence of about three decades, an outbreak of CHIK with sporadic cases of dengue is being reported from different parts of India. A total of 30 serum samples collected from 2 different medical centers were processed for RNA isolation followed by CDNA synthesis and RT-PCR. 12 samples were positive in RT-PCR but only 4 were tested positive for ELISA and 8 positive samples were failed to react in ELISA. The RT-PCR positive samples further confirmed as positive using Real time RT-PCR assays.

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